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Title: SOME PROPERTIES OF DIAMINE OXIDASE FROM PISUM SATIVUM

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Partial purification of diamine oxidase from pea seedlings, Pisum sativum, was accomplished by homogenization of 8-10 day etiolated epicotyl tissue, followed by ammonium sulfate fractionation and DEAE Sephadex column chromatography. The preparation thus obtained was purified 50 fold.

Some properties of this enzyme were investigated. A radio-tracer method was adapted for assay of diamine oxidase with tryptamine. The oxidation product of tryptamine was characterized by sodium borohydride reduction and subsequent repeated crystallizations of the reduced species with unlabeled tryptophol. The Michaelis constants for tryptamine and putrescine were determined with purified diamine oxidase; the values obtained at pH 8 were 4×10^{-4} M for tryptamine and 7.4×10^{-5} M for putrescine.

The titration of diamine oxidase was accomplished with several hydrazines. Beta-hydroxyethylhydrazine (BOH) gave a more pronounced inhibitory effect than 1,1-dimethylhydrazine (UDMH) or hydrazine. The rates of inhibition of diamine oxidase with these inhibitors were examined and the initial rapid interaction of hydrazines with the enzyme was found to be second-order and dependent upon inhibitor concentration. The inhibition of diamine oxidase activity by these hydrazines was not reversed by dialysis.

SOME PROPERTIES OF DIAMINE OXIDASE FROM PISUM SATIVUM

By

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Typed by D. Neketin for _____ Edith Fusayo Yamasaki

To my parents

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SOME PROPERTIES OF DIAMINE OXIDASE FROM PISUM SATIVUM

INTRODUCTION

Since the discovery of an enzyme system in plants which

oxidized di- and monoamines in in vitro assays (Werle and Pechman,

1949), much work has been done on the purification and characteri-

zation of the enzyme plant amine oxidase (Kenten and Mann, 1952;

Mann, 1955, 1961; Mann and Smithies, 1955; Hill and Mann, 1962,

1963, 1964; Hill, 1966; Hasse and Maisack, 1955; Clarke and Mann,

1957; Werle and Hartung, 1956; Werle, Beaucamp and Schirren, 1959;

Werle, Trautschold and Aures, 1961; Goryachenkova, 1956a, 1956b;

and Uspenskaia and Goryachenkova, 1958).

The plant enzyme investigated by Hill and Mann (1962) de-

monstrated a greater rate of oxidation of diamines than monoamines.

Therefore, it was designated as pea seedling diamine oxidase

(diamine-oxygen oxidoreductase EC 1.4.3.6).

Kenten and Mann (1952) had shown that extracts of pea

seedlings were active on a variety of monoamines and diamines,

with maximal rates of amine oxidation with 1,4-diaminobutane (putres-

cine) and 1,5-diaminopentane (cadaverine) as substrates. Subse-

quently, a highly purified diamine oxidase preparation (Hill and

Mann, 1964) was used to obtain relative rates of oxidation of various

amines. The purified enzyme displayed rates with each substrate

which were comparable to those observed with the crude pea seedling

extract (Kenten and Mann, 1952), suggesting the presence of a single

amine oxidase. Werle, Trautschold and Aures (1961) determined the substrate specificity of their diamine oxidase preparations and found that cadaverine was the most rapidly oxidized amine of those tested as substrates.

To facilitate the study of the mechanism of action of diamine oxidase, Mann (1961), Uspenskaia and Goryachenkova (1958), and Werle, Trautschold and Aures (1961) worked extensively to obtain relatively pure diamine oxidase preparations. Mann (1961) procured a stable pea seedling diamine oxidase preparation, purified 880 fold, and found a concomitant increase in copper content during purifi- cation. The enzyme contained 0.08-0.09% copper; a concentrated solution of the enzyme was pink, displaying a broad maximum absorption band at approximately 500 mμ. The magnitude of this peak was small, however, in comparison with the absorption at 280 mμ. Werle, Trautschold and Aures (1961) purified diamine oxidase from pea seedlings, 1000 to 1200 fold. The rose-colored preparation displayed a shoulder at 500 mμ in the visible absorption spectrum. Uspenskaia and Goryachenkova (1958) obtained rather labile preparations of pea seedling diamine oxidase, purified 550 fold by the application of electrophoretic techniques.

Goryachenkova (1956a) found that partially purified diamine oxidase could be protected from inactivation at pH 4.5 - 5.0 if

dialysis were carried out in the presence of pyridoxal phosphate and flavin adenine dinucleotide. After Werle and Pechmann (1949) suggested that a flavin component or pyridoxal phosphate might be a coenzyme for diamine oxidase, many workers attempted to establish the identity of the cofactor or cofactors. Mann (1961), Werle, Trautschold and Aures (1961), and Hill and Mann (1964) were unable to find evidence for the presence of pyridoxal phosphate as the prosthetic group.

From spectrophotometric studies, it was believed that pyridoxal phosphate was present in pig kidney diamine oxidase (Mondovi et al., 1964) and subsequently, it was demonstrated to be present in pig kidney diamine oxidase as a prosthetic group (Mondovi et al., 1967c).

Mondovi et al. (1967b) in the purification of diamine oxidase from pig kidney demonstrated that the absorption spectrum of the purified enzyme was quite similar to that observed for pea seedling diamine oxidase, having absorption maxima at 405 mμ and 500 mμ in the visible region. Purified 3200 fold, this diamine oxidase was pink-yellow in color, containing 11 to 12 mμmoles of copper per mg of protein. These results suggest that the pig kidney diamine oxidase is very similar to pea seedling diamine oxidase.

In studying the properties of the pea seedling diamine oxidase, Mann (1961) used hydrazine as a carbonyl reagent and examined the spectra of the reactions between hydrazine and the enzyme. Absorption maxima at 330 mμ and 420 mμ were found to replace the broad band at 500 mμ. From these results, Mann (1961) suggested an interaction of the hydrazine with a carbonyl component of the enzyme.

Hydrazine and several alkyl hydrazines have been found to inhibit the activity of diamine oxidases of various origins (Schuler, 1952; Werle, Beaucamp and Schirren, 1959; Werle, Trautschold and Aures, 1961). Much work has been done on the inhibition of monoamine oxidase (monoamine-oxygen oxidoreductase EC 1.4.3.4) by certain substituted hydrazines (Davison, 1957; Green, 1962, 1964; and Smith, Weissbach and Udenfriend, 1963). Barsky et al. (1959) showed that inhibition of monoamine oxidase by hydrazine derivatives was irreversible. Davison (1957) demonstrated that the reaction of iproniazid with monoamine oxidase was irreversible and the inhibition was progressive with time, being first-order in the absence of substrate. Isopropylhydrazine was found to behave in a similar manner to iproniazid, but having a more pronounced inhibitory effect. It was proposed (Davison, 1957) that the iproniazid attached to the active site of the enzyme was oxidized to the isopropylidene derivative since oxygen was required for the occurrence of irreversible inhibition.

Smith, Weissbach, and Udenfriend (1963) utilized radioactive

iproniazid to study the mechanism of inhibition. They demonstrated

that the ^{14}C-isopropyl group of the inhibitor was bound to the mono-

amine oxidase and that the non-dialyzable radioactivity of the com-

pletely inhibited enzyme represented 0.14 mμmole of inhibitor per mg

of total protein. It was suggested that the iproniazid molecule was

converted to isopropylhydrazine, which in turn inhibited the enzyme.

The work of Aldridge (1950, 1953) on irreversible inhibition

of cholinesterase by various substituted esters of phosphoric acid

revealed that inhibition of this enzyme was dependent upon the time

of contact of enzyme and inhibitor. Bimolecular rate constants were

calculated on the assumption that the reaction was pseudo-first order.

The reaction mechanism postulated for these irreversible inhibition

reactions was that the enzyme and inhibitor reacted in bimolecular

fashion in a slow step, forming a complex which was converted to

an irreversibly inhibited enzyme in a unimolecular fast step. This

type of irreversible inhibition can be compared to the reaction of

diisopropylfluorophosphate (DFP) with chymotrypsin, as studied by

Jansen, Nutting and Balls (1949). They demonstrated that the reaction

of DFP with the enzyme was extremely rapid. Using radioactive

phosphorous (^{32}P) in the inhibitor DFP, they found 1.1 moles of

phosphorous bound per mole of chymostrypsin, based on the molecular

weight of 27,000. A crystalline DFP-inhibited chymotrypsin pre-

paration remained inactive after dialysis and recrystallization.

Much work on the effect of auxins on the growth of plants has led to the use of certain hydrazine derivatives as growth retardants. A possible mechanism for the activity of these plant growth retardants has been suggested (Reed, 1965; Reed, Moore and Anderson, 1965). Because of the discovery of tryptamine in certain plant tissues, it had been postulated that the plant diamine oxidase might play a role in the formation of auxins, specifically, indoleacetic acid from tryptamine (Reed, 1965). Since previous work with various hydrazines and pea seedling homogenates (Reed, 1965; Reed, Moore and Anderson, 1965) and with in vivo experiments using rats (Reed, Dost and Wang, 1965) indicated the very potent effectiveness of these compounds as inhibitors of amine oxidase activity, a study was initiated to purify pea seedling diamine oxidase and to characterize the enzyme, with possible titration of the enzyme with several hydrazine inhibitors.

In previous work with the time-dependent inhibition of monoamine oxidase by a hydrazine derivative, iproniazid, (Davison, 1957) it was shown that the reaction was first-order. Davison (1957) noted that isopropylhydrazine was a more potent inhibitor of monoamine oxidase activity, but the kinetics of inhibition with this particular hydrazine were not investigated. Smith, Weissbach and

Udenfriend (1963) speculated that isopropylhydrazine, and not the

hydrazine derivative iproniazid, was probably the active inhibitor

of monoamine oxidase activity. Therefore, in the study of the pea

seedling diamine oxidase, it was decided to use hydrazines, rather

than the hydrazide derivatives, and to investigate the inhibition of

the enzyme by beta-hydroxyethylhydrazine (BOH), 1,1-dimethyl-

hydrazine (UDMH), and hydrazine, the parent compound.

EXPERIMENTAL PROCEDURE

Materials

Pisum sativum, var. Alaska, seeds were purchased from Northrup, King & Company. Catalase (2X crystallized from bovine liver) was from Sigma Chemical Company. Ammonium sulfate was special enzyme grade from Mann Research Laboratories.DEAE Sephadex A-50, capacity 3.5 ± 0.5 meq, was from Pharmacia. Pyridoxal 5-phosphate, tryptamine hydrochloride, and putrescine dihydrochloride were obtained from Sigma Chemical Company. Indoleacetaldehyde sodium bisulfite was from Calbiochem. Tryptophol was from Regis Chemical Company. Sodium borohydride was from Matheson, Coleman and Bell. 1, 4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) and p-terphenyl ($\emptyset_3$) of scintillation grade were from Packard Instrument Company. Tryptamine 2-^{14}C bisuccinate, varying in specific activity from 2.73 mC/mmole to 10.2 mC/mmole, putrescine-1, 4-^{14}C dihydrochloride, specific activity of 9.1 mC/ mmole, and cadaverine-1, 5-^{14}C dihydrochloride, specific activity of 1.29 mC/mmole were from New England Nuclear Corporation. Germination towels, standard weight, were obtained from Anchor Paper Company. Hydrazine (anhydrous) 95+% and 1, 1-dimethylhydrazine (anhydrous) 99+% (UDMH) were from Matheson, Coleman and Bell. Beta-hydroxyethylhydrazine (BOH) was used from two sources. The

inhibition studies done with purified diamine oxidase were with 82.4% BOH, a gift from Dr. S. I. Cohen of the Squibb Company, and the studies with partially purified diamine oxidase were with BOH from K&K Laboratories, assumed to be of high purity.

Purified pea seedling diamine oxidase, reported to have the specific activity of 48 (Hill and Mann, 1964) and a protein concentration of 14 mg/ml, was the kind gift of Dr. P. J. G. Mann (Rothamsted Experimental Station, Harpenden, England). The protein concentration was redetermined in our laboratory by a micro-Kjeldahl method (Lang, 1958) and found to be 20 mg/ml, based on the assumption that the nitrogen content of the protein was 16%. For the experiments reported here, the protein concentration of 20 mg/ml is used for the purified diamine oxidase.

Methods

Buffers used were prepared from H_3BO_3 and KH_2PO_4 or only KH_2PO_4 and the desired pH values were obtained by titration with KOH. For the borate-phosphate buffers, sufficient amounts of boric acid and potassium dihydrogen phosphate were weighed to give a final concentration of 33 mM in borate and 33 mM in phosphate species when brought into solution with glass-distilled water.

All determinations of radioactivity were performed with a liquid scintillation spectrometer. The counting solution was prepared

from 30 mg POPOP and 3 g p-terphenyl per liter of toluene. Counting

vials having a volume capacity of 24 ml were used for all determinations

of radioactivity.

All enzyme assays were performed in duplicate.

Protein determinations, unless otherwise indicated, were

performed by the method of Warburg and Christian (1942).

All temperature values are in centigrade units.

Concentrations denoted by brackets in figures represent molar

concentrations.

Solutions of inhibitors were prepared by weighing desired

aliquots of BOH, UDMH, or hydrazine into 100 ml volumetric flasks

and diluting with glass-distilled water to a final concentration of

4×10^{-2} M. Subsequent dilutions were made with an appropriate

buffer.

Enzyme Assays

For the enzyme assay with tryptamine a modified procedure of

the method of Wurtman and Axelrod (1963) was employed. The method

is based upon the radioassay of the toluene-extractable ^{14}C-labeled

aldehyde product of amine oxidase activity when a ^{14}C labeled amine

is used as a substrate. In a typical assay tryptamine $2-^{14}$C, 240,

000 dpm and 40 mμmoles, was added to 20 μg of catalase and 100 μl

of enzyme preparation to give a final assay volume of 400 μl 33 mM

borate-phosphate buffer, pH 8, contained in a 10 ml glass conical

centrifuge tube. After a 10 minute incubation period at 25°, the

reaction was terminated by the addition of 0.25 ml of 2N HCl, and

extraction was accomplished with 5 ml toluene by vigorous shaking,

then centrifuging to separate the organic and aqueous phases. Four

ml of the organic phase were pipetted into 10 ml of counting solution

and counted. Catalase was used only in the assay of partially

purified diamine oxidase and purified diamine oxidase. Boiled enzyme

preparations were used for control assays to determine the amount

of radioactive substrate that was extractable in this method.

One unit of enzyme activity is defined as that amount of

enzyme required to oxidize one μmole of tryptamine per minute at 25°

with tryptamine concentration at 1×10^{-4}M. Specific activity of the

partially purified diamine oxidase is defined as units per mg protein.

For the enzyme assay with putrescine (1, 4-diaminobutane) a

modified procedure of the method of Okuyama and Kobayashi (1961) was

used. Putrescine-1, 4-^{14}C with unlabeled putrescine to give desired

substrate concentration was incubated with 100 μl of enzyme pre-

paration, 20 μg of catalase to give the final reaction volume of 400 μl,

33 mM borate-phosphate pH 8 or 67 mM phosphate buffer pH 7. The

reaction was allowed to proceed for the desire incubation times, then

terminated by the addition of 100 mg of powdered sodium bicarbonate.

Immediate extraction of the product, Δ^1-pyrroline, was performed

with toluene and counting was done as in the assay with tryptamine.

Boiled enzyme preparations were used to determine the amount of

radioactivity extractable by this procedure which was not due to en-

zyme activity.

Enzyme Purification

The summary of the procedure for a typical purification of pea

seedling diamine oxidase is given in Table 1. All operations, unless

otherwise indicated, were carried out at 6-7°, and all centrifugations

were at 12,100 x g for 20 minutes.

Step 1, Extraction: Etiolated pea seedlings (Pisum sativum,

var. Alaska), 508 g, were harvested after 8-10 days growth in ger-

mination towels at 20°. The seedlings, exclusive of the roots and

cotyledons, were homogenized in a Waring Blendor for several minutes

with 0.01 M potassium phosphate buffer, pH7 (1 ml of buffer per 1 g

of fresh tissue). The homogenate was strained through several layers

of cheesecloth to obtain a preparation which was centrifuged. The

light-brown supernatant fluid had a volume of 850 ml and a protein

concentration of 9.2 mg/ml.

Step 2, Ammonium sulfate fractionation: Solid ammonium

sulfate (114 g/l) was added slowly to the supernatant with constant

stirring. Concentrated ammonia was added to readjust the pH to 7.

After stirring for an additional 30 minutes, the precipitate was dis-

carded after separation by centrifugation and decantation of the super-

natant. Additional solid ammonium sulfate (450 g/l) was added to

this supernatant liquid and a white, flocculent precipitate was

obtained. After adjusting the pH to 7 with concentrated ammonia and

allowing the preparation to stand overnight at 4°, the mixture was

centrifuged. The precipitate was suspended in 100 ml of 0.01 M

phosphate buffer, pH 7 and either frozen at -10° for storage or im-

mediately used in the following step after overnight dialysis against

one liter of 0.01 M phosphate buffer, pH 7 with several changes of

buffer.

Step 3, DEAE Sephadex A-50 column chromatography: The

dialyzed solution (130 ml) was placed on a column (4 x 42.5 cm) of

DEAE Sephadex A-50 which had been equilibrated with 0.01 M phos-

phate buffer, pH 7. After the enzyme solution was adsorbed, a

yellow band about 10 cm in length was observed at the top of the

column. Elution of the enzyme was accomplished with the equilibra-

ting buffer and the enzyme activity was obtained in the initial effluent,

corresponding to 63% of the column volume. The active fractions

were combined. The resulting solution, having about 0.1 mg/ml

of protein was stored at 4° and was found to retain its activity for

several months. The preparation at this stage of purity is designated

as partially purified diamine oxidase.

TABLE I. Partial Purification of Pea Seedling Diamine Oxidase

Step	Volume ml	Protein g	Units	Specific activity units/mg protein $\times 10^4$
1. Homogenate supernatant	850	7.80	2.19	2.80
2. $(NH_4)_2SO_4$ precipitate	130	1.57	0.924	5.87
3. DEAE Sephadex column chromatography	225	0.037	0.522	141

Characterization of Tryptamine Oxidation Product

In order to characterize the tryptamine oxidation product,

partially purified diamine oxidase, 60.7 μg, tryptamine 2-^{14}C,

71.4 mμmoles containing 1.62 x 10^6 dpm, and catalase 140 μg were

reacted for 40 minutes at 25° in a final reaction volume of 2.8 ml

which was 33 mM borate-phosphate pH 8.0. At the end of the in-

cubation period, 0.4 ml aliquots were pipetted within one minute

into assay tubes, containing either 0.25 ml of 2N HCl or 5 mg sodium

borohydride.

After 30 minutes, 0.25 ml of 2N HCl were added to those tubes

containing NaBH$_4$ in order to decompose the excess NaBH$_4$. Addition

of 50 mg of crystalline tryptophol (recrystallized twice from toluene

using petroleum ether b.p. 60° - 80° fraction) was followed by ex-

traction with 5 ml of toluene by shaking, then centrifuging the reaction

tubes to separate the organic and aqueous layers. One ml of the

toluene layer and an additional 3 ml of toluene were pipetted into

10 ml of counting solution and counted in a liquid scintillation spectro-

meter. The remaining organic layer was transferred to another conical

centrifuge tube and petroleum ether b.p. 60° - 80° fraction was added

until a white crystalline precipitate formed. These crystals were

filtered off with a sintered glass funnel with suction and rinsed with

additional petroleum ether. After drying, a weighed portion of the

crystals was dissolved in 10 ml of counting solution with added 4 ml

of toluene and counted. The remaining crystals were re-dissolved in

toluene and after the second and third recrystallizations, weighed

samples were counted in the same manner as before.

To determine the amount of toluene-extractable radioactivity

from the acidified reaction mixture, 0.4 ml of the original reaction

mixture was pipetted into 0.25 ml of 2N HCl and extracted with 5 ml

of toluene in the presence and absence of non-radioactive indole-

acetaldehyde. The unlabeled indoleacetaldehyde was generated from

20 mg of recrystallized indoleacetaldehyde $NaHSO_3$, according to

the procedures of Larsen and Klungsöyr (1964) and added as a toluene

solution to the acidified reaction mixture. After extraction of the

reaction mixture, a 1 ml aliquot of the toluene phase was counted in

the same manner as the toluene extract from the sodium borohydride

reduction procedure. Boiled enzyme solution was used for controls

to determine the amount of radioactivity extractable with toluene due

to non-enzymic reactions.

RESULTS

Km Determinations

Purified diamine oxidase was diluted 5×10^4 fold with 33 mM

borate-phosphate buffer pH 8.0 before assaying with putrescine-1,

$4-^{14}C$ in order that the measured enzymatic activity would reflect

a meaningful dependence upon substrate concentration. Figure 1

shows the Lineweaver-Burk reciprocal plot giving a value of 7.4 x

10^{-5} M for the Michaelis constant for putrescine at pH 8.0. Utilizing

a 10^3 fold dilution of an ammonium sulfate precipitate from step 2

in the purification scheme, enzyme activity was measured with

varying putrescine concentration in a final reaction volume of 400 μl,

67 mM phosphate buffer pH 7.0. Figure 2 shows that the Km value

for putrescine can be determined at pH 7.0 as 7.2×10^{-4} M.

Using tryptamine as substrate, purified diamine oxidase was

diluted 5×10^2 fold before assaying in the presence of 20 μg catalase.

A reciprocal plot (Figure 4) gave a value of 4×10^{-4} M for the Michaelis

constant at pH 8.0. Partially purified pea seedling diamine oxidase

obtained from step 3 of the purification procedure was also assayed

with tryptamine and from a reciprocal plot (Figure 3) a Km value of 5.3 x

10^{-4} M was calculated.
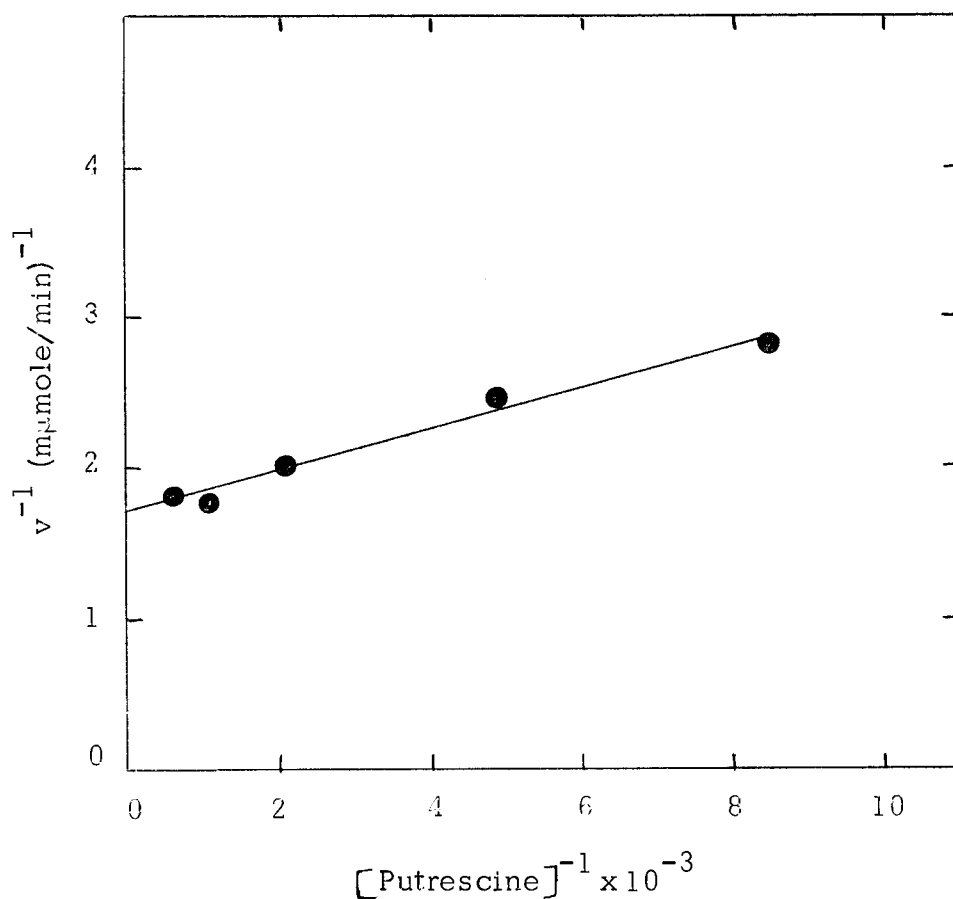
Figure 1. Lineweaver-Burk plot of the rate of putrescine oxi-
dation at pH 8.0 by purified diamine oxidase. Putrescine-1,4-^{14}C
250, 600 dpm with added unlabeled putrescine to give the desired sub-
strate concentrations were used to assay the activity of 0.04 μg of
purified diamine oxidase in the presence of 20 μg of catalase in a total
assay volume of 400 μl, 33 mM borate-phosphate pH8 buffer. The 15
minute incubation period at 25° was followed by extraction as described
under Methods for the putrescine assay.
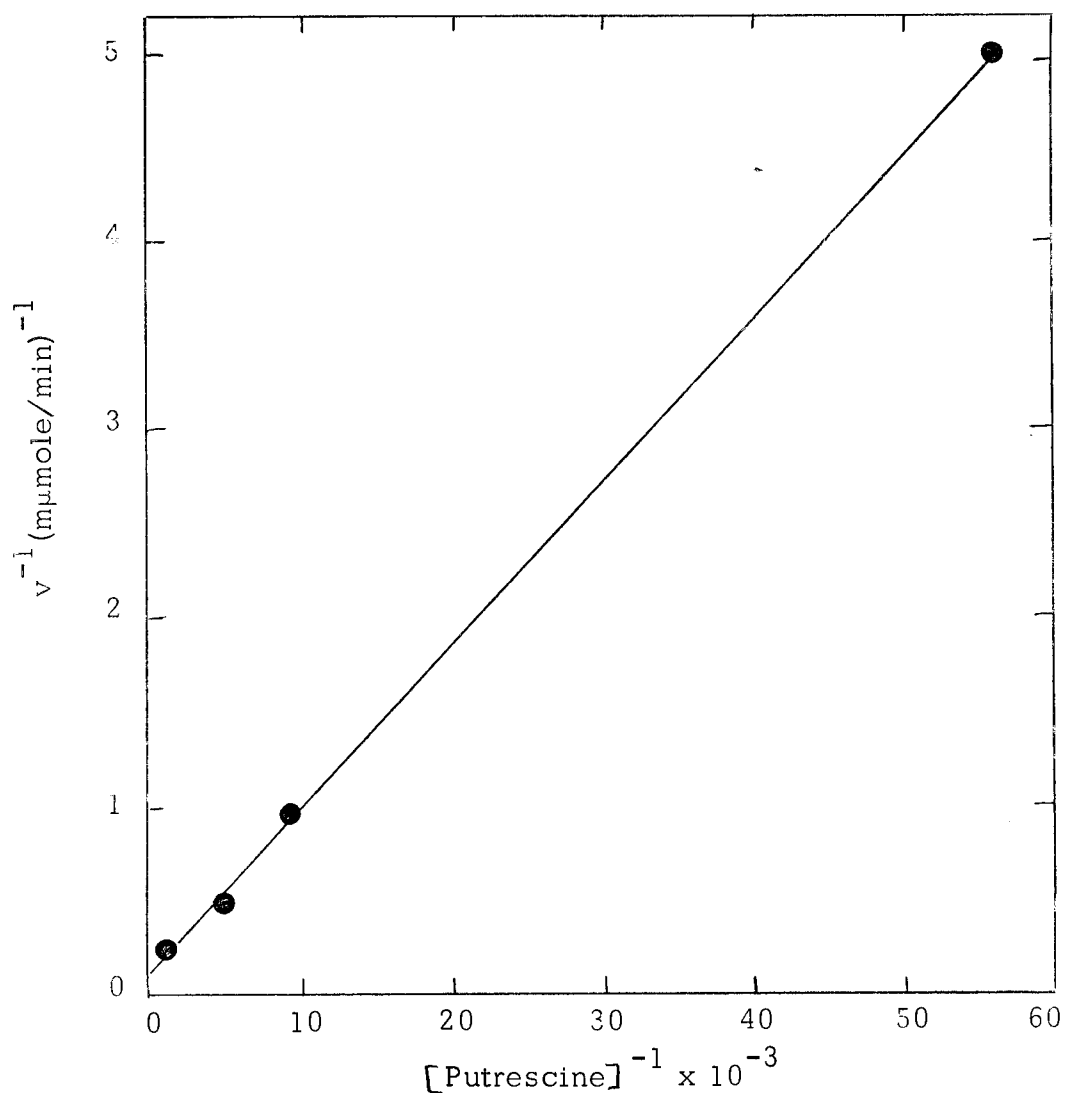
Figure 2. Lineweaver-Burk plot of the rate of putrescine oxidation at pH 7.0 by partially purified diamine oxidase. Putrescine-1, 4-^{14}C 145, 800 dpm with additional unlabeled putrescine to give the desired substrate concentrations were used to assay the activity of 7.8 µg of diamine oxidase (from step 2. of the purification procedure) in a total assay volume of 400 µl, 67 mM phosphate buffer, pH 7.0. The 0.5 minute incubation period at 25° was followed by extraction as described under Methods for the putrescine assay.
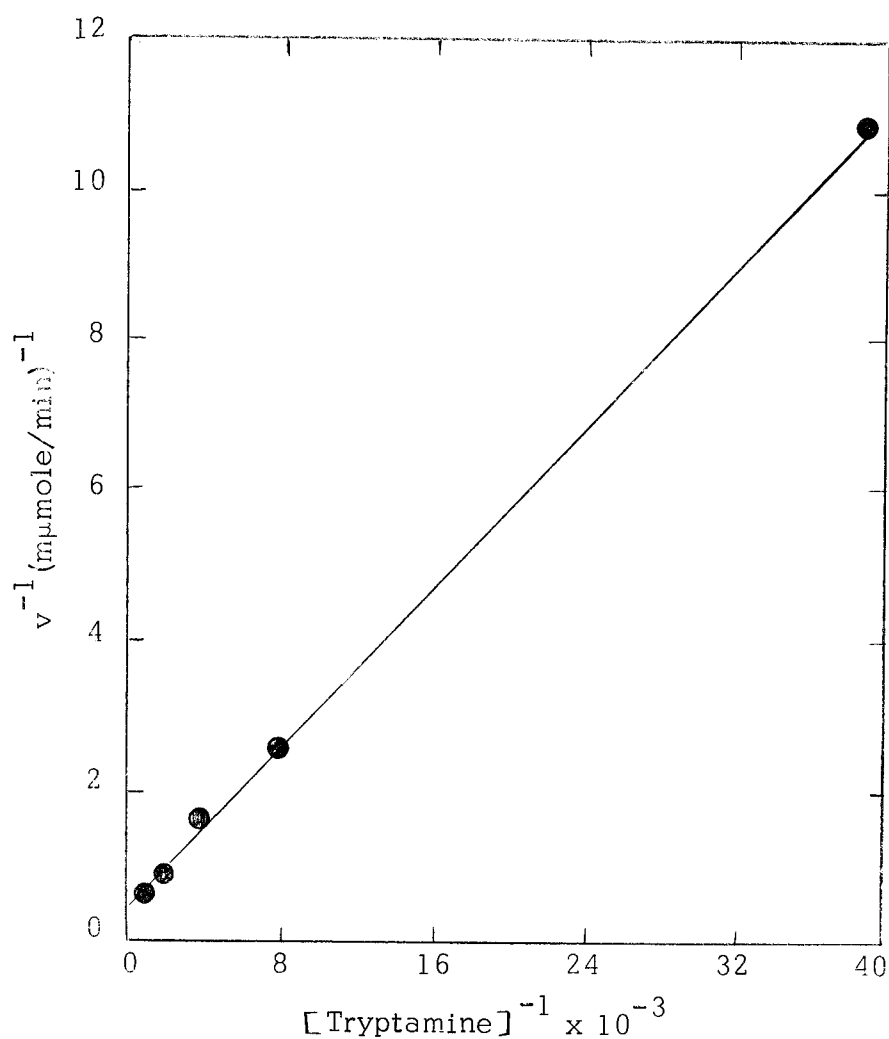
Figure 3. Lineweaver-Burk plot of the rate of tryptamine oxidation at pH 8.0 by partially purified diamine oxidase. Tryptamine 2-^{14}C 232, 800 dpm and unlabeled tryptamine to give the desired substrate concentrations were used to assay the activity of 4.3 μg of partially purified diamine oxidase (from step 3. in the purification procedure) in the presence of 20 μg of catalase in a total assay volume of 400 μl, 33 mM borate-phosphate pH 8.0 buffer. The 10 minute incubation period at 25°was followed by extraction as described under Methods for the tryptamine assay.

Figure 4. Lineweaver–Burk plot of the rate of tryptamine oxi-
dation at pH 8.0 by purified diamine oxidase. Tryptamine $2-^{14}C$ 216,700
dpm with unlabeled tryptamine to give the desired substrate concentra-
tions were used to assay the activity of 4.0 μg of purified diamine oxi-
dase in the presence of 20 μg of catalase in a total assay volume of
400 μl, 33 mM borate-phosphate pH 8.0 buffer. The 15 minute incuba-
tion period at 25° was followed by extraction as described under Methods
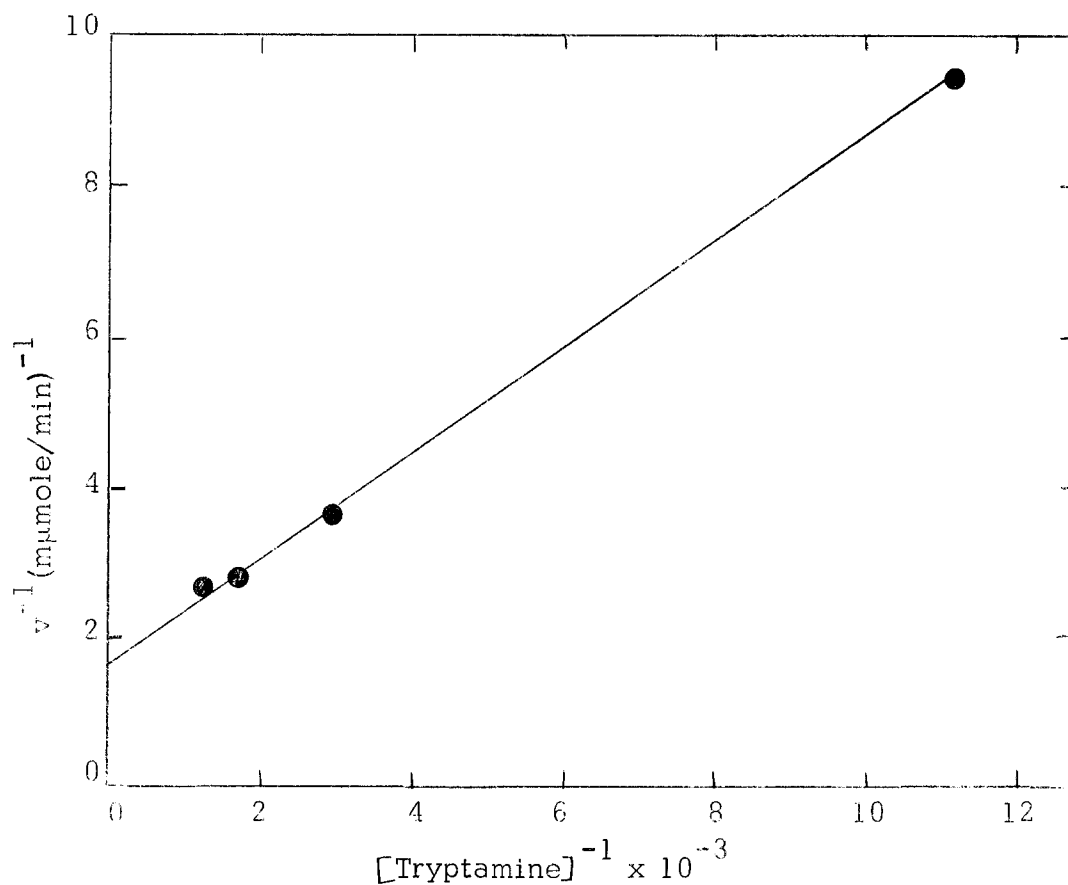for the tryptamine assay.

Effect of Enzyme Concentration and Time on Tryptamine Oxidation

Purified diamine oxidase, diluted 5×10^3 fold from stock solution, was used in varying concentrations to note the effect of enzyme concentration on product formation (Figure 5). With increasing concentration of enzyme there is a proportionate, linear increase in product formation.

The effect of incubation time on tryptamine conversion to product was measured with purified diamine oxidase. Figure 6 shows that the linear relationship extends to 15 minutes, after which time deviation from linearity is observed. It was found that the amount of product formed determined that substrate depletion probably affected the results for incubation periods beyond 15 minutes. Pea seedlings homogenate prepared from etiolated epicotyl tissue, 3 mg fresh tissue per ml of homogenate, was assayed at two tryptamine concentrations for varying incubation periods as shown in Figure 7. It is seen that at the higher substrate concentration of 8.4×10^{-4}M the initial linear relationship is not maintained, whereas, at the lower substrate con-centration, 9×10^{-5}M, linearity is observed to 60 minutes.

Effect of Enzyme Concentration and Time on Putrescine Oxidation

Purified diamine oxidase, diluted 5×10^4 fold from stock solution, was assayed at varying concentrations. Figure 8 displays a typical linear relationship between the Δ^1-pyrroline formed and the

Figure 5. Effect of enzyme concentration on the rate of trypta-
mine oxidation by purified diamine oxidase. Reaction mixtures contain
ing tryptamine 2-[14]C 216, 700 dpm, 20 μg catalase and purified diamine
oxidase varying from 0.2 to 0.8 μg were incubated for 15 minutes at
25° in a total assay volume of 400 μl, 33 mM borate-phosphate buffer
pH 8.0. Extraction of the radioactive product was as described under
Methods.
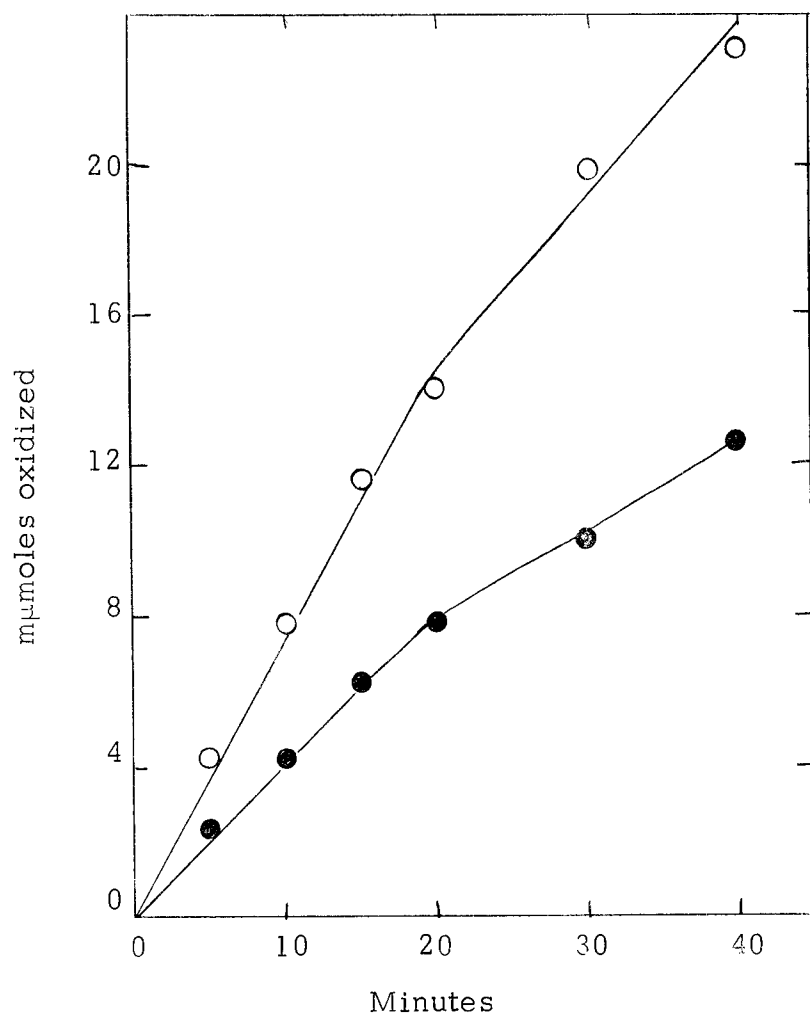
Figure 6. Effect of incubation time on the rate of tryptamine oxidation by purified diamine oxidase. Reaction mixtures containing tryptamine 2-^{14}C 216, 700 dpm, 20 μg catalase and purified diamine oxidase (●) 2μg and (o) 4μg were incubated for varying time periods at 25° in a total assay volume of 400 μl of 33 mM borate-phosphate pH 8.0 buffer, followed by extraction as described under Methods.
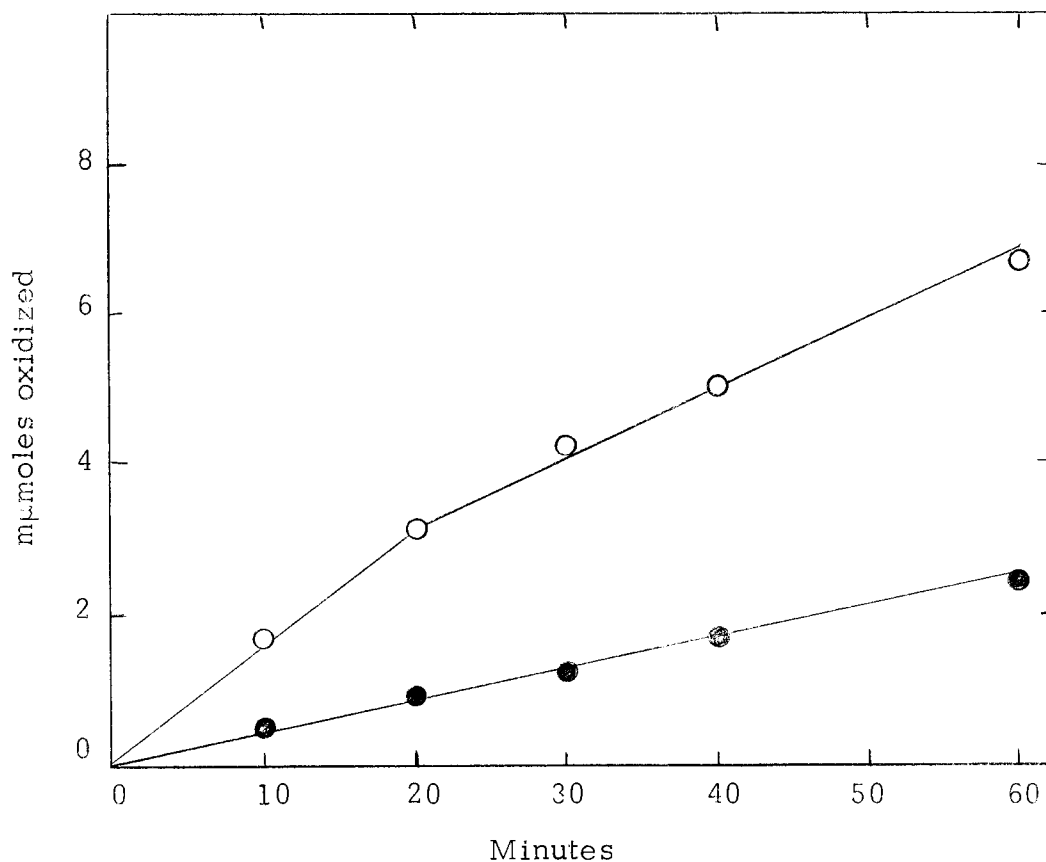
Figure 7. Effect of incubation time on the rate of tryptamine oxidation by pea seedling homogenate. Reaction mixtures containing 0.3 mg (based on fresh tissue weight) of 10 day pea seedling epicotyl homogenate were incubated with tryptamine $2\text{-}^{14}C$ 216, 700 dpm and (o) 8.4×10^{-4} M and (●) 9×10^{-5} M total substrate concentration for varying times, followed by extraction as described under Methods.
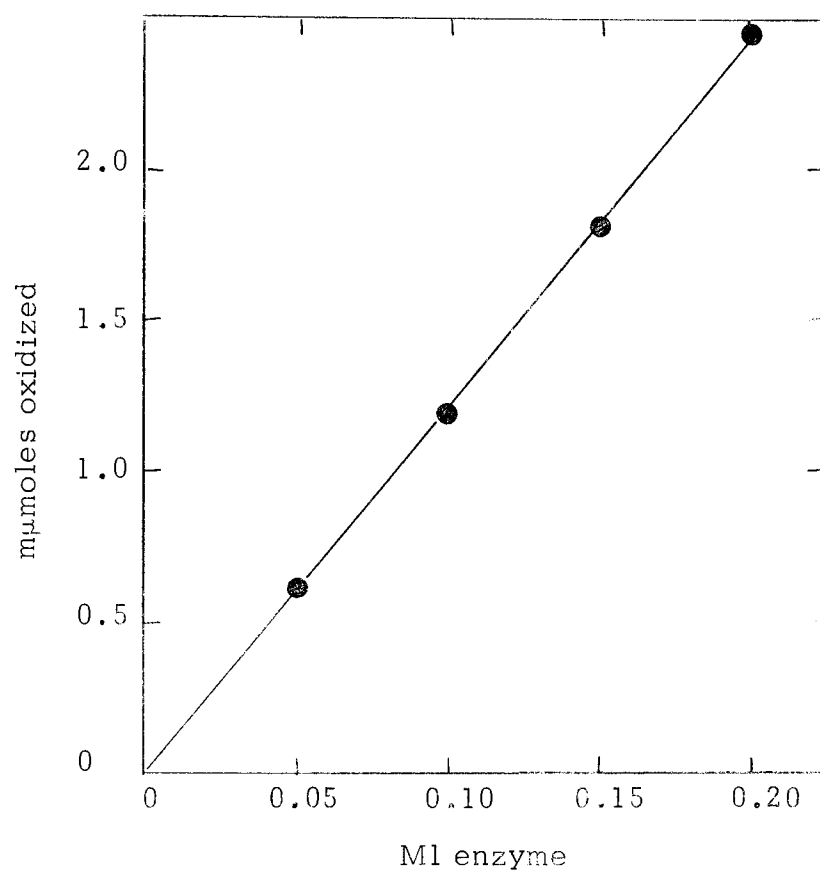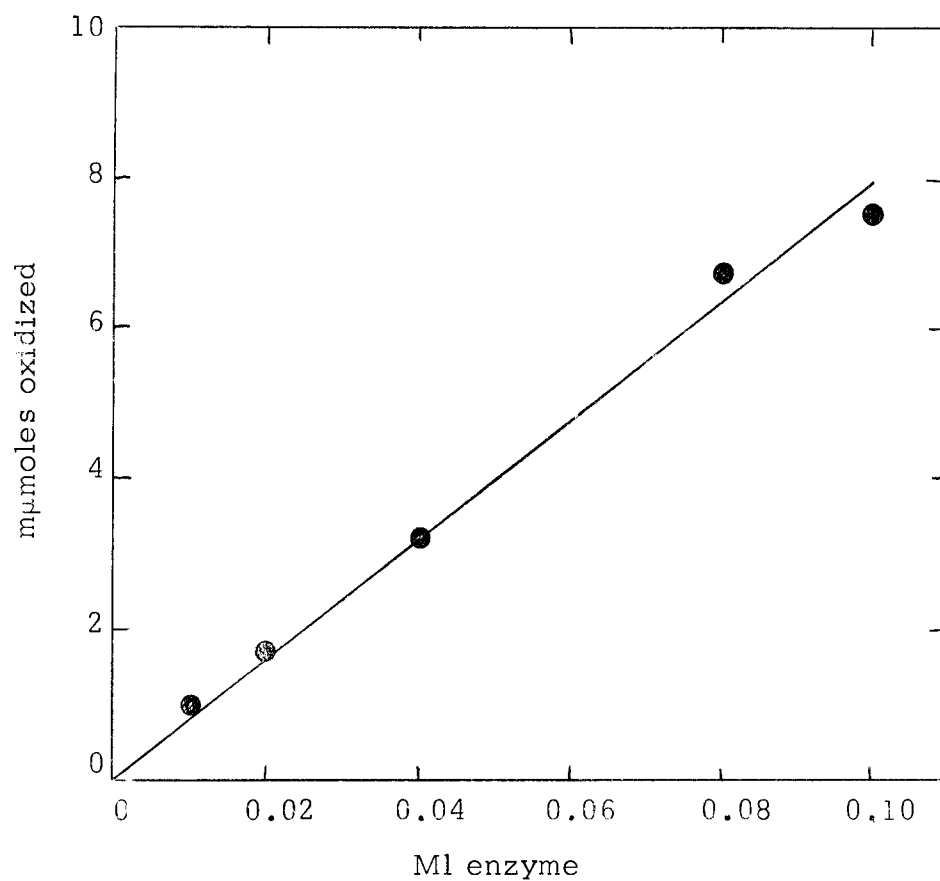
Figure 8. Effect of enzyme concentration on the rate of putres-
cine oxidation by purified diamine oxidase. Reaction mixtures contain-
ing putrescine-1,4-^{14}C, 2.06 x 10^{-4} M containing 250, 600 dpm, 20μg
catalase, and purified diamine oxidase varying from 0.004 μg to 0.04
μg in protein were assayed as described for figure 1.

amount of enzyme. In preliminary experiments with purified enzyme, lesser dilutions of the enzyme resulted in non-linear relationship between product formation and enzyme concentration; these results were due to the very great rate of putrescine oxidation and subsequent depletion of substrate.

Pea seedling homogenate also displayed linearity of product formation with increasing enzyme concentration with putrescine as substrate. These results are shown in Figure 9.

The effect of time on putrescine oxidation by purified diamine oxidase, diluted 5×10^4 fold, is shown in Figure 10. There was found a constant amount of radioactivity extractable, which was not due to non-enzymatic action, which appeared within 10 seconds of the reaction time. This radioactivity displaces the linear curve such that it does not pass through the origin. It was surmised that product inhibition may have caused this phenomenon; therefore, an experiment was performed by incubating the enzyme with unlabeled putrescine $(1.8 \times 10^{-4} M)$ for 15 minutes before assay with putrescine-1, $4-^{14}C$ for varying time periods. Figure 11 shows that the linear relationship is maintained in an additional 10 minutes of incubation time with labeled substrate, but the curve does not pass through the origin. Thus, it was concluded that an impurity in the radioactive substrate was present which was rapidly oxidized to a toluene-extractable
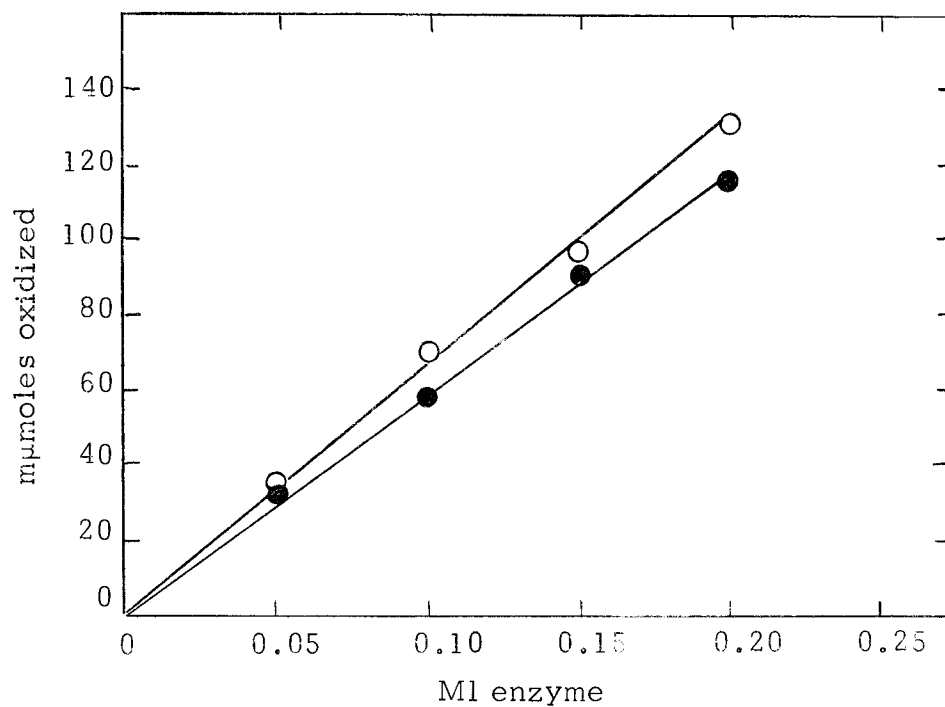
Figure 9. Effect of enzyme concentration on the rate of putrescine oxidation by pea seedling homogenate. Reaction mixtures containing ?.15 to 0.6 mg (based on fresh tissue weight) of 10 day pea seedling epicotyl homogenate and putrescine-1,4-^{14}C 199, 800 dpm at (o) 3.9 x 10^{-4} M and (•) 4.6 x 10^{-4} M total substrate concentration were assayed as described for figure 1.
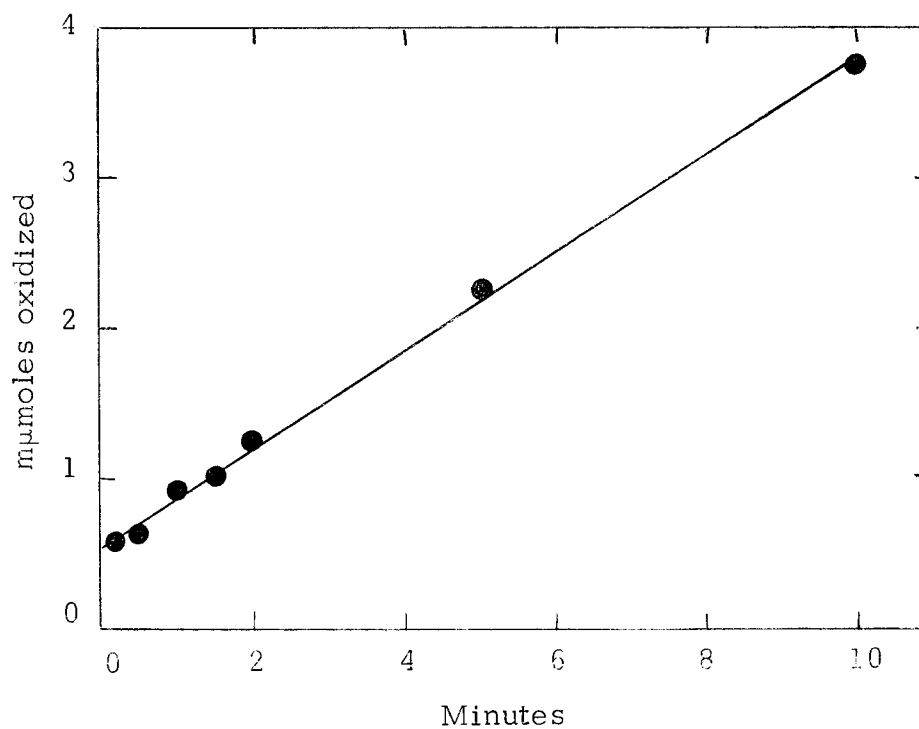
Figure 10. Effect of incubation time on the rate of putrescine
oxidation by purified diamine oxidase. Assay of 0.04 µg of purified
diamine oxidase was carried out with putrescine-1,4-^{14}C 250, 600 dpm
and 2.06 x 10^{-4} M in the presence of 20 µg of catalase as described
for figure 1. with the exception of varying incubation periods.
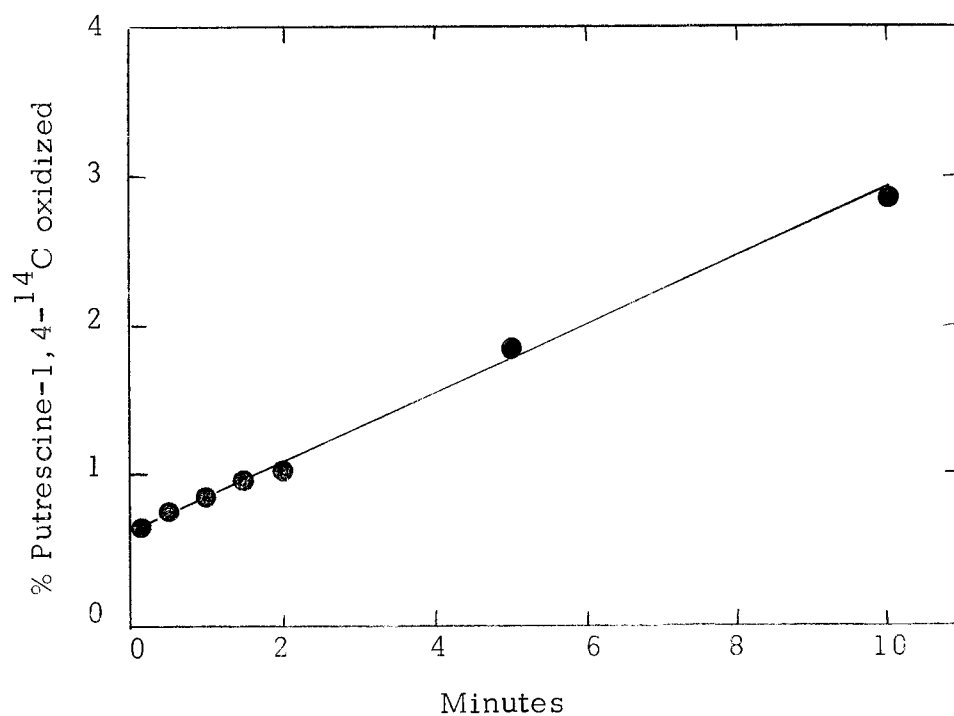
Figure 11. Effect of incubation time and product formation on the rate of putrescine oxidation by purified diamine oxidase. Putrescine -1,4-^{14}C 250, 600 dpm was added to 0.04 μg of purified diamine oxidase and 70 mμmoles of unlabeled putrescine and 20 μg of catalase 15 minutes after the beginning of incubation at 25°. The reaction was terminated at varying time periods after the addition of labeled putrescine, followed by extraction as described under Methods.

product and that the phenomenon was not due to occurrence of product

inhibition.

Effect of Time on Cadaverine Oxidation

Purified diamine oxidase was diluted sufficiently to be used

in assays with cadaverine-1, 5-^{14}C which were performed in the

same manner as the assay for putrescine oxidation as described in

Methods. Figure 12 shows that with increasing incubation time

there is obtained a rapid increase in extractable radioactivity in

the initial period of the assay; these results were quite similar to

those obtained with the oxidation of putrescine-1, 4-^{14}C.

Characterization of Tryptamine Oxidation Product

Sodium borohydride reduction yielded a total radioactivity

of 167, 800 dpm in the toluene extract (Table II) from the aliquot of

incubation mixture. The radioactivity extracted in the organic phase

from the control assay with the boiled enzyme preparation was 16, 900

dpm; therefore, the total radioactivity due to enzymatic activity was

150, 700 dpm. It is believed that the increased concentration of

borate ions in the reaction mixture formed from the hydrolysis of

$NaBH_4$ increased the total amount of tryptamine extractable over and

above that amount which was extracted in the control assays with

boiled enzyme preparations and tryptamine 2-^{14}C. Determination

of the specific activity of the crystals obtained gave the dpm/mg
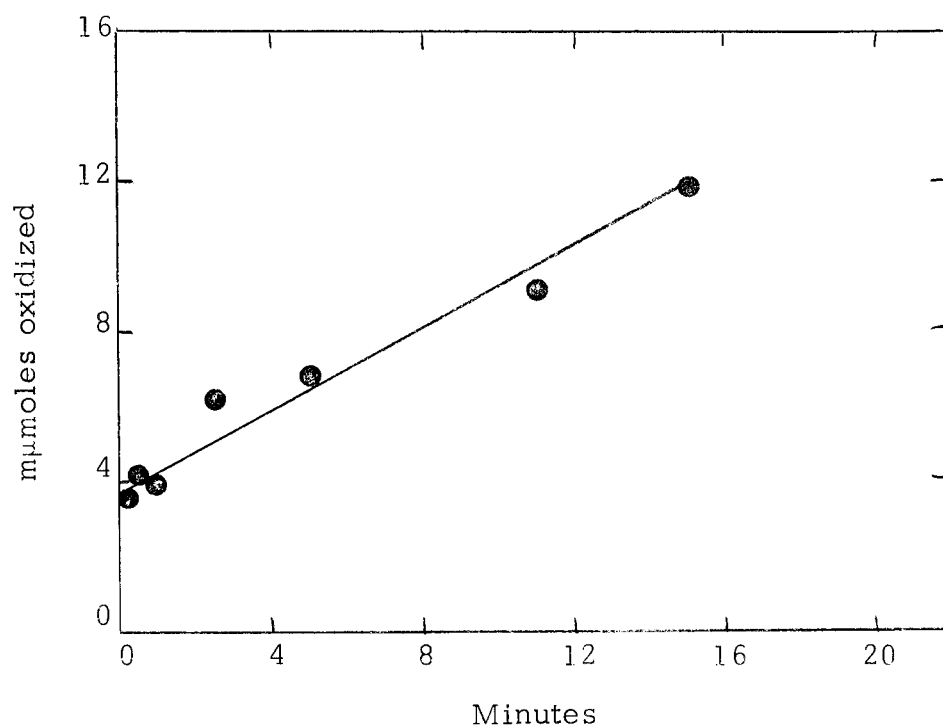
Figure 12. Effect of incubation time on cadaverine oxidation by purified diamine oxidase. Cadaverine-1,5-^{14}C 2.35 x 10^{-4}M containing 269, 300 dpm was used in assay of activity of 0.4 μg puri- fied diamine oxidase in the presence of 20 μg of catalase for varying time periods at 25° with total assay volume of 400 μl, 33 mM borate- phosphate pH 8.0 buffer. Extraction procedure followed that described for the putrescine assay under Methods.

values shown in Table II. The total radioactivity of the recrystallized product was calculated on the basis of 50 mg unlabeled tryptophol added after reduction of the reaction mixture and before extraction with toluene. The average value for total radioactivity of the recrystallized tryptophol was 155,400 dpm.

The values for toluene-extractable radioactivity from acidified assay mixtures were 142,700 dpm for the extractions in the absence of carrier indoleacetaldehyde and 150,100 dpm in the presence of carrier. Thus, the addition of unlabeled indoleacetaldehyde increased the extraction efficiency of the ^{14}C-labeled product by five percent.

The amount of radioactivity repeatedly recrystallized with tryptophol corresponded very well with the total radioactivity in the toluene phase prior to crystallization and also to the amount of radioactivity extractable in toluene in the presence of carrier indoleacetaldehyde.

Oxygen Requirement for Tryptamine Conversion to Product

Since dilute partially purified diamine oxidase preparations were found to be easily inactivated by frothing of buffered solutions, it was decided to saturate both the enzyme and assay media with nitrogen or air for 30 minutes using a gas flow rate such that a fine stream of bubbles was obtained without concomitant frothing of solutions. Table III shows that in the presence of air, the partially

TABLE II. Characterization of Tryptamine Oxidation Product

Toluene extract of reaction mixture after the following	Extracted Radioactivity	
	Total DPM	Ave. DPM - Blank
1. No addition of carrier indoleacetaldehyde	142,600	
	146,900	142,700
2. Addition of carrier indoleacetaldehyde	150,300	
	154,100	150,100
3. Incubation of boiled enzyme	2,060	
4. NaBH$_4$ reduction of reaction mixture and addition of 50 mg tryptophol	167,800	150,900
5. NaBH$_4$ reduction of reaction mixture containing boiled enzyme	16,900	

Recyrstallizations of #4 extract		Specific activity $\dfrac{DPM}{mg}$
First	157,600	3,140
Second	154,300	3,080
Third	154,300	3,080

purified diamine oxidase had eight times the activity as that obtained

in the presence of nitrogen as the gas phase. Undoubtedly, the

reaction under the nitrogen atmosphere was not entirely diminished

because of traces of oxygen.

TABLE III. Oxygen Requirement for Diamine Oxidase Activity

Gas atmosphere	Product formed (mμmoles)
Nitrogen	0.48
	0.36
Air	3.53
	3.15

Partially purified diamine oxidase 8.6 μg was incubated for 10 minutes at 23° with tryptamine 2-^{14}C, 232,000 dpm and 50.2 mμmoles, in a total reaction volume of 400 μl, 33 mM borate-phosphate buffer pH 8. To remove traces of oxygen from the source. nitrogen gas was first bubbled through alkaline pyrogallol (Umbreit, Burris, and Stauffer, 1964). Compressed air, filtered through cotton, was used as the air gas phase. With continued gas flow through the substrate in serum-capped conical centrifuge tubes, an aliquot of the enzyme preparation was added by hypodermic needle and syringe to initiate reaction.

Effect of pH on Tryptamine Oxidation by Partially Purified Diamine
Oxidase

To test for pH stability, partially purified diamine oxidase
was diluted 10 fold with 0.01 M phosphate buffers of varying pH and
incubated for 20 minutes at 3°. Figure 13 shows that with subsequent
assay of these preparations at pH 8, the enzyme preparation exposed
to pH 7.9 was twice as active as that subjected to pH 5.8. The
general tendency was observed that the enzyme displayed greater
stability in the pH range near 7.9.

To determine the effect of pH on enzymatic activity, 10 fold
diluted enzyme preparations, using 33 mM borate-phosphate buffers
of varying pH, were assayed at the various pH values. Data in
Figure 14 indicates that there was very little enzymatic activity at
values below pH 7, but with increasing pH a concomitant increase
in product formation was observed. The greatest activity was found
at pH 9.

Although the greatest enzymatic activity of the partially
purified diamine oxidase with tryptamine was at pH 9, it was decided
to use pH 8 for the assay because of the greater stability of the
enzyme at that pH.
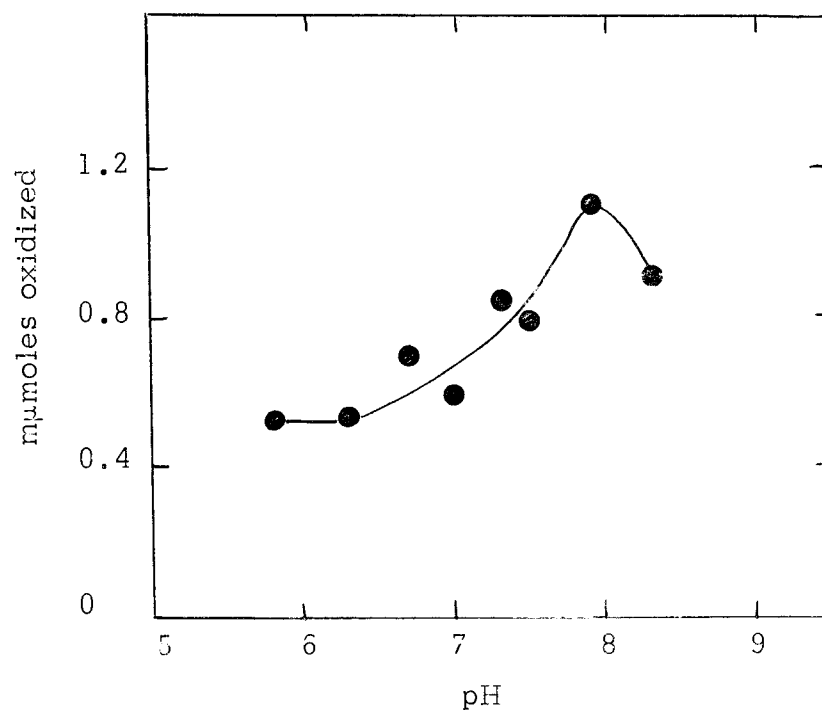
Figure 13. Effect of pH on enzyme stability. Each reaction mixture contained 20 μg of catalase, tryptamine 2-^{14}C 232, 000dpm and 1.26 x 10^{-4}M, and partially purified diamine oxidase 0.43 μg which had been exposed to varying pH for 20 minutes at 3°. The total assay volume was 400 μl, 33 mM borate-phosphate pH 8.0 buffer. After a reaction period of 10 minutes at 25°, extraction was carried out as de-scribed under Methods.
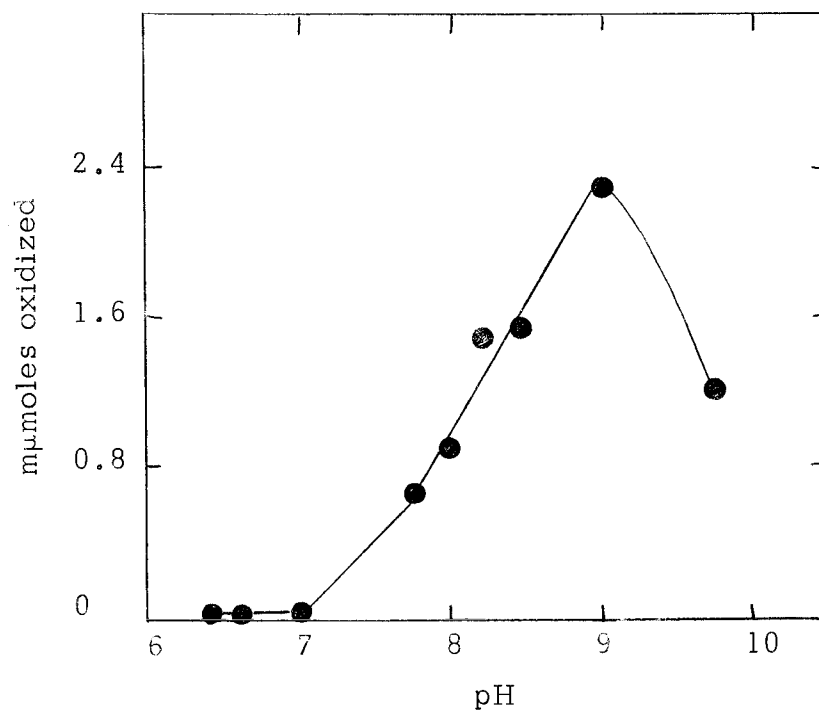
Figure 14. Effect of pH on the rate of tryptamine oxidation by partially purified diamine oxidase. Reaction mixtures contained 20 μg of catalase, tryptamine $2-^{14}C$ 232, 000 dpm and 1.26×10^{-4} M, and partially purified diamine oxidase 0.43 μg which was diluted with buffer of appropriate pH immediately before assay for 10 minutes at 25°. The total assay volume was 400 μl, 33 mM borate-phosphate buffers of varying pH. Extraction was carried out as described under Methods.

Effect of Temperature on Stability of Partially Purified Diamine Oxidase

Table IV lists the values of mμmoles of tryptamine oxidized by enzyme preparations which had been previously exposed to various temperatures. The exposure to 60° did not inactivate the enzyme, but exposures to 75° and 100° destroyed enzymatic activity. Partially purified diamine oxidase displayed little sensitivity to changes in temperature unless 60° was exceeded.

TABLE IV. Temperature Stability of Diamine Oxidase

Temperature	Product formed (mμmoles)
5	3.8
17	3.8
25	3.4
30	3.0
40	3.1
60	3.2
75	0.02
100	0

Partially purified diamine oxidase 4.3 μg was incubated for 10 minutes at the temperatures indicated, then assayed at 25° in a reaction mixture containing tryptamine 2-^{14}C, 50.2 mμmoles and 232,000 dpm, and 20 μg of catalase, after a prior equilibration for 20 minutes at 25°.

Effect of Catalase on Tryptamine Oxidation

Using varying concentrations of catalase, assays were
performed with purified diamine oxidase as outlined in Methods for
the assay with tryptamine as the substrate. Table V shows the
activity of purified diamine oxidase with varying concentrations of
added catalase. These results demonstrate a dependence of diamine
oxidase activity on the presence of catalase. The amount of catalase
selected to be added to assays of purified and partially purified
diamine oxidase was 20 μg since the partially purified preparation
was found to respond in like manner to the presence of catalase as
the purified diamine oxidase.

TABLE V. Effect of Catalase on Diamine Oxidase Activity

Catalase μg	Product formed (mμmoles)
0	5.2
2	9.4
10	12.8
15	13.8
40	13.8

Each reaction mixture consisted of 4 μg of purified diamine
oxidase, tryptamine 2-^{14}C, 40.8 mμmoles containing 195,800 dpm,
and catalase in a total volume of 400 μl, 33 mM borate-phosphate
buffer pH 8. Incubation was for 15 minutes at 25°.

Effect of Pyridoxal 5-phosphate and Cu (II) on Tryptamine Oxidation by Partially Purified Diamine Oxidase

The results in Table VI show that dialyzed diamine oxidase retained 92% of the non-dialyzed enzyme activity. Cu (II) was found to have an inhibitory effect upon enzyme activity. With the dialyzed preparations both 10^{-5} and 10^{-6} M Cu (II) caused a decrease in enzyme activity, atlhough the 10^{-6} M Cu (II) produced only a 20% inhibition. Addition of 10^{-4} M pyridoxal phosphate to the dialyzed preparation gave a 6% reduction in activity, whereas with the non-dialyzed enzyme, a 30% reduction in activity was found . At 10^{-5} M, the pyridoxal phosphate was found to have a lesser inhibitory effect than 10^{-4} M upon the enzyme activity. The effect of adding both 10^{-5} M Cu (II) and 10^{-5} M pyridoxal phosphate appeared to be one of inhibition produced by 10^{-5} M Cu (II).

Inhibition Studies of Diamine Oxidase with Hydrazine Compounds

Inhibition experiments were performed with partially purified and purified diamine oxidase preparations.

Titration of Partially Purified Diamine Oxidase

It is shown in Table VII that enzyme activity in the presence of increasing concentrations of hydrazines decreased sharply. BCH displayed the greatest ability to inhibit diamine oxidase activity and UDMH appeared to have a lesser ability to inhibit than BCH or hydrazine.

Reaction Mixture	Addition Concentration (M)	Product Formed (mμmoles)
Enzyme	—	4.3
" + pyridoxal phosphate	10^{-4}	3.0
" + pyridoxal phosphate	10^{-5}	4.1
" + Cu (II)	10^{-4}	0.5
" + Cu (II)	10^{-5}	1.0
" + pyridoxal phosphate + Cu(II)	10^{-5} 10^{-5}	0.9
Dialyzed Enzyme	—	4.0
" + pyridoxal phosphate	10^{-4}	3.7
" + pyridoxal phosphate	10^{-5}	3.9
" + Cu (II)	10^{-5}	0.8
" + Cu (II)	10^{-6}	3.2

Assays were performed in the presence and absence of 10^{-4}M, 10^{-5}M pyridoxal phosphate or Cu (II) ions. All additions were made to enzyme which was added last to initiate the reaction. Dialysis was performed for 17 hours against 33 mM borate-phosphate buffer pH 8. Partially purified diamine oxidase 4.3 μg was incubated for 10 minutes at 25° with tryptamine 2-^{14}C (232,800 dpm and 50.3 mμmoles), with the additions noted, in a final reaction volume of 400 μl, 33 mM borate-phosphate buffer pH 8.

TABLE VII. Zero Time Inhibition of Diamine Oxidase by Hydrazines

Inhibitor	Inhibitor Concentration (M)	% Inhibition
BOH	1×10^{-5}	98
	1×10^{-6}	90
	1×10^{-7}	43
	3.3×10^{-8}	38
	1×10^{-8}	10
	3.3×10^{-9}	13
UDMH	1×10^{-5}	80
	1×10^{-6}	48
	1×10^{-7}	8
	3.3×10^{-8}	23
	1×10^{-8}	24
	3.3×10^{-9}	23
Hydrazine	1×10^{-5}	98
	1×10^{-6}	74
	1×10^{-7}	18
	3.3×10^{-8}	6
	1×10^{-8}	4
	3.3×10^{-9}	14

Inhibitor solutions were prepared such that a 100 µl aliquot incubated in a final assay volume of 400 µl would yield the given concentrations. Partially purified diamine oxidase, 4.3 µg, was added to a mixture of tryptamine $2-^{14}C$, $1.26 \times 10^{-4}M$ and 232,800 dpm, 20 µg of catalase and 100 µl of the appropriate inhibitor concentration and assayed for 10 minutes at 25°. Control assays were performed with the reaction mixtures as indicated with the exculsion of inhibitor.

Titration of Purified Diamine Oxidase

Studies of inhibition of purified diamine oxidase demonstrated that with prior incubation of enzyme with BOH the enzyme was effectively titrated to 94% inhibition when the ratio of moles BOH to moles of enzyme (based on protein concentration and the molecular weight of 96,000 according to Hill and Mann, 1964) was 0.95 (Table VIII). From a plot of inhibition vs negative log molar concentration (Figure 15) notable differences were seen between the effects of BOH and UDMH. It appears that the curve for UDMH, obtained by a 15 minute prior incubation of UDMH with enzyme before assay of activity, is sigmoid, but that for BOH only a portion of a curve is displayed which possibly would appear sigmoid if the inhibition of enzyme activity were examined at lower inhibitor concentrations. It is apparent that lower concentrations of BOH than UDMH were required to attain 50% inhibition of purified enzyme activity. It is noted that the BOH used in the experiments with the purified diamine oxidase was of 82.4% purity and that a disubstituted compound of the β-hydroxyethylhydrazine is present as an impurity. If this compound exerts as great an inhibitory effect as BOH, then the shape of the curve for BOH inhibition in Figure 15 might be altered somewhat.

Titrations performed with purified diamine oxidase and UDMH or hydrazine without prior incubation before assay of enzyme activity

TABLE VIII. Titration of Diamine Oxidase by Hydrazines

Inhibitor Concentration(M)	moles	Ratio of Inhibitor: Enzyme	BOH(0)	*Inhibitor and % Inhibition		
				UDMH (0)	UDMH(15)	Hydrazine(15)
1×10^{-5}	4×10^{-9}	95	–	94	99	98
1×10^{-6}	4×10^{-10}	9.5	100	46	86	79
3.3×10^{-7}	1.3×10^{-10}	3.1	99	8	59	–
1×10^{-7}	4×10^{-11}	0.95	94	0	19	15
3.3×10^{-8}	1.3×10^{-11}	0.31	66	0	0	9
1×10^{-8}	4×10^{-12}	0.10	34	0	0	2

* Numbers in parentheses denote the time in minutes the enzyme was preincubated with the inhibitor before assay with substrate.

Purified diamine oxidase, 4.2×10^{-11} moles calculated on the basis of nitrogen content and the molecular weight of 96,000, was either preincubated with the inhibitor for 15 minutes before assay or was added at zero time to inhibitor solutions contained in the assay mixtures as described in Methods for the assay with tryptamine. The assay with substrate was for 15 minutes at 25°. The percent inhibition values were calculated on the basis of control assays performed in the absence of inhibitor.
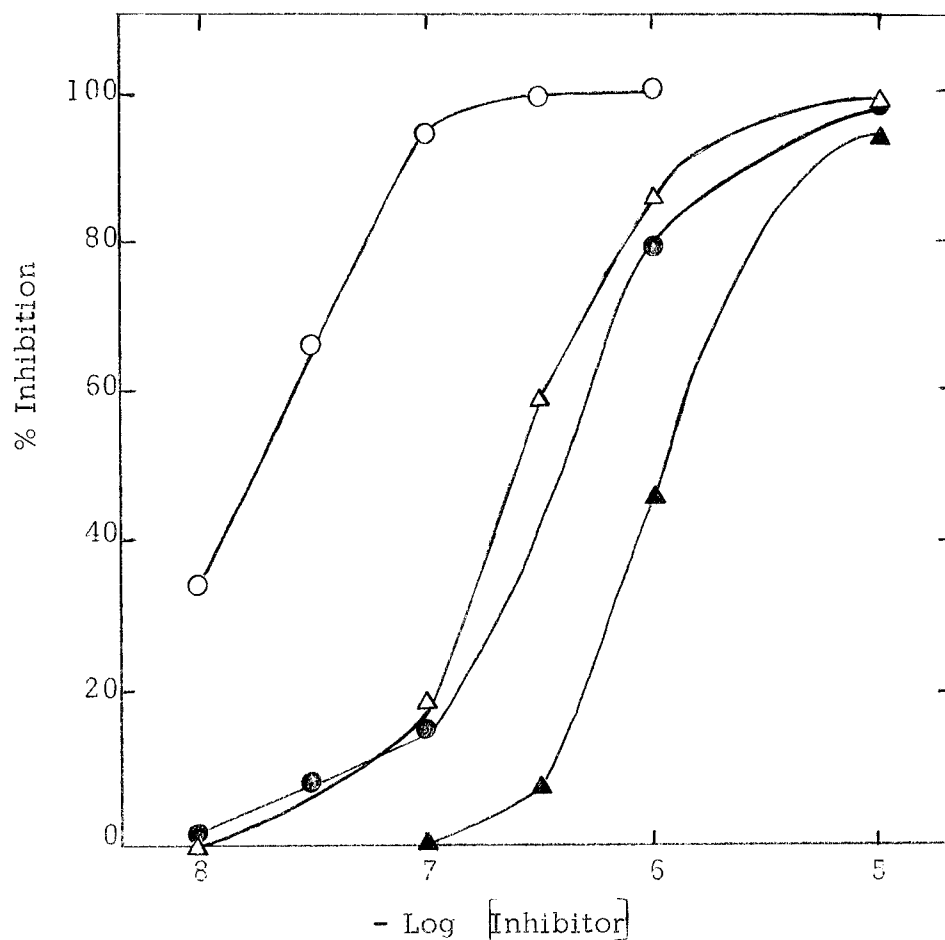
Figure 15. Effect of inhibitor concentration on the rate of tryptamine oxidation by purified diamine oxidase. (o) BOH or (Δ) UDMH was preincubated with 4 μg of purified diamine oxidase for 15 minutes before assay with tryptamine. (●) Hydrazine or (▲) UDMH was added to tryptamine before addition of 4 μg of purified diamine oxidase. Tryptamine $2-^{14}C$ 242, 300 dpm and $1 \times 10^{-4}M$ and 20 μg of catalase were used for each assay at 25° in a total reaction volume of 400 μl, 33 mM borate-phosphate pH 8.0 buffer. Extraction of the assay mixtures was performed as described under Methods.

are shown in Table VIII and Figure 15. Sigmoid curves are found for these two inhibitors. With the lower concentrations of UDMH there was noted a slight stimulation of enzymatic activity over control assays performed in the absence of inhibitor. Hydrazine when added with substrate to the enzyme shows a greater inhibitory effect than UDMH.

Dialysis of Inhibited Partially Purified Diamine Oxidase

Dialysis experiments were performed with partially purified diamine oxidase which has been incubated for 2 hours with solutions of hydrazines and assayed for remaining activity before being dialyzed. Several concentrations of the inhibitors were used. Because preliminary experiments indicated that maximum inhibition was obtained within 2 hours with the various inhibitors when incubated with partially purified diamine oxidase, this time period was employed. The results in Table IX for the six inhibited enzyme preparations show that there was no significant recovery of enzyme activity after 17-18 hours dialysis. If the inhibitor did not form a stable complex with the enzyme, dialysis would have restored a major portion of the activity, comparable to dialyzed control preparations.

TABLE IX. Effect of Dialysis on the Inhibition of Diamine Oxidase by Hydrazines

Inhibitor	Concentration (M)	% Inhibition Before Dialysis	% Inhibition After Dialysis
BOH	1×10^{-7}	99	96
	3.3×10^{-8}	83	78
UDMH	1×10^{-7}	96	97
	3.3×10^{-8}	67	68
Hydrazine	1×10^{-7}	91	88
	3.3×10^{-8}	40	26

Inhibitors were prepared such that a 100µl aliquot would yield the given concentrations in the assay mixture. Partially purified diamine oxidase 4.3 µg was used for each assay with tryptamine 2-^{14}C, 1.26 x 10^{-4}M and 232,000dpm, and 20 µg of catalase. Dialysis against 100 volumes of 33mM borate-phosphate pH 8 buffer with one change in buffer was at 6° for 17-18 hours. Controls were assayed in the absence of inhibitor and were dialyzed for the same period of time.

Time Course of Inhibition of Partially Purified Diamine Oxidase

The time course of inhibition of diamine oxidase by hydrazines was followed for each hydrazine concentration by incubating the inhibitor and enzyme for the desired time periods before assay of remaining activity. The time course of inhibition of partially purified diamine oxidase with hydrazine, UDMH, or BOH is plotted in Figure 16. The curves show that at 10^{-7} M final assay concentration the initial rates of inhibition were quite rapid, then decreased until an apparent constant amount of inhibition, which did not decrease during the 2 hour time period examined, was obtained for each inhibitor. Hydrazine and UDMH display rather similar curves. An apparent constant rate of inhibition for either hydrazine or UDMH occurs at a later time than for BOH. After 10 minutes of interaction of BOH and enzyme, there was found 90% inhibition, but at least 30 minutes of interaction of enzyme with either UDMH or hydrazine was required to achieve similar inhibition of enzymatic activity.

Using a graphical method for analysis of the order of the inhibition reaction, the best fit of the data was obtained for a second-order rate equation when the reactants were approximately of the same concentration. For reaction times up to 10 minutes, the plot of reciprocal percent remaining activity vs time gave the curves shown in Figures 17 a, b, c. Deviations from this type of second-order plot appear to be dependent upon the type of inhibitor.
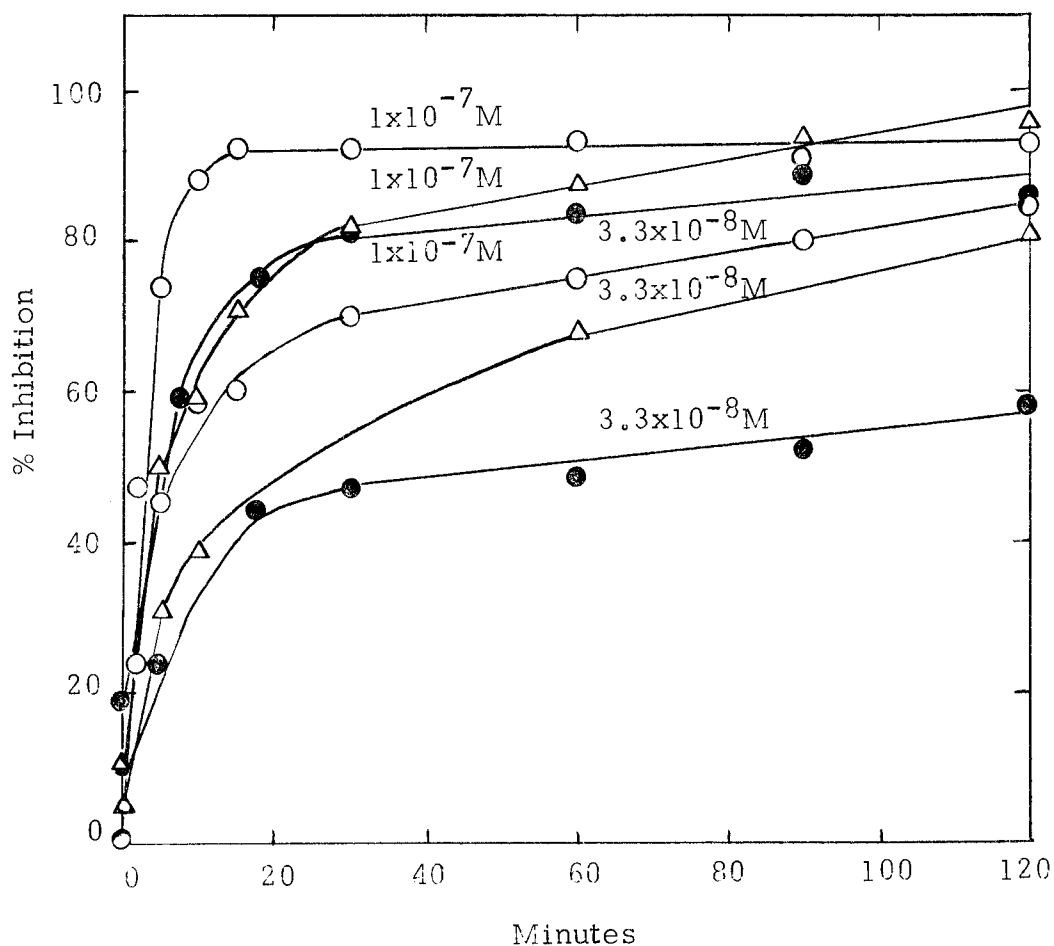
Figure 16. Percent inhibition of the rate of tryptamine oxidation
vs time. (o) BOH, (●)hydrazine, and (△) UDMH were preincubated
for varying time periods with 4.3 μg of partially purified diamine oxidase,
then assayed with tryptamine 2-^{14}C 232, 800 dpm and 1.28 x 10^{-4}M
in the presence of 20 μg of catalase as described for figure 3.

Figure 17. Determination of second-order rate constants for inhibition of partially purified diamine oxidase by various hydrazines. The data from figure 16 are recalculated for 1/% remaining activity and plotted against time.

Figure 17a. BOH at a final concentration of 1×10^{-7}M and 4.3 μg of partially purified diamine oxidase.
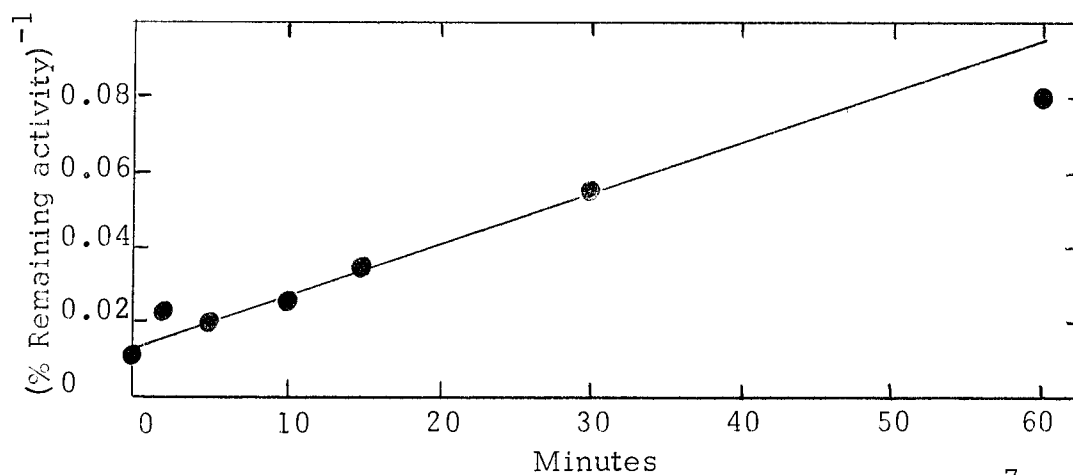
Figure 17b. UDMH at a final concentration of 1×10^{-7}M and 4.3 μg of partially purified diamine oxidase.
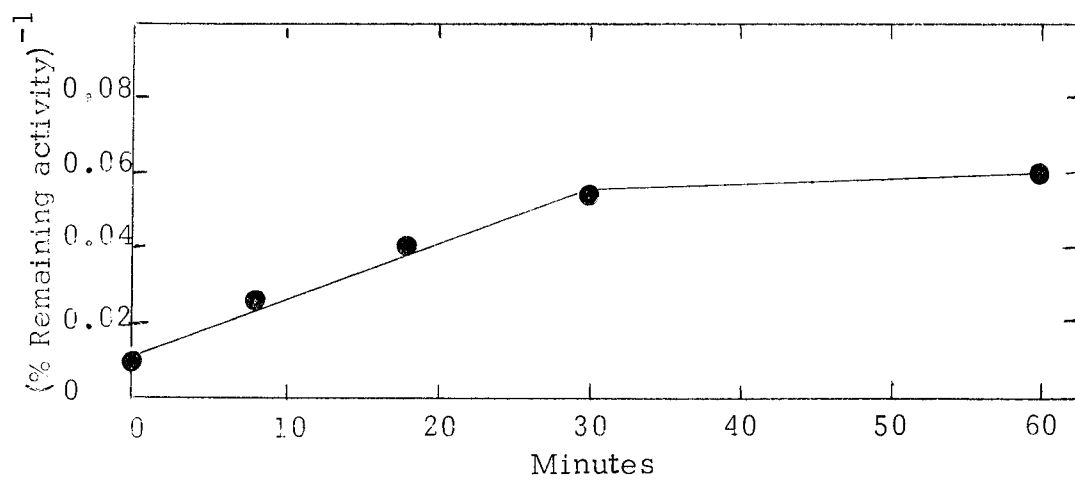
Figure 17c. Hydrazine at a final concentration of 1×10^{-7}M and 4.3 μg of partially purified diamine oxidase.

BOH at 10^{-7}M (Figure 17a) maintains linearity to 15 minutes,

and hydrazine at 10^{-7}M (Figure 17c) displays a linear response beyond

20 minutes, after which time a randomness of points is obtained.

UDMH at 10^{-7}M (Figure 17b) appears to maintain linearity for a

longer period of time than either BOH or hydrazine.

Time Course of Inhibition of Purified Diamine Oxidase

The time course of inhibition of purified diamine oxidase was

followed with UDMH at 3.3×10^{-7}M and 3.3×10^{-8}M and with BOH

at 10^{-7}M and 3.3×10^{-8}M. By plotting the data (Figure 18) such

that the percent inhibition is compared with the time of prior incu-

bation of enzyme with inhibitor (in the absence of substrate), the

rate of inhibition was found to be dependent upon the inhibitor concen-

tration and the time of incubation of inhibitor with enzyme. Using

the graphical method employed with the partially purified diamine

oxidase, the reciprocal of the percent remaining activity plotted vs

time (Figures 19 a, b,) shows a linear relationship for both UDMH

and BOH. These results imply that the initial reaction occurring

when inhibitor was mixed with enzyme was dependent upon both

enzyme and inhibitor concentrations. The results indicated that the

concentration of enzyme employed must be comparable to that of

inhibitor; from the calculated value of moles of purified enzyme used,

this idea is substantiated.
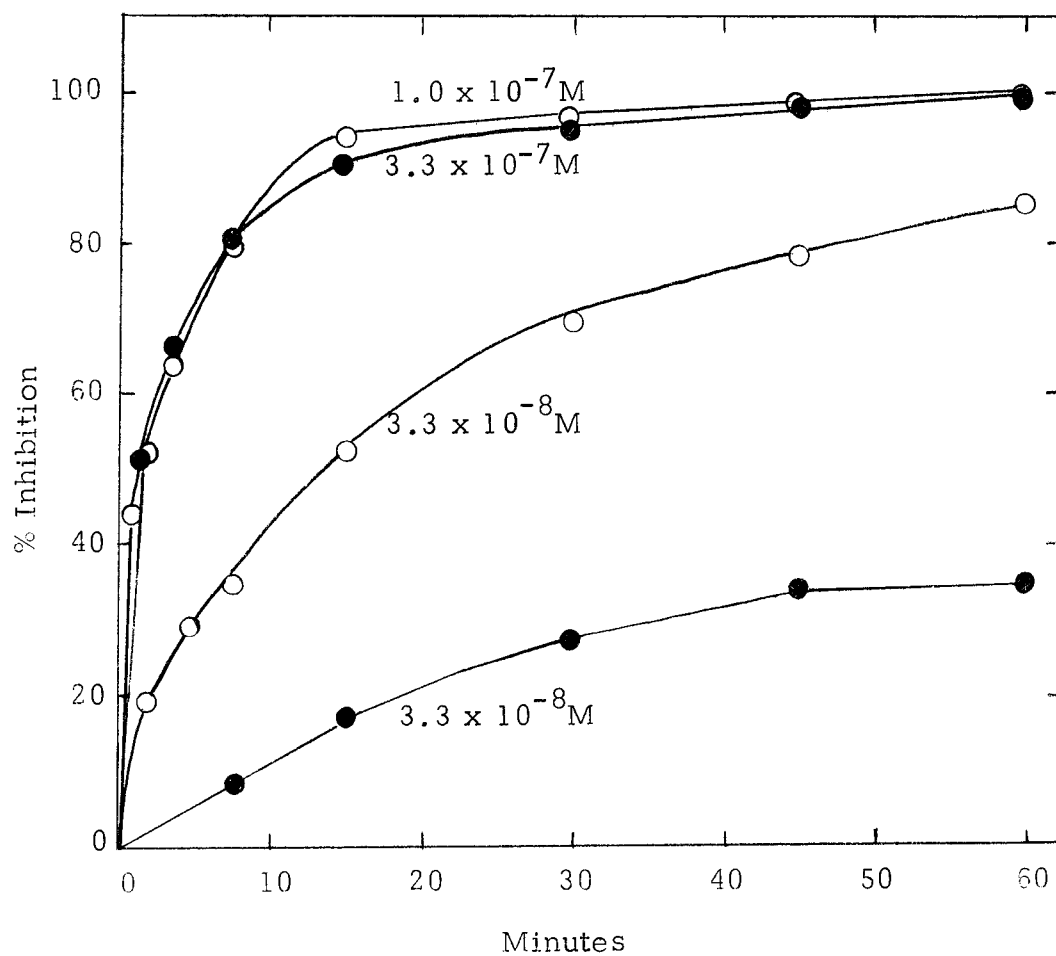
Figure 18. Percent inhibition of the rate of tryptamine oxidation by purified diamine oxidase vs time. (o) BOH and (●) UDMH were preincubated for varying time periods with 4 μg of purified diamine oxidase, then assayed with tryptamine 2-[14]C 216, 700 dpm for 15 minutes at 25° in the presence of 20 μg of catalase as described in figure 16.
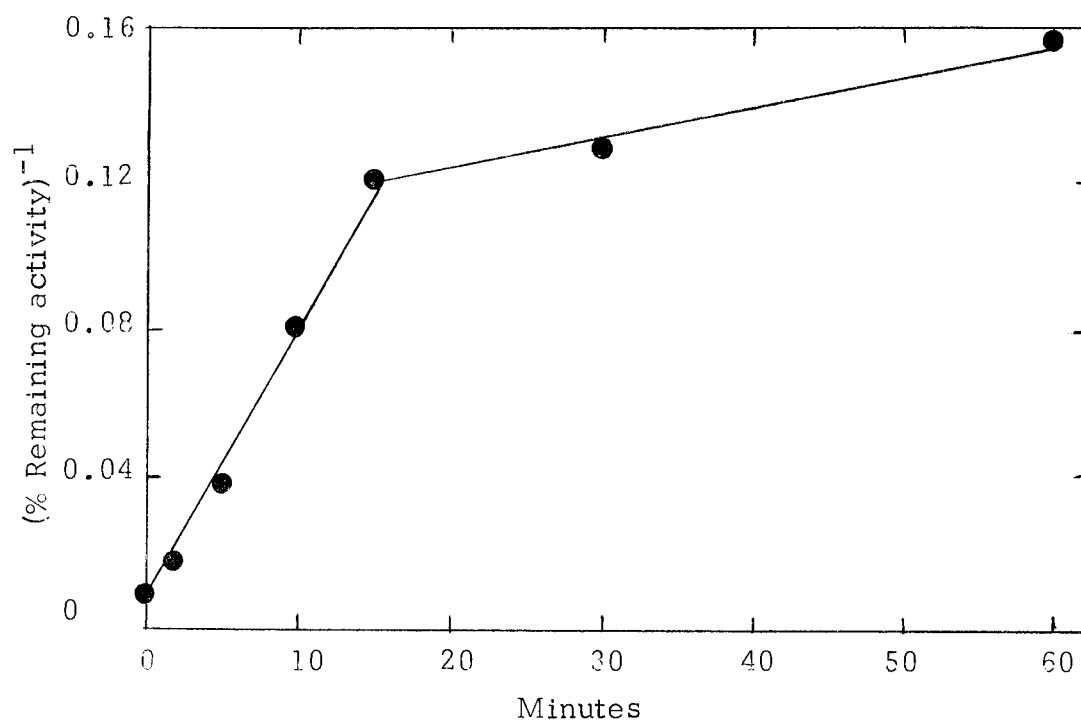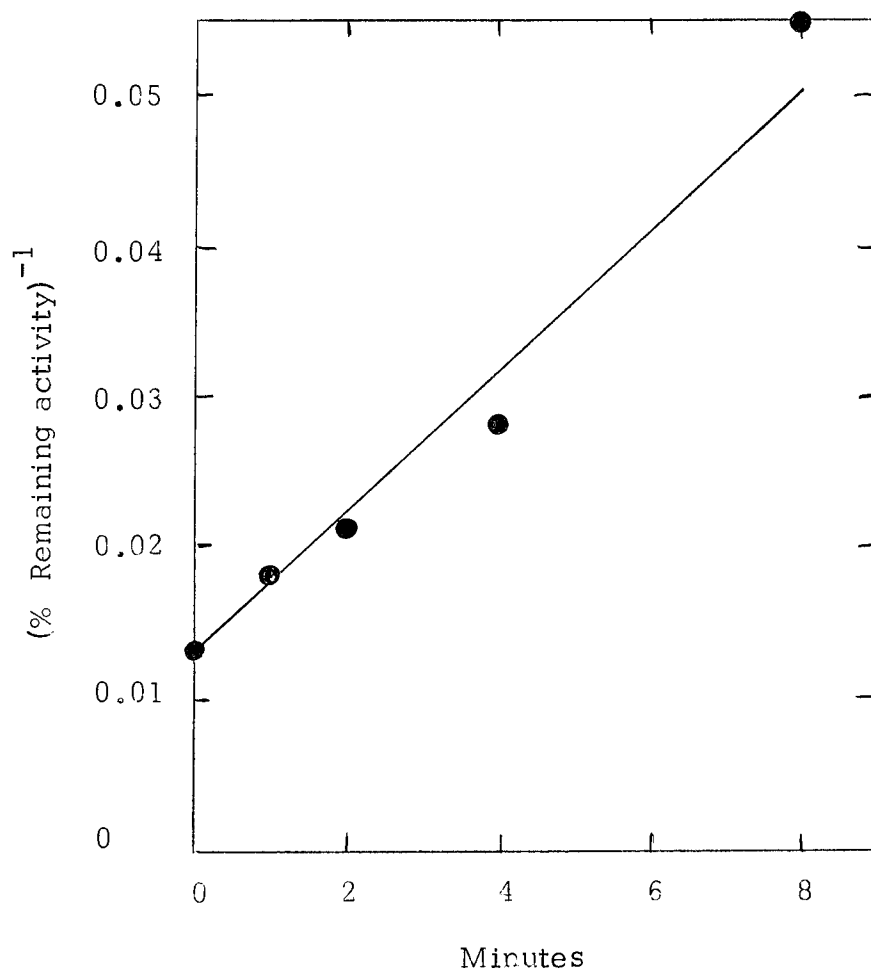
Figure 19. Determination of second-order rate constants for in-hibition of purified diamine oxidase by various hydrazines. The data from figure 18 are recalculated for 1/% remaining activity and plotted against time.

Figure 19a. BOH at a final concentration of 1×10^{-7} M and 4 µg of purified diamine oxidase.
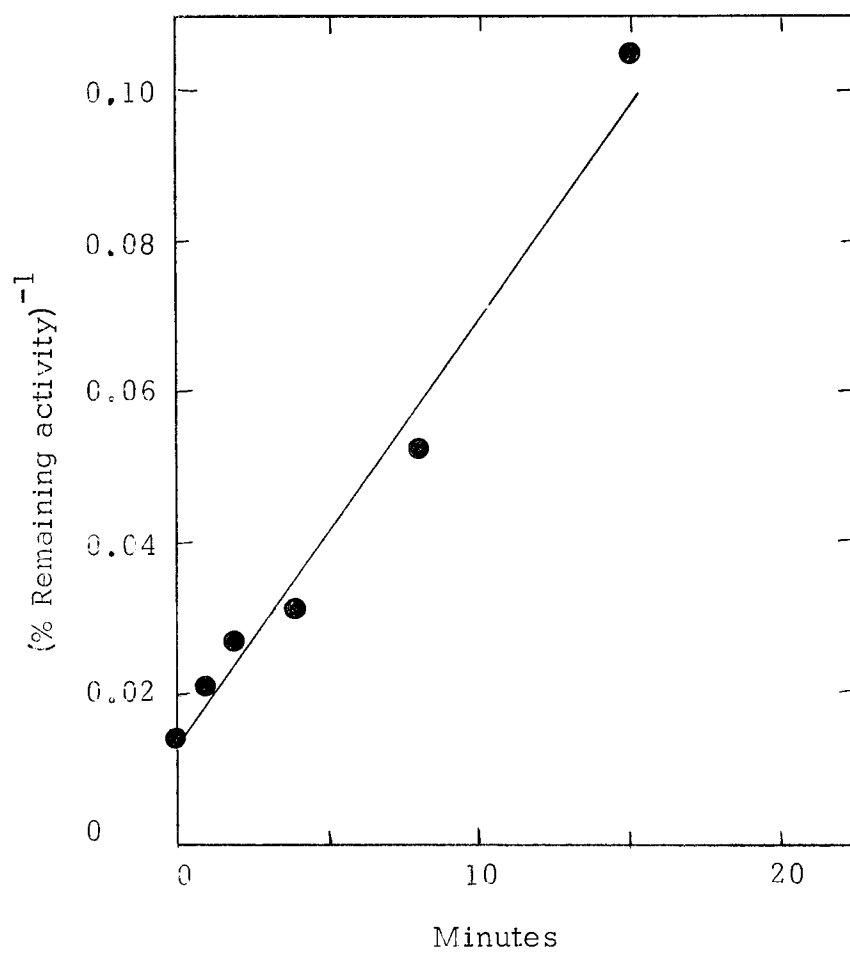
Figure 19b. UDMH at a final concentration of 3.3×10^{-7}M and 4 μg of purified diamine oxidase.

The second order rate constants from Figures 17 a, b, c, and Figures 19 a, b, are given in Table X. The slope of the linear curves in the figures mentioned was divided by the intercept on the ordinate and the initial concentration of the inhibitor in order to obtain specific rate constants having units of $M^{-1}sec^{-1}$ (Frost and Pearson, 1961).

TABLE X. Rate Constants for the Inhibition of Diamine Oxidase by Hydrazines

| Diamine Oxidase Preparation	Inhibitor	Concentration (M)	Rate Constant $M^{-1}sec^{-1}$
Purified (4μg)	BOH	1×10^{-7}	6.3×10^{4}
	UDMH	3.3×10^{-7}	1.8×10^{4}
Partially Purified (4.3 μg)	BOH	1×10^{-7}	1.2×10^{6}
	UDMH	1×10^{-7}	2.0×10^{4}
	Hydrazine	1×10^{-7}	2.2×10^{4}

All reaction mixtures had a total volume of 400 μl, 33 mM borate-phosphate pH 8 buffer. Remaining enzyme activity was determined with tryptamine $2-^{14}C$ as the substrate.

DISCUSSION

Since pea seedlings are a rich source of diamine oxidase, purification attempts were begun, utilizing the methods of Mann (1955, 1961). When it became apparent that enzyme activity could not be recovered in the same proportionate yields as reported by Mann (1955, 1961), other methods were attempted. The purification scheme outlined in the results gave only 50 fold purification of the pea seedling diamine oxidase; however, it was deemed of sufficient purity to be used in defining some of the properties of pea seedling diamine oxidase and in characterizing some reactions of diamine oxidase with hydrazine compounds.

The assay for the pea seedling diamine oxidase involved the sensitive radiotracer assay method developed by Wurtman and Axelrod (1963). It was noted that in the published procedure the product or products which might be obtained from the amine oxidase activity on tryptamine had not been characterized sufficiently. The validy of paper chromatography in acidic and basic solvent systems to characterize indoleacetaldehyde as the oxidation product of tryptamine is open to some question, as this compound is know to exhibit extreme lability under widely varying pH (Gray, 1959). The applicability of the radiotracer assay of Wurtman and Axelrod(1963) for the assay of small quantities of amine oxidase activity such as are encountered

in inhibitor studies with hydrazine compounds is demonstrated by the experiments described in Results.

The studies with purified pea seedling diamine oxidase and partially purified diamine oxidase indicated that both preparations measured identical enzymatic activity. The Km values obtained for tryptamine oxidation are very similar and the studies performed with putrescine as substrate, both with purified and partially purified enzymes, indicated the preference of the enzyme for this diamine over that of tryptamine. Because of the great rapidity with which putrescine is oxidized, it was decided to use tryptamine-2-^{14}C for the general assay since it would have been necessary to perform large dilutions to permit assay of activity with putrescine-1, 4-^{14}C for preparations at any step in the enzyme purification and with the purified diamine oxidase.

The radiotracer assay method permitted the determination of an oxygen requirement for the diamine oxidase activity which would have been impossible by the manometric techniques utilized by Kenten and Mann (1952), Mann (1955, 1961) and Werle, Trautschold and Aures (1961).

Putrescine, when used as substrate for pea seedling diamine oxidase, is very rapidly oxidized. In experiments describing product

formation with varying substrate concentration Kenten and Mann

(1952) demonstrated that the initial reaction velocity reached a max-

imum rate at a putrescine concentration of 10^{-3}M. In experiments

measuring the oxidation of varying concentrations of putrescine at

pH 8, a Km value of 7.4×10^{-5}M was found for purified diamine

oxidase, which is in agreement with the results of Kenten and Mann

(1952) in that 1,4-diaminobutane is a more suitable substrate for

this enzyme than monoamines. In preliminary experiments it had

been observed that the oxidation of putrescine proceeded 10 times

faster than the oxidation of tryptamine by partially purified diamine

oxidase when the same pH, temperature, and substrate concentration

were employed.

The reaction of amine oxidases on amine substrates has been

recognized as $RCH_2NH_2 + H_2O + O_2 = RCHO + H_2O_2 + NH_3$. The

influence of hydrogen peroxide formation on diamine oxidase activity

has been investigated by Kenten and Mann (1952), Mann (1955), and

Mondovi et al. (1967a). Suggesting that hydrogen peroxide had an

inactivating effect only when the enzyme was in the reduced state,

Mann (1955) determined that the presence of catalase in the assay

media would partially prevent inactivation of the enzyme. The results

obtained in the assay of purified diamine oxidase with varying con-

centrations of catalase (Table IV) corroborate the findings of the

earlier investigators.

The characterization of product from tryptamine oxidation by pea seedling amine oxidase had been carried out by Clarke and Mann (1957). The inherent lability of some indole compounds to acidic or basic solvents and the difficulty in obtaining specific and characteristic color reactions of indoleacetaldehyde demonostrated that an unequivocal method for the indentification of indoleacetaldehyde was in order. Indoleacetaldehyde $2\text{-}^{14}C$ was the product in the radiotracer assay as described in Methods, because a sodium borohydride reduction, which is relatively specific for carbonyl groups, resulted in tryptophol $2\text{-}^{14}C$ which could be recrystallized with unlabeled carrier tryptophol. Since constant specific activity of radioactivity per weight of crystallized material was obtained, the product of the diamine oxidase activity on tryptamine was confirmed as indoleacetaldehyde. Table II gave a good correlation between the product obtained in toluene extracts of acidified reaction mixtures and the recrystallized tryptophol resulting from the sodium borohydride reduction of the reaction mixture. The results indicated that indoleacetaldehyde, indeed, was the product of diamine oxidase activity on tryptamine.

That the cofactors for the pea seedling amine oxidase are pyridoxal phosphate and flavin adenine dinucleotide had been reported by Werle and Pechmann (1949) and Goryachenkova (1956a), but further studies by Mann (1961), Hill and Mann (1964) and Hill (1966) show that the spectrum of the purified diamine oxidase does not correspond

with spectra of known pyridoxal phosphate or flavin-containing enzymes. Further work on the nature of the carbonyl component of the diamine oxidase is currently being carried out by investigators at Rothamsted Experimental Station (Hill, 1967). The results described in the work with partially purified diamine oxidase show that neither pyridoxal phosphate nor Cu(II) ions enhance enzymatic activity which is in accord with the results of Mann (1961). In original preparations of purified diamine oxidase (Mann, 1961) there was found an inhibition of enzymatic activity by Cu(II) ions at 10^{-5} M and no stimulation of enzyme activity at lower concentrations of Cu (II) ions.

The wide substrate specificity of pea seedling diamine oxidase was demonstrated by Kenten and Mann (1952), Mann (1955), Hill and Mann (1964), and Werle, Trautschold and Aures (1961). It is suggested that the explanation of preferential oxidation of diamines because of proper chain length and basic groups may be related to the hydrophobic nature surrounding active sites of certain enzymes (Hofstee, 1967). If a stablization of certain hydrophobic areas of the enzyme were obtained by the fit of a particular substrate of appropriate chain length with corresponding hydrophobic nature, then, the enzyme rate of reaction would be enhanced. This could account, in part, for the rapid preferential oxidation of diamines by pea seedling diamine oxidase.

The variation in pH of the assay would presumably affect the rate of oxidation due to de-protonation or protonation of substrate and also the enzyme molecule, either at the active site or elsewhere on the protein. Hare (1928) was among the first investigators to examine the pH effect on enzyme reactions; with tyramine oxidase the pH effect was studied in order to be able to distinguish between the effect of pH on substrate or on the enzyme molecule. The experiments performed with the partially purified diamine oxidase show that the pH optimum of tryptamine oxidation (Figure 14) is slightly higher than that observed by Clarke and Mann (1957).

The oxidation of tryptamine at high and low substrate concentrations and the oxidation of tryptamine with varying times of incubation, using several enzyme concentrations as in Figure 6, indicated that product inhibition does occur where linearity of product formation with time no longer holds. Clarke and Mann(1957) reported enzyme inactivation or inhibition at high tryptamine concentration, but it was not established whether hydrogen peroxide caused the inactivation or whether an inhibitory effect was induced by a postulated formation of a compound between tryptamine and its oxidation product. In order to maintain substrate concentration at a level where product inhibition would not be significant, the concentration of tryptamine used in the assays was maintained at approximately 1×10^{-4}M.

The assays performed with putrescine as substrate were dependent upon the extraction of the cyclized product, Δ^1-pyrroline. The products of oxidation of putrescine and cadaverine have been well characterized by Mann and Smithies (1955), Hasse and Maisack (1955), and Okuyama and Kobayashi (1961); therefore, no further work was done on the characterization of the products obtained from diamine oxidase activity on these substrates.

The titration curves for both purified and partially purified diamine oxidase demonstrated the potent inhibitory effects of BOH, UDMH, and hydrazine. It appears as though BOH is the best inhibitor of pea seedling diamine oxidase of the three inhibitors used. Also, the greater rate of inhibition by BOH reflected by a larger second-order rate constant suggests that this inhibitor has a greater affinity for the active site. Several mechanisms can be proposed to explain the phenomenon. One is that the inhibition of the enzyme may require the inhibitor to undergo decomposition in the process of forming an inhibitor-enzyme complex, and BOH possibly is more susceptible to decomposition than hydrazine or UDMH. Another plausible explanation is that the active site has a greater affinity for molecules of certain chain length and structure, explaining the greater rate of oxidation of putrescine and cadaverine than tryptamine and other arylamines or alkylamines of greater chain length than putrescine and cadaverine.

If the enzyme were to require a proper fit of substrate to the active

site, and if the fit were dependent upon the substrate possessing

polar groups at both ends of the molecule, then BOH of the three in-

hibitors would most resemble putrescine, a preferred substrate for

the pea seedling diamine oxidase. Hence, this particular inhibitor

perhaps would be readily attached to the enzyme active site, forming

an inactive enzyme-inhibitor complex which does not decompose to

form product. The results from the dialysis experiments would indic-

ate that the reversal of the inhibition does not take place to give free

enzyme and inhibitor. If any dissociation of the enzyme-inhibitor

occurred, detection under the experimental conditions employed was

not possible.

The time course of inhibition of purified and partially purified

diamine oxidase demonstrated that the enzyme, once inhibited, dis-

plays a constant amount of inhibition after the maximal degree of in-

hibition is obtained. Because hydrazine and UDMH display little

structural resemblance to putrescine, it is apparent that the inhibition

cannot be related only to the theory of proper juxtaposition at the

active site of the enzyme of a molecule possessing two polar groups

with suitable chain length.

From the results of plotting reciprocal remaining activity <u>vs</u>

time, a second-order reaction was postulated as occurring in the in-

itial phase of inhibition. The deviation from second-order kinetics

may reflect several variables. One explanation is that a multi-step

inhibition process could involve a subsequent reaction which does

not involve second-order kinetics. Other explanations might be that

the inhibitor may be decomposed due to instability in the environment

of the enzyme, or that the free inhibitor is depleted in the firm bond-

ing with the enzyme, thus, influencing the concentration of one of

the reactants.

Since the inhibition reaction observed with the pea seedling

diamine oxidase indicates that the rate of inhibition is dependent

upon inhibitor concentration, the experiments of irreversible inhibi-

tion of cholinesterase by various substituted esters of phosphoric

acid may not be strictly comparable. The work of Jansen, Nutting

and Balls (1949) with the inhibition of chymotrypsin by DFP which

was found to be a very rapid and stoichiometric reaction might be more

comparable to the observations obtained from the inhibition of diamine

oxidase with the various hydrazines. The relation between stability

of some of the irreversible inhibitors of cholinesterase and reactivity

as inhibitor was examined by Aldridge and Davison (1952), and it

was found that the greater the stability of inhibitor to hydrolysis,

the lower its inhibitory power. It is possible that such a relationship

exists between the various hydrazines in that BOH was postulated

to be more susceptible to decomposition than UDMH.

It appears likely that the mechanism of inhibition may involve,

in part, hydrazone formation between the substituted hydrazine and a

carbonyl component of the diamine oxidase. Taylor and Jenkens

(1966) used phenylhydrazine to inhibit the activity of leucine amino-

transferase which was shown to contain one mole of bound pyridoxal

phosphate per mole of enzyme. By calculating the molar ratios of

phenylhydrazine to enzyme from the data presented in the work of

Taylor and Jenkins (1966), it was found that 96% inhibition of enzyme

activity is obtained, after a prior 15 minute incubation of enzyme and

inhibitor, when 30 moles of phenylhydrazine were present for each

mole of enzyme; 66% inhibition was obtained with 3 moles of inhibitor

per mole of enzyme.

Table VIII listed the molar ratios of inhibitor to enzyme and the

corresponding percent inhibiton obtained after prior incubation of BOH

or UDMH with the purified enzyme for a 15 minute period. There was

found 99% inhibition of enzymatic activity with the ratio of 3.1 moles

of BOH to one mole of enzyme; with the ratio of 0.95 there was obtain-

ed 94% inhibition. In contrast, with a 15 minute prior incubation of

3.1 moles of UDMH with one mole of enzyme, there was noted only

59% inhibition of enzymatic activity. At a molar ratio of 0.95

19% inhibition was found with UDMH. From these results and those

reported for phenylhydrazine inhibition of leucine aminotransferase,

it is seen that bulky groups, such as phenyl, may have affected the

reactivity of the inhibitor with the active site.

It is suggested that hydrazone formation does play a part in

the inhibition of diamine oxidase by hydrazines, but since no evidence

has been obtained to suggest that pyridoxal 5-phosphate is definitely

present in the pea seedling diamine oxidase, it is postulated that

it is the unknown carbonyl component (Hill, 1966) in the enzyme which

is highly reactive to hydrazines. It is very likely that a subsequent

reaction occurs in the inhibition after the postulated hydrazone form-

ation. The exact mechanism of inhibition of diamine oxidase by the

various hydrazine compounds remains to be solved. The experiments

described with the purified and partially purified diamine oxidase

show that inhibition is rapidly attained with BOH, UDMH, and

hydrazine, and that inhibition once attained is not demonstrably re-

versed. The inhibition appears to be dependent not only upon enzyme

concentration but also upon inhibitor concentration when the amount

of enzyme closely approximates the amount of inhibitor.

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