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Some kinetics of the oxygen requirement of amine oxidases have been investigated using bovine plasma monoamine oxidase (PAO) and pea seedling diamine oxidase (DAO). A system is described for studying enzyme reactions under controlled oxygen tension and in an O_2-free N_2 atmosphere.

O_2 Km values were determined at two different levels of amine concentration for both PAO and DAO. The apparent O_2 Km values were found to vary with amine concentration and were in the order of 10^{-4} to 10^{-6} M depending on the enzyme and amine present in the reaction mixture.

Ratios of moles of aldehyde product formed per mole of enzyme were determined under anaerobic conditions for PAO and DAO. The molar ratios were found to be approximately one mole of aldehyde per mole of enzyme in an O_2-free atmosphere for both enzymes.

This suggests that the enzymes may produce a mole equivalent of aldehyde in the absence of oxygen.

Kinetic Studies of the Oxygen Requirement of Some Amine Oxidases

by

Robert Malcolm Swindell

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KINETIC STUDIES OF THE OXYGEN REQUIREMENT OF SOME AMINE OXIDASES

INTRODUCTION

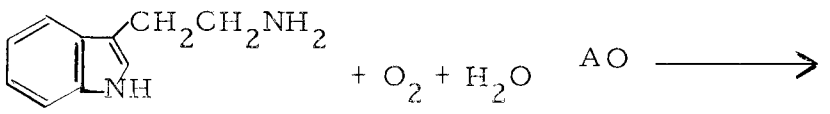
Although many studies have been made on amine oxidases (AO) isolated from plants and animals and several amine oxidase reviews have been published, some of which are by Blaschko (1953, 1963) and Zeller (1963), very little work has been done on the role of oxygen in the enzyme-substrate reaction. The reaction has been established as an oxidative deamination of amines according to the following equation:

$$RCH_2NH_2 + O_2 + H_2O \xrightarrow{AO} RCHO + NH_3 + H_2O_2$$

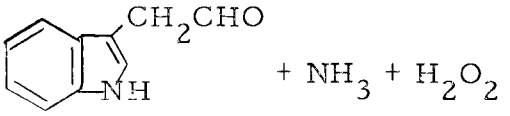
One can see from the equation that one mole of oxygen is required for every mole of aldehyde produced. If catalase is present, as it usually is in vivo or in a partially purified preparation, then the H_2O_2 is converted to $H_2O + \frac{1}{2}O_2$. These studies would show a requirement of only one-half of a mole of oxygen for each mole of aldehyde formed which gives as the overall reaction:

$$RCH_2NH_2 + \tfrac{1}{2}O_2 \xrightarrow[CAT]{AO} RCHO + NH_3$$

For example with tryptamine, one of the substrates used in this research, the reaction would be the following:



tryptamine



indoleacetaldehyde

As early as 1937, Philpot (1937) found that the reaction rate of tyramine oxidase from rat liver increased with an increase in the oxygen tension. That same year Kohn (1937), using three different oxygen tensions (100%, 20%, and 1.5%), showed that the rate of reaction of tyramine oxidase increased with increased oxygen up to 100% whereas xanthine oxidase (from milk) was essentially saturated at 20%. In both of these studies no attempt was made to purify the enzyme.

Blaschko (1953) stated that the activity of amine oxidase as measured by the manometric technique was dependent upon the partial pressure of oxygen in the gas phase, and for this reason 100% O_2 was usually used. Since the activity was less at 20%, he considered this as an indication that the enzyme in living tissue was very sensitive to change in oxygen tension, or more likely, that in a living cell molecular oxygen was not the electron acceptor. Instead he thought that some other electron acceptor was interposed between

the enzyme and oxygen (Blaschko, 1952). Zubrzycki and Staudinger (1967) came to a similar conclusion when using rabbit liver mitochondria and isoamyl amine; they found a Km for O_2 of 7×10^{-4} M which was so high that they also thought it unlikely that O_2 was the natural intercellular electron acceptor. The pO_2 of mitochondria has been estimated to be no more than 1 mm Hg (1.4×10^{-6} M at 37°C (Frieden, Osaki, and Kobayashi, 1965).

Frieden, Osaki and Kobayashi (1965) in a comprehensive examination of copper-containing enzymes and the role of oxygen, list Km's of O_2 for some oxidases, but they have no values for amine oxidase or diamine oxidase. They admitted that the present knowledge (1965) of how oxygen reacts with copper-containing proteins is "primitive and incomplete".

In 1961, Novick presented a paper on amine oxidases at a Federation of American Societies for Experimental Biology meeting, and in the abstract of his paper (Novick, 1961) he stated that the Km's of amines change in response to alterations in oxygen tension. He found the magnitude of the change to be similar to the rate change, and that the affinity for oxygen was low. He estimated the O_2 Km's to be in the range of 150-350% for several substrates. Interestingly, in a publication five years later on the effect of oxygen tension (Novick, 1966), he shows no data on Km values. Working with mitochochondrial preparations of various tissues of rats, mice, and

guinea pigs, he noted an increase in monoamine oxidase (MAO) activity when the oxygen tension was increased from 20% to 100%. When using tryptamine as substrate, he found the ratio of activity (100% O_2/20% O_2) in rat liver to be 2.1 whereas with tyramine, the ratio was 3.3. He also found different ratios depending on tissue, with a range of 1.4 to 3.8 for tryptamine.

Novick (1966) also studied the effect of oxygen tension on the inhibition of MAO by iproniazid, d,l-amphetamine, and trans-2-phenylcyclopropylamine. He found that for rat liver MAO, the percent inhibition was the same at 20 and 100% oxygen tension. More studies need to be made on this aspect, particularly under anaerobic conditions for a better understanding of the mechanism of inhibitor interaction with amine oxidases.

Since very little work has been done on O_2-dependent reaction kinetics of amine oxidases, an attempt was made in this study to examine certain aspects of the oxygen requirement of some amine oxidases. Two different types of amine oxidases were employed and which are of interest in this laboratory--bovine plasma monoamine oxidase (PAO) and pea seedling diamine oxidase (DAO). While all of the research mentioned up to this point has been on mitochondrial MAO, bovine PAO is termed a "soluble" enzyme, and experiments in progress in this laboratory indicate that the pea seedling DAO may be in the microsomal fraction of the cell. Although the

mitochondrial and the soluble amine oxidases are similar in that they both catalyze an oxidative deamination of amines, they are quite different in many respects. The mitochondrial MAO have been found to be flavoenzymes and are bright yellow in color, but DAO and PAO are pink in color and have an aldehyde, possibly pyridoxal phosphate, instead of flavin as a prosthetic group. Both DAO and PAO are copper-containing proteins, while there is some argument as to whether mitochondrial MAO requires copper. Part of the problem of studying MAO is the difficulty in purifying the enzyme as it is membrane bound and hard to solubilize. Erwin and Hellerman (1967) prepared highly purified mitochondrial MAO from bovine kidney. They found four to five equivalents of active flavin per atom of copper, which they thought to be an indication, along with inhibitor studies with chelating agents, that copper was not essential. In contrast Nara and Yasunobu (1965) found copper to be present and essential in purified preparations of beef liver MAO by showing a linear relationship between copper content and specific activity during purification of the enzyme. The MAO isolated from mitochondria also appear to have essential sulfhydryl groups, yet neither PAO nor DAO is sensitive to sulfhydryl reagents (Kapeller-Adler, 1963). As one can see, PAO and DAO are markedly different physiochemically from MAO, yet despite the obvious differences in substrate specificities of PAO and DAO, they appear similar to each other in

that both apparently have the same cofactors.

The purification and properties of pea seedling DAO have been studied extensively by Hill and Mann at the Rothamsted Experimental Station, Harpenden, England (Hill, 1967; Hill and Mann, 1962, 1964; Kenten and Mann, 1952; and Mann, 1955, 1961) and by Reed and his coworkers here at Oregon State University (Reed, 1965; Reed, Dost, and Wang, 1965; Reed, Moore and Anderson, 1965). The enzyme demonstrated a greater oxidation rate of diamines than monoamines and was designated as diamine-oxygen oxidoreductase EC 1.4.3.6. Bovine plasma monoamine oxidase has been purified and characterized by Yasunobu and coworkers at the University of Hawaii (Nara and Yasunobu, 1965; Yamada and Yasunobu, 1962a, 1962b, 1963; Yamada et al., 1963).

McEwen, Cullen, and Sober (1966), working with rabbit plasma monoamine oxidase have determined from kinetic data a speculative mechanism for the initial stages of the deamination reaction to be as shown in Figure 1. According to this mechanism, the aldehyde is produced before oxygen is involved in the mechanism. If this is true, then under anaerobic conditions the enzyme should produce from a large excess of substrate, one mole of aldehyde per mole of enzyme. Studies have been made anaerobically on pea seedling DAO by Mann (1961) and on bovine PAO by Yamada and Yasunobu (1962b). However, in both cases they looked at the spectral changes that

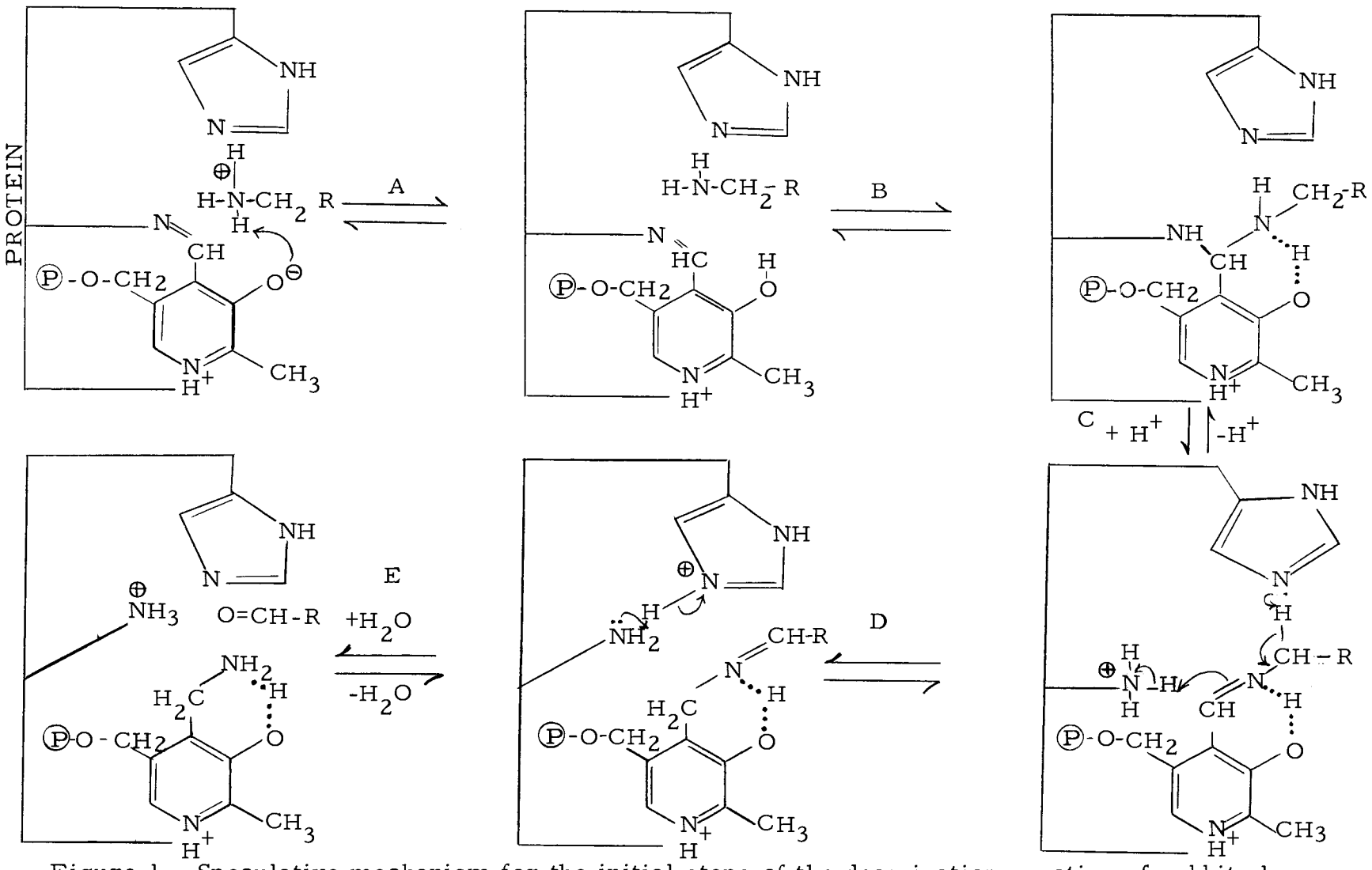


Figure 1. Speculative mechanism for the initial steps of the deamination reaction of rabbit plasma amine oxidase (McEwen, Cullen, and Sober, 1966).

occurred, but they did not publish any data indicating whether the aldehyde was formed. Yamada and Yasunobu (1962b) found that the absorption maximum at 480 nm was bleached by the anaerobic addition of substrate, and the absorption spectrum of the copper-free enzyme showed a maximum at 380 nm which was not altered by the anaerobic addition of substrate. They also found that Cu(II) could be added back to the Cu-free enzyme with a shift of the maximum band from 380 nm back to 480 nm. From these data, along with data on the inhibition by various carbonyl reagents, they made the interpretation that the band at 380 nm may indicate the presence of pyridoxal phosphate, and that a Cu(II)-pyridoxal phosphate complex may be at the active site which gives a maximum at 480 nm. Interaction of this complex with the substrate would then form a colorless intermediate. Mann (1961) found similar results with DAO. Under anaerobic conditions, he found that the pink color disappeared upon the addition of putrescine which could be restored by the addition of oxygen. He also suggested that, since hydrazine reacts irreversibly with the enzyme with loss of color and activity, and the primary reaction product absorbs maximally at 420 nm, the pink color is due to an aromatic carbonyl such as pyridoxal phospate. Hill and Mann (1964) showed that the spectra were the same under anaerobic conditions for several different substrates. Also the Cu-free enzyme was inactive, but the activity could be restored by the addition of

Cu(II). Yamada and Yasunobu (1962b) and Yamada et al. (1963) showed that copper was essential and also showed by chemical means using neocuproine and by electron spin resonance that there was no valence change of cupric copper during the reaction. It is interesting to note that McEwen, Cullen, and Sober (1966) do not implicate copper in their model (page 6) due to lack of information concerning the role of copper at the active site. They also do not show any interaction of molecular oxygen with the model, nor how ammonia and hydrogen peroxide are formed. Since all cupro-protein enzymes utilize only molecular oxygen as the electron acceptor (Mahler and Cordes, 1966), the interaction of O_2 with the enzyme should be at the site containing the copper.

In this study it was decided to use a sensitive radiotracer enzyme assay in a special anaerobic system (described in the materials and methods section) to see if this partial mechanism was correct and if the aldehyde is formed in the absence of oxygen with a molar ratio of aldehyde to enzyme under these conditions. Other kinetic studies were undertaken to determine the Km values for O_2, using pea seedling DAO and bovine PAO in the hope of learning more about the mechanism of interaction of O_2 with the enzyme.

MATERIALS AND METHODS

Materials

Pisum sativum var. Alaska seeds were purchased from Northrup, King and Company. Ammonium sulfate was a special enzyme grade from Mann Research Laboratories. L(+) ascorbic acid was from Matheson, Coleman, and Bell Company. Tryptamine hydrochloride, putrescine dihydrochloride, methylene blue, catalase (2× crystallized from bovine liver), and DEAE cellulose were from Sigma Chemical Company. Benzylamine was from Calbiochem. 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) and p-terphenyl of scintillation grade were from Packard Instrument Company. Tryptamine 2-^{14}C bisuccinate, specific activity of 10.3 mC/mmole and putrescine dihydrochloride, specific activity of 5.22 mC/mmole were from New England Nuclear Corporation. Benzylamine 7-^{14}C hydrochloride, specific activity of 1.6 mC/mmole was from Mallinckrodt Nuclear. Tanks of compressed air and pre-purified nitrogen were from National Cylinder Gas Division of Chemetron Corporation. Analyzed tanks of O_2 containing 1.07% and 108 ppm O_2 and a balance of N_2 were from Matheson Company. Laboratory reagents for buffers, cleaning solutions, and other chemical solutions were reagent grade unless otherwise specified. Deionized glass distilled water was used for all solutions.

Methods

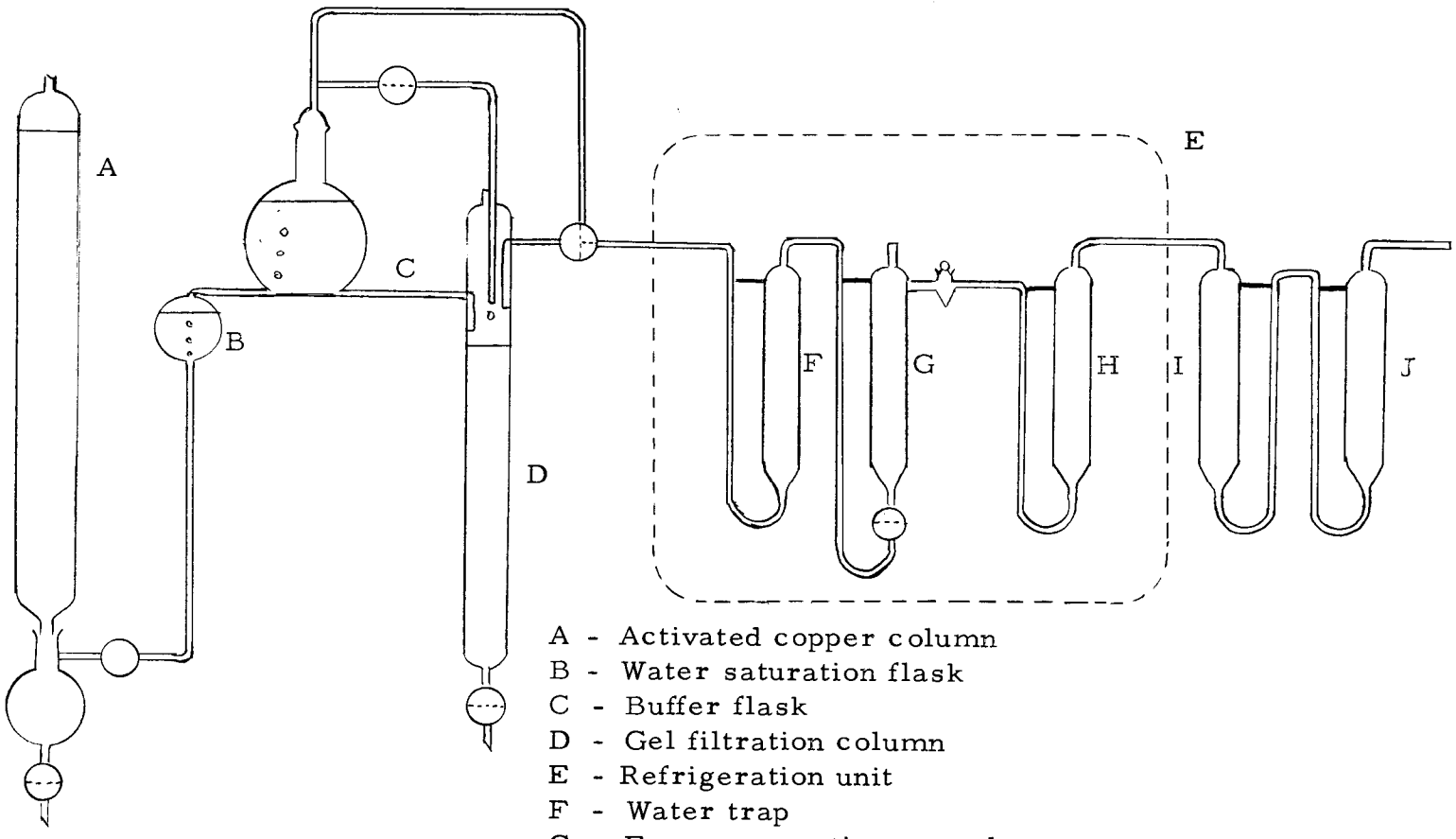
System for O_2-free N_2 and Controlled O_2 Tension Experiments

Since most of the research presented here required a system in which oxygen tension could be varied, a suitable procedure was needed for varying the amount of oxygen dissolved in an aqueous solution.

The first attempt was to use conical glass centrifuge tubes equipped with rubber serum stoppers as the reaction vessels. The enzyme in 0.4 ml. of phosphate buffer was carefully placed in the bottom of the tube so that no liquid adhered to the sides of the tube. The substrate, containing a known amount of radioactive (^{14}C) and unlabeled amine, was dried onto a strip of filter paper ($\frac{1}{2}'' \times 1''$) and placed in the tube above the enzyme solution. A rubber serum stopper was then inserted to close off the vessel. Next, a 24 gauge needle was inserted into the centrifuge tube through the rubber stopper. The needle was connected to a vacuum pump and to a source of oxygen-free nitrogen (pre-purified from a commercial tank passed through alkaline pyrogallol) by way of a three-way stopcock. Thus, one could draw the gas out of the tube and then immediately fill the tube with N_2. After evacuating and filling several times, one could

either run the reaction in a N_2 atmosphere or with an O_2-N_2 mixture. The mixture was made by diluting pure O_2 with the N_2 mentioned above by means of syringes hooked together with a three-way stopcock (with capillary bore and luer-lock fittings). The reaction could be started at any time by tilting the tube, which allowed the enzyme solution to flow onto the substrate paper. The reaction could be terminated by the addition of HCl through the serum stopper.

Although the approach was sound in principle, the system was not useful because of leakage and diffusion of air through the serum stopper and adsorbed oxygen on the walls of the reaction vessel. Therefore, the system shown in the diagram in Figure 2 was used in these studies. The gas continually passed over the enzyme at 1 to 2°C and bubbled through the substrate until an equilibrium was established. The enzyme was thus protected from denaturation by frothing as it equilibrated with the O_2-N_2 atmosphere. For the anaerobic studies, pre-purified N_2 was passed over a hot activated copper column (described on page 21). All connections up to the column were $\frac{1}{4}$" or 1/8" OD copper tubing connected with brass Swagelok fittings. All connections between glass apparatus were made with nylon Swagelok connectors joining $\frac{1}{4}$" OD glass capillary tubing. Because the resulting gas was anhydrous and would evaporate the sample, a round flask containing glass-distilled water was a part of the system just after the copper column to resaturate the gas with



A - Activated copper column
B - Water saturation flask
C - Buffer flask
D - Gel filtration column
E - Refrigeration unit
F - Water trap
G - Enzyme-reaction vessel
H - Substrate vessel
I - Methylene Blue and air trap
J - Methylene Blue O_2 detector vessel

Figure 2. Diagram of system for running enzyme reactions in an O_2-free N_2 atmosphere.

water. The gas then bubbled through a second flask containing buffer which could flow out of the flask onto the top of the gel filtration column. The gas stream flowed out of the top of the flask and then branched to flow through the buffer in the top of the column and through the system in the refrigeration unit. The refrigeration unit consisted of a styrofoam cooler with copper cooling coils along the bottom connected to a refrigerated circulating water bath. An antifreeze solution at -11°C was circulated through the coils. A fan kept the air inside the unit at a constant 1 to 2°C while the gas was flowing through the vessels. Three vessels which are described below were placed in this refrigerating unit.

The first vessel was a 15 ml. centrifuge tube sealed at each end with $\frac{1}{4}$" OD capillary tubing (see Figure 3a). This vessel serves as a trap for water which condenses out of the gas as the gas temperature drops from room temperature to 1 to 2°C. Otherwise, the condensation would collect in the enzyme reaction vessel and change the concentration of the reactants. The next vessel is the enzyme reaction vessel (see Figure 3b). During the equilibration time, while the gas is flowing in the system, the enzyme is held in the side arm of the vessel. To initiate reaction, the substrate is forced into the enzyme reaction vessel onto the enzyme solution which transfers the mixture to the main body of the reaction vessel. The mixture is agitated with a small teflon-coated magnet in the reaction vessel

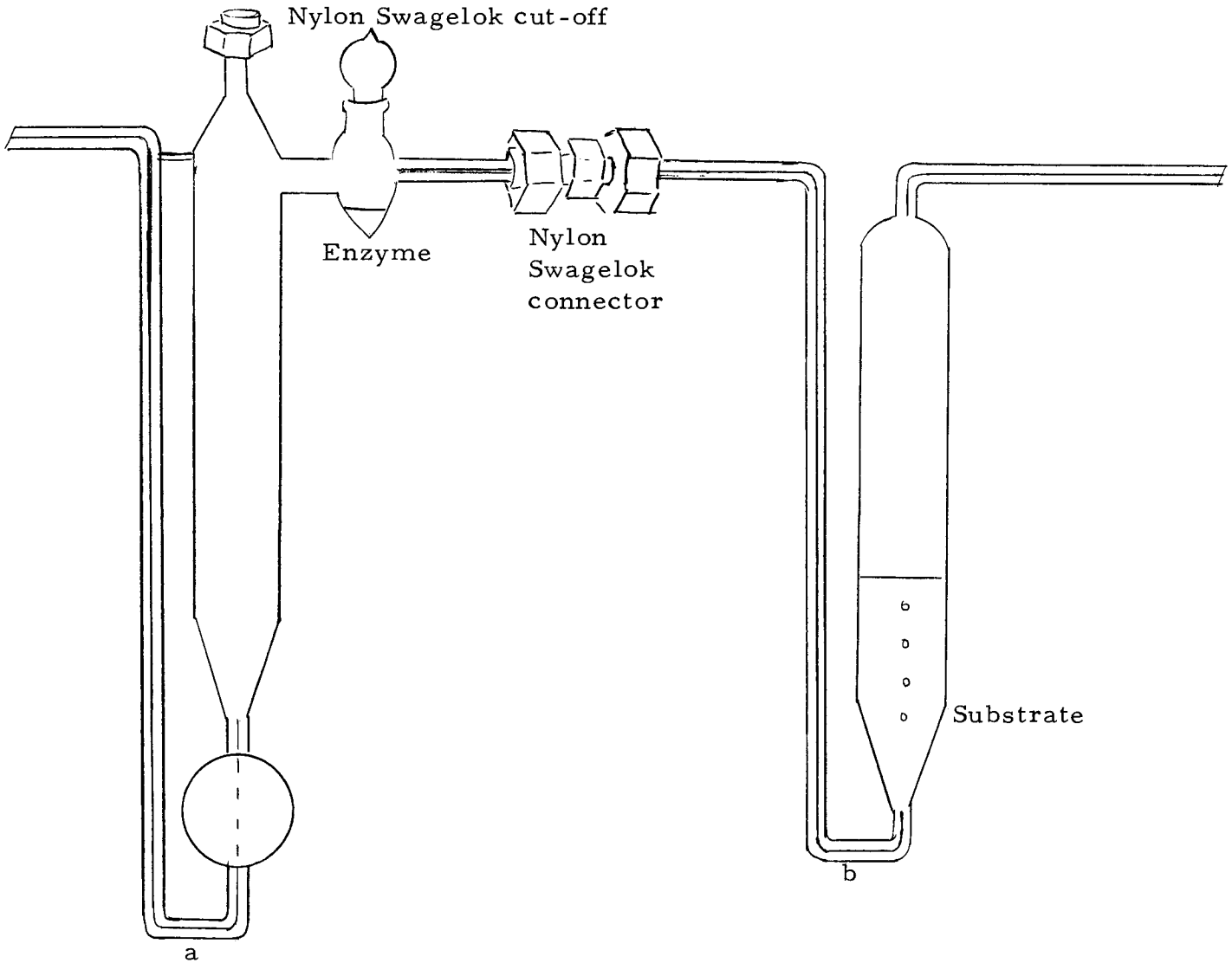


Figure 3. Diagrams of a-enzyme-reaction vessel and b-substrate vessel.

by means of an external magnet. After the preset amount of incubation time, the reaction is stopped by both heating the reaction mixture to its boiling point with a Bunsen burner and with the addition of HCl in the case of reactions with tryptamine or benzylamine as the substrate. The third vessel is the same as the water trap and contains the substrate. The last two vessels are outside of the refrigerating unit. The first of these is a vessel which is reversed to prevent the methylene blue solution in the last vessel from transferring into the substrate vessel when substrate is forced into the reaction vessel. The last vessel contains a methylene blue solution (discussed on page 22) and serves as an indication of O_2 being present by its color, and also serves as a trap to prevent air from entering the system when the substrate is added to the enzyme. When the time for reaction is reached, the stopcock on the enzyme-reaction vessel is turned to stop the gas flow, and, by means of a syringe, pressure is applied to the end of the methylene blue vessel. This forces the methylene blue into the inverted vessel and the substrate into the enzyme-reaction vessel. The reaction is then carried out according to one of the enzyme assays described below. When an O_2-N_2 mixture was desired for a particular oxygen tension rather than having an O_2-free N_2 system, then a tank containing a known amount of oxygen was mixed with the nitrogen through a Swagelok universal tee. So as to span a wide range of oxygen tensions, three

different concentrations of oxygen in nitrogen gas mixtures were purchased. A compressed air tank, a 1.07% and a 108 ppm oxygen tanks were used to mix with the nitrogen. A flow meter was connected into the oxygen circuit and at the end of the system. For low flow rates, a soap bubble meter was used, but for higher flows a rota meter with a stainless steel ball was used (Manostat Corporation #36-541-03). Thus, with the oxygen flow and the total flow monitored, one could calculate the oxygen tension and the O_2 concentration in the enzyme and substrate solutions.

Enzyme Assays

A modified procedure of the method of Wurtman and Axelrod (1963) was used for the enzyme assays with tryptamine and benzylamine. In this procedure, the radioactive aldehyde product is extractable with toluene from an acidified aqueous reaction medium, leaving the unreacted amine in the aqueous layer. In a typical reaction 0.1 μc (220,000 dpm) of tryptamine 2-^{14}C or benzylamine 7-^{14}C was dissolved in 1 ml. of 0.033 M borate-phosphate buffer at pH 8 or 0.067 M phosphate buffer at pH 7.1 and unlabelled amine added to give a total amount of substrate concentration of 200 mμ moles. The desired amount of enzyme in 0.1 ml. of 0.033 M borate-phosphate buffer was added to the substrate. After ten minutes incubation at 25°C, the reaction was terminated by heating the solution to

boiling with a Bunsen burner. The solution was then acidified with 0.35 ml. of 0.2 N HCl. The acidification step alone is sufficient to stop the reaction, but with the anaerobic system described on pages 11-16, it was desirable to stop the reaction before opening the vessel to the air. The acidified solution was then transferred to a 10 ml. conical ground glass-stoppered centrifuge tube and 5 ml. of toluene were added. The product was extracted with vigorous shaking and the layers separated by centrifugation. Four ml. of the toluene layer were then pipetted into 10 ml. of the fluor solution (30 mg POPOP plus 3 mg p-terphenyl per liter of toluene) and counted in a liquid scintillation counter (Packard Tri Carb 3375). A blank in which 0.1 ml. buffer was substituted for the enzyme was used to determine the amount of radioactive substrate that was extractable by this method.

A modified procedure of the method of Okuyama and Kobayashi (1961) was utilized for the assay with putrescine (1,4-diamino-butane). As with the method for tryptamine and benzylamine, the substrate was comprised of labeled (putrescine 1,4-^{14}C) and unlabeled substrate to obtain the desired substrate concentration in 1.0 ml. Then 0.1 ml. of enzyme was added and the solution incubated at 25°C for ten minutes. The reaction was terminated by heating to boiling with a Bunsen burner. The aldehyde formed cyclizes to the product Δ^1-pyrroline, which cannot be efficiently extracted with toluene from an

acid medium. Therefore, 200 mg. of sodium bicarbonate were added to saturate the solution and to give a pH of about 8, which allowed maximum extraction efficiency with toluene. The remainder of the procedure is identical to that described above for tryptamine and benzylamine.

The spectrophotometric assay of Tabor, Tabor and Rosenthal (1954) using benzylamine as substrate, was used without modification except that 1 ml. quantities were used instead of 3 ml., no catalase was added, and the incubation temperature was 25°C.

Enzyme Purification

Bovine plasma amine oxidase (PAO) was obtained from young steer blood collected at a nearby slaughterhouse and purified by Dr. D. J. Reed in this laboratory by the method of Yamada and Yasunobu (1962a) as modified by Hucko and Reed (1968).

Purified pea seedling diamine oxidase (DAO) was a gift from Dr. P. J. G. Mann of Rothamsted Experimental Station, Harpenden, England. It was purified by the method of Mann (1961).

Partial purification of pea seedling diamine oxidase (ppDAO) was performed by the following modified method of Mann's procedure (1955).

Step 1--Extraction: Etiolated pea seedlings (Pisum sativum, var. Alaska) were harvested after eight to ten days

growth in germination towels at 20° C. The roots and cotyledons were removed and the top part of the seedlings, the epicotyls (about 800 g. per run), were wrapped in cheesecloth, and the juice extracted with a Carver Hydraulic Press, using a force up to 10,000 psi. About half of the total weight could be recovered as juice. The residue was re-extracted with a volume of 0.01 M phosphate buffer equal to 50 ml. per 100 g. of tissue. The buffer was then added to the juice and cooled in an ice bath. From this point on during the purification, the temperature was kept at 0-2° C.

Step 2-- $(NH_4)_2SO_4$ fractionation: Solid $(NH_4)_2SO_4$ was added (114 g/l) and stirred until all of the $(NH_4)_2SO_4$ dissolved. The solution was centrifuged at 12,000 g for 15 minutes and the precipitate discarded. To the clear solution, additional ammonium sulfate was added to give a final concentration of 450 g/l of original volume of solution. This solution was stirred for several hours to allow the complete solubilizing of the $(NH_4)_2SO_4$. The solution was again centrifuged at 12,000 g for 15 minutes. The supernatant was discarded and the precipitate dissolved in 100 ml. of 0.01 M phosphate buffer at pH 7.

Step 3-- Chloroform-ethanol precipitate: 30 ml. of a 1:2 (v/v) mixture of chloroform-ethanol held at about -10°C was added slowly with stirring to the solution in a salt-ice bath to keep the temperature from rising above 5°C. The precipitate which formed was centrifuged down at 2000 g and discarded along with the organic layer. The supernatant was dialyzed for 24 hours against three changes of 0.01 M phosphate buffer pH 7.1.

Step 4-- DEAE Cellulose Column Chromatography: Several dialyzed solutions were combined and ammonium sulfate added (500 g/l) to precipitate the protein. The precipitate was separated by centrifugation and decantation and redissolved in 25 ml. of 0.01 M. phosphate buffer. The 25 ml. was placed on a column (2 × 23 cm) of DEAE cellulose which had been equilibrated with 0.01 M phosphate buffer. Elution was performed with the equilibration buffer. The active fractions were combined and either stored at 4°C or frozen. The preparation at this point will be designated as partially purified diamine oxidase (ppDAO).

Activated Copper Column for Removal of O_2 from N_2

An activated copper column was prepared by the method of Meyer and Ronge (1939). In this procedure $CuCl_2$ was dissolved in water and an equal weight of infusorial earth added. The copper was precipitated out as copper hydroxide on the infusorial earth by adding NaOH. The precipitate was washed, filtered, and cut into 5 mm pieces. The pieces were dried at 150-180°C overnight, passed over a wide mesh screen to remove dust, and then packed into the column. The column was an E-C coated heating tube (Arthur H. Thomas Co. cat. #6136-P). These columns have a transparent electrical conduction heating film coated on the glass so that the color of the column can be observed while the column is heated. By adjusting the voltage across the two zones of film, the temperature was set at 195°C in an insulating glass jacket surrounding the column ($\sim$200°C in the column). The column is activated by flushing with hydrogen until the column becomes dark violet in color. The column is now ready for use. One can easily tell how much of the column has been oxidized by O_2 by observing the color change. The column turns brown as the copper is converted to cupric oxide with a distinct yellow abscission layer of copper oxide in between the unused dark violet copper and the used brown copper oxide. The column can be regenerated repeatedly by sweeping with H_2

while being heated to 200°C. Nitrogen gas purified of O_2 in the above described manner is reported to have less than 4×10^{-7} atm. of O_2 at 1 atm. pressure (Meyer and Ronge, 1939). This would result in a concentration of dissolved O_2 of less than 5.2×10^{-10} M at 25°C in the solutions used in the anaerobic system.

Oxygen Detector

A solution of methylene blue and ascorbic acid was prepared according to the method of Oster and Wotherspoon (1954) and used as a detector of small quantities of O_2 for the anaerobic studies. These workers found that a 10^{-5} M concentration of methylene blue in a 10^{-3} M ascorbic acid solution could be photoreduced by visible light to the leuco base. The color would not return in the absence of O_2. The method was sufficiently sensitive to show that gas from a commercial N_2 tank would gradually restore the color but pre-purified N_2 passed over hot copper would not. About 8 to 10 ml. of this solution was placed in the last vessel in the anaerobic system described earlier in this section. A high intensity desk lamp was used to photoreduce the dye. The purified N_2 gas was passed through the system until the color could no longer be restored. The solution could be used for many experiments since the color could be photo-reduced several times.

RESULTS

Km Determinations

Two concentrations of the amine substrate were used with a constant concentration of enzyme to determine the O_2 Km values of the amine oxidase preparations. The two levels were considered necessary to determine if there was any effect of amine concentration on the O_2 Km value. At least four experimental points were measured in the range of 0.34 to 20% O_2 tension for each substrate concentration.

Km Determinations for Pea Seedling Diamine Oxidase

The O_2 Km values for DAO were determined with tryptamine as the substrate. In the first set of experiments, 205 mμ moles of tryptamine containing 131,000 dpm of tryptamine 2-^{14}C, were dissolved in 1 ml. of 0.033 M borate-phosphate buffer (B-P) buffer at pH 8. DAO, 0.05 mg, in 0.1 ml. of B-P buffer was used in all experiments. The solutions were equilibrated with an O_2-N_2 mixture for a minimum of eight hours in the system described on pages 11 to 16 and then assayed as described in the methods section. The oxygen tensions used were 20, 9.7, 4.65, 2.25, and 1.07% O_2 with a balance of nitrogen.

The $[O_2]$ in the solutions was calculated from the equations:

$$X_{(O_2 \text{ in solution})} = P \text{ in mmHg}/K$$

$$[O_2] = X_{(O_2 \text{ in solution})} \times X_{(\text{total})} \times [H_2O]$$

In these equations, X is the mole fraction, P is the partial pressure of oxygen, and K is Henry's law constant. Henry's law constant was determined to be 3.25×10^7 at 25°C (Jones, 1962).

The cpm obtained in the enzyme assays was converted to dpm and from which mμ moles of aldehyde produced per minute were calculated by using the following equations:

$$\text{dpm} = (\text{cpm-blank})/\text{counter efficiency}$$

$$\text{m}\mu \text{ moles per minute} = \text{dpm} \times M/F \times I \times R \times T$$

In these equations, M is the total mμ moles of substrate, F is the fraction of toluene counted (4/5), I is the incubation time, R is the recovery efficiency of the system, and T is the total dpm in the substrate.

Figure 4 shows the Lineweaver-Burk reciprocal plot of v^{-1} versus $[O_2]^{-1}$. From the points a least squares line was determined which fits the equation, $v^{-1} = 2.48 \times 10^{-6}[O_2]^{-1} + 0.0612$. From this line, the Km was determined to be 4.05×10^{-5} M. Since each point on the line represents only a single run, a correlation coefficient was calculated for the points to see how well they represent a linear relationship. The correlation coefficient was

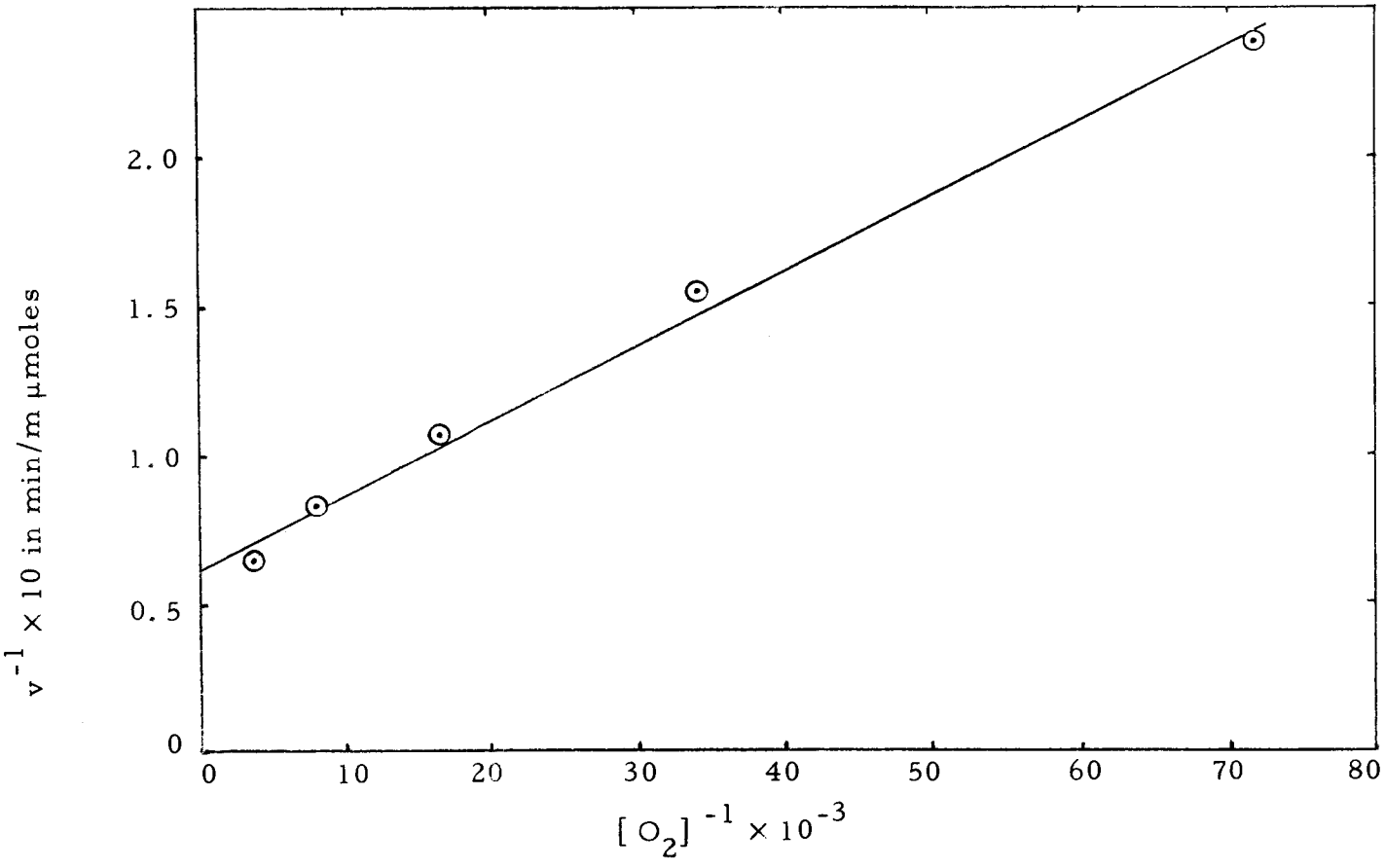


Figure 4. Lineweaver-Burk plot of the influence of oxygen tension on the rate of tryptamine oxidation by diamine oxidase (DAO) at pH 8. Tryptamine, 205 m µmoles, and 0.05 mg of DAO were incubated in a total volume of 1.1 ml. (For a discussion of Lineweaver-Burk plots, see Mahler and Cordes, 1966, p. 228).

determined to be 0.996 which indicates a good linear relationship between the points and indicates that the least squares line is a good representation of the points.

In the next set of experiments, the tryptamine concentration was changed to 1000 mμ moles and 262,000 dpm per 1.0 ml. The enzyme concentration was held constant at 0.05 mg per 0.1 ml. The experiments were performed as described above and in the methods section. The oxygen tensions used were 20, 8.2, 3.1, 2.7, and 1.07% O_2 with a balance of nitrogen. The Lineweaver-Burk plot is shown in Figure 5. The line fits the equation $v^{-1} = 2.44 \times 10^{-6} [O_2]^{-1} + 0.03$, and from this the Km was determined to be 8.15×10^{-5} M. The correlation coefficient was determined to be 0.999. As the substrate concentration increased from 205 to 1000 mμ moles, the apparent O_2 Km value of DAO also increased as did V_{max}. It should be noted here that the Km value for tryptamine (4×10^{-4} M) (Yamasaki, 1967) is in between the two concentrations of tryptamine used in these experiments (2×10^{-4} M and 1×10^{-3} M).

The partially purified DAO (ppDAO) was utilized to find the O_2 Km using tryptamine and putrescine as substrates. In the experiments with tryptamine, 205 mμ moles of tryptamine containing 131,000 dpm of tryptamine 2-^{14}C in 1.0 ml. of B-P buffer at pH 9 were used in the assays. The ppDAO at a concentration of 0.113 mg of protein (9.5% active) per 0.1 ml. was used in all experiments.

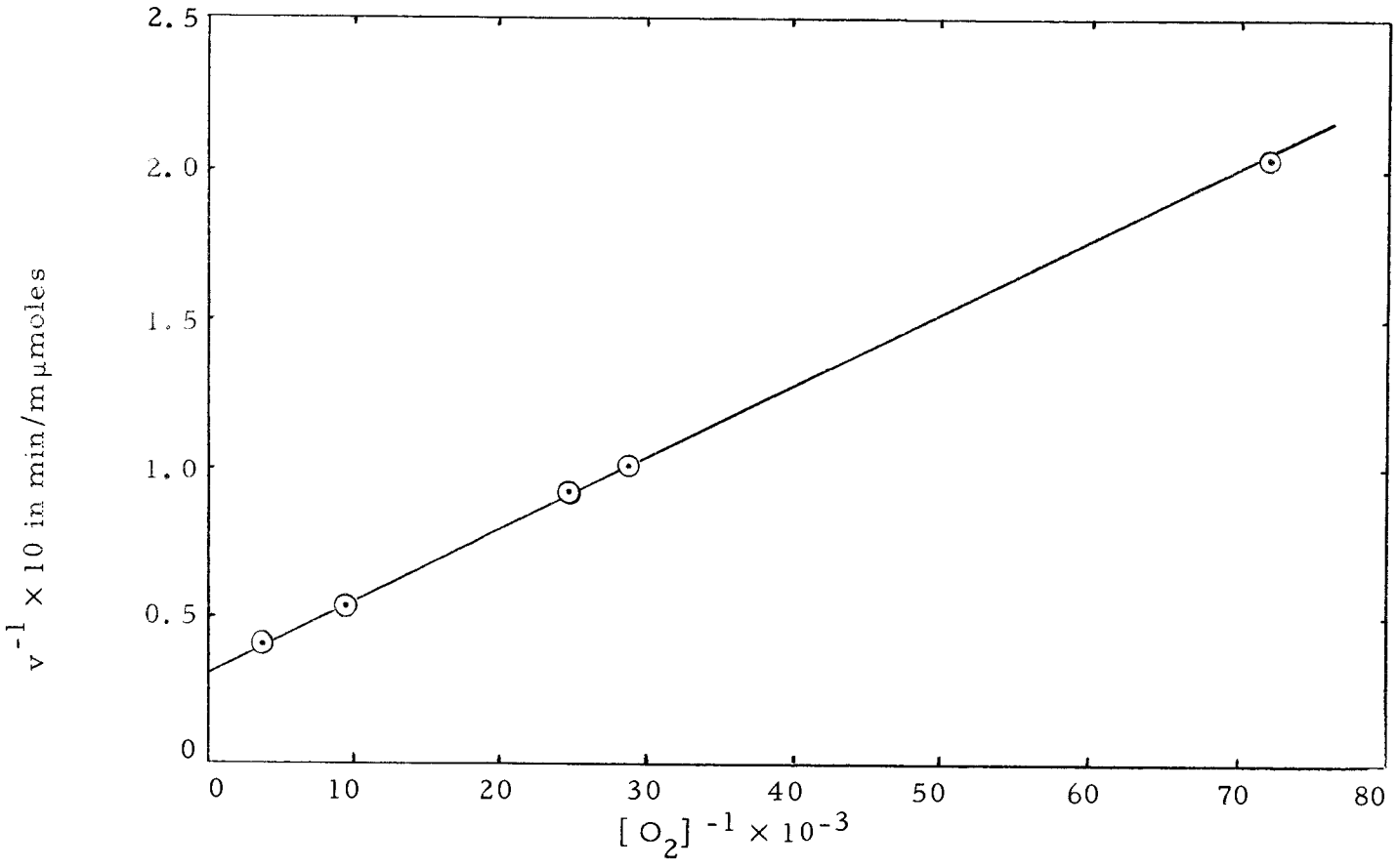


Figure 5. Lineweaver-Burk plot of the influence of oxygen tension on the rate of tryptamine oxidation by diamine oxidase (DAO) at pH 8. Tryptamine, 1000 mμmoles, and 0.05 mg of DAO were incubated in a total volume of 1.1 ml.

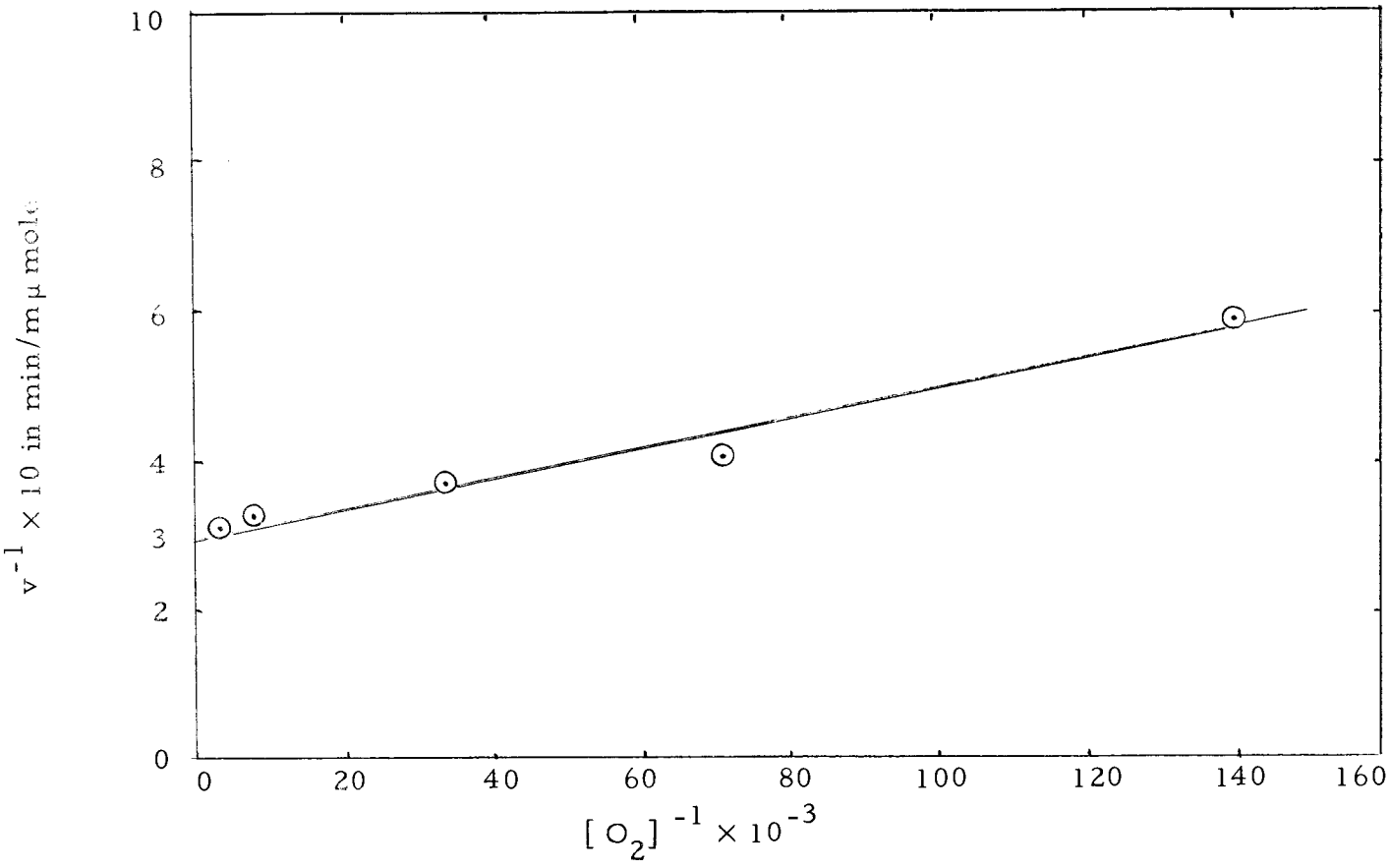


Figure 6. Lineweaver-Burk plot of the influence of oxygen tension on the rate of tryptamine oxidation by partially purified diamine oxidase (ppDAO) at pH 8. Tryptamine, 205 mμ moles, and 0.113 mg of ppDAO were incubated in a total volume of 1.1 ml.

The oxygen tensions used to equilibrate the system were 20, 9.7, 2.25, 1.07, and 0.55% O_2. The Lineweaver-Burk reciprocal points have a correlation coefficient of 0.979 and the least squares line fits the equation, $v^{-1} = 1.97 \times 10^{-6} [O_2]^{-1} + 0.297$. The O_2 Km value from this line is 6.62×10^{-6} M. This value for the O_2 Km is less than that with purified DAO at the same substrate concentration.

The O_2 Km value was found to be 5.26×10^{-4} M when putrescine was used at a concentration of 210 mμ moles containing 128,600 dpm per 1.0 ml. of B-P buffer at pH 8. The enzyme concentration was the same as above (0.113 mg/0.1 ml.). The oxygen tensions used were 20, 1.07, 1.04, 0.72, and 0.34% O_2. The Lineweaver-Burk plot is shown in Figure 7 and the line fits the equation, $v^{-1} = 2.38 \times 10^{-6} [O_2]^{-1} + 0.0045$. The correlation coefficient of the points is 0.993. The ppDAO is much more active with putrescine than with tryptamine, and these data show the activity to be more sensitive to changes in oxygen tension with a higher Km value when compared with the same concentration of enzyme and tryptamine.

Km Determinations for Bovine Plasma Amine Oxidase

For the studies with PAO at two levels of substrate, 0.067 M phosphate buffer at pH 7.1 was used instead of borate-phosphate buffer at pH 8. In the first set of experiments, benzylamine was

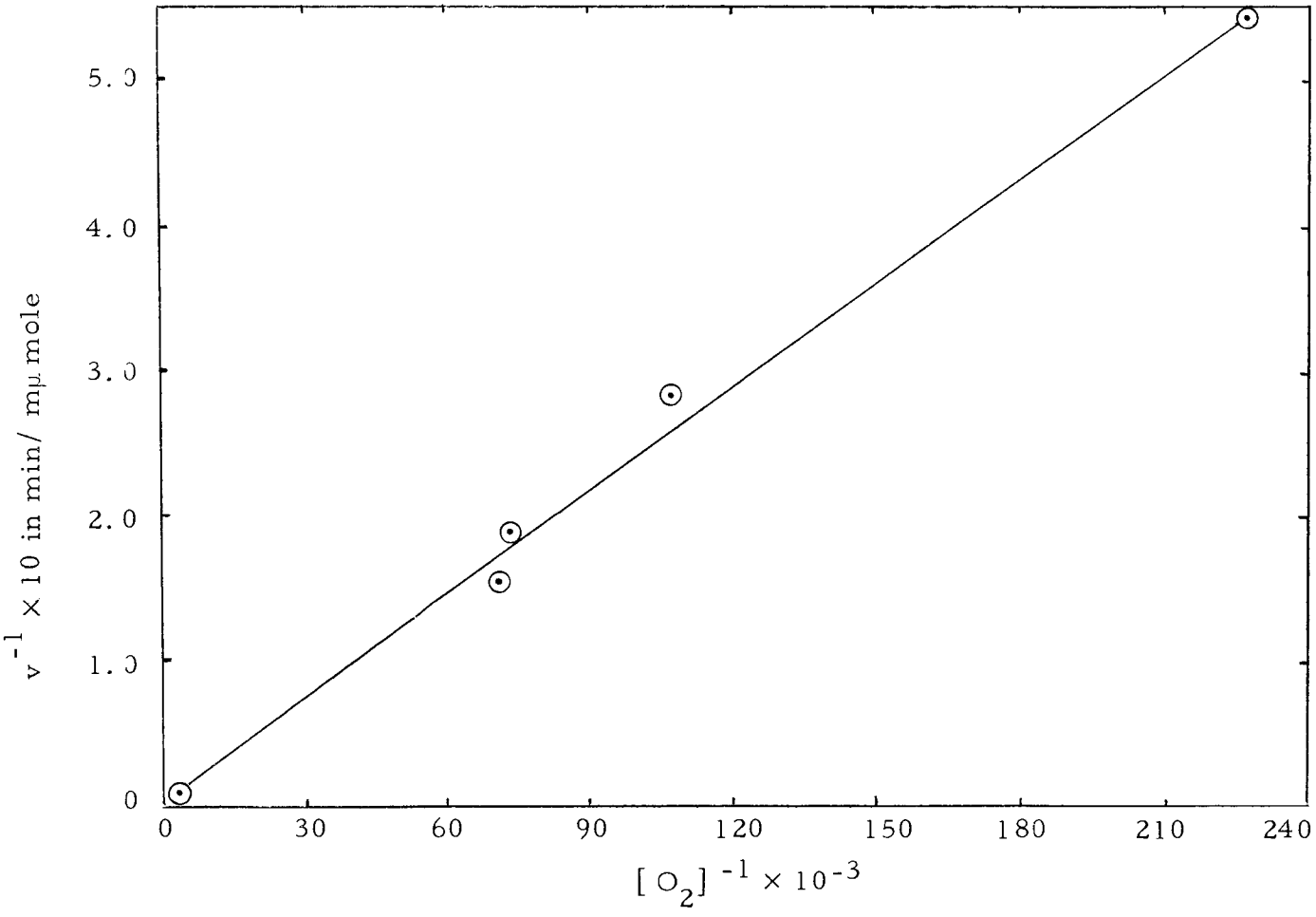


Figure 7. Lineweaver-Burk plot of the influence of oxygen tension on the rate of putrescine oxidation by partially purified diamine oxidase (ppDAO) at pH 8. Putrescine, 210 mμ moles, and 0.113 mg of ppDAO were incubated in a total volume of 1.1 ml.

used at a concentration of 3300 mμ moles containing 2.3×10^6 dpm of benzylamine 7-^{14}C per 1.0 ml. The same substrate concentration is used in the spectrophotometric assay of Tabor, Tabor, and Rosenthal (1954). PAO at a concentration of 0.138 mg (49% active) per 0.1 ml. was used. The system was equilibrated with 20, 4.65, 2.25, and 1.07% O_2. The reciprocal plot of v^{-1} versus $[O_2]^{-1}$ is shown in Figure 8. The points have a correlation coefficient of 0.999 and the least squares line fits the equation, $v^{-1} = 3.44 \times 10^{-6}[O_2]^{-1} + 0.105$. From this line, the Km was calculated to be 3.29×10^{-5} M.

The second benzylamine concentration (330 mμ moles plus 230,000 dpm) was a 1/10 dilution of the one previously used. The enzyme concentration was kept the same (0.138 mg per 0.1 ml.). The oxygen tensions used were 20, 8.2, 3.1, 2.7, and 1.07% O_2. The reciprocal plot is shown in Figure 9, and the points have a correlation coefficient of 0.991. The least squares line fits the equation, $v^{-1} = 3.48 \times 10^{-6}[O_2]^{-1} + 0.522$. From these data, the Km was calculated to be 6.67×10^{-6} M. Similar to the data for DAO, the O_2 Km value and V_{max} for PAO increased with increase in benzylamine concentration (from 330 to 3300 mμ moles per 1.0 ml.).

Table 1 contains a summary of O_2 Km values reported in this thesis. At each substrate concentration, a point at 0.0108% O_2 was determined, but the velocity found was, in all cases, too high to fit

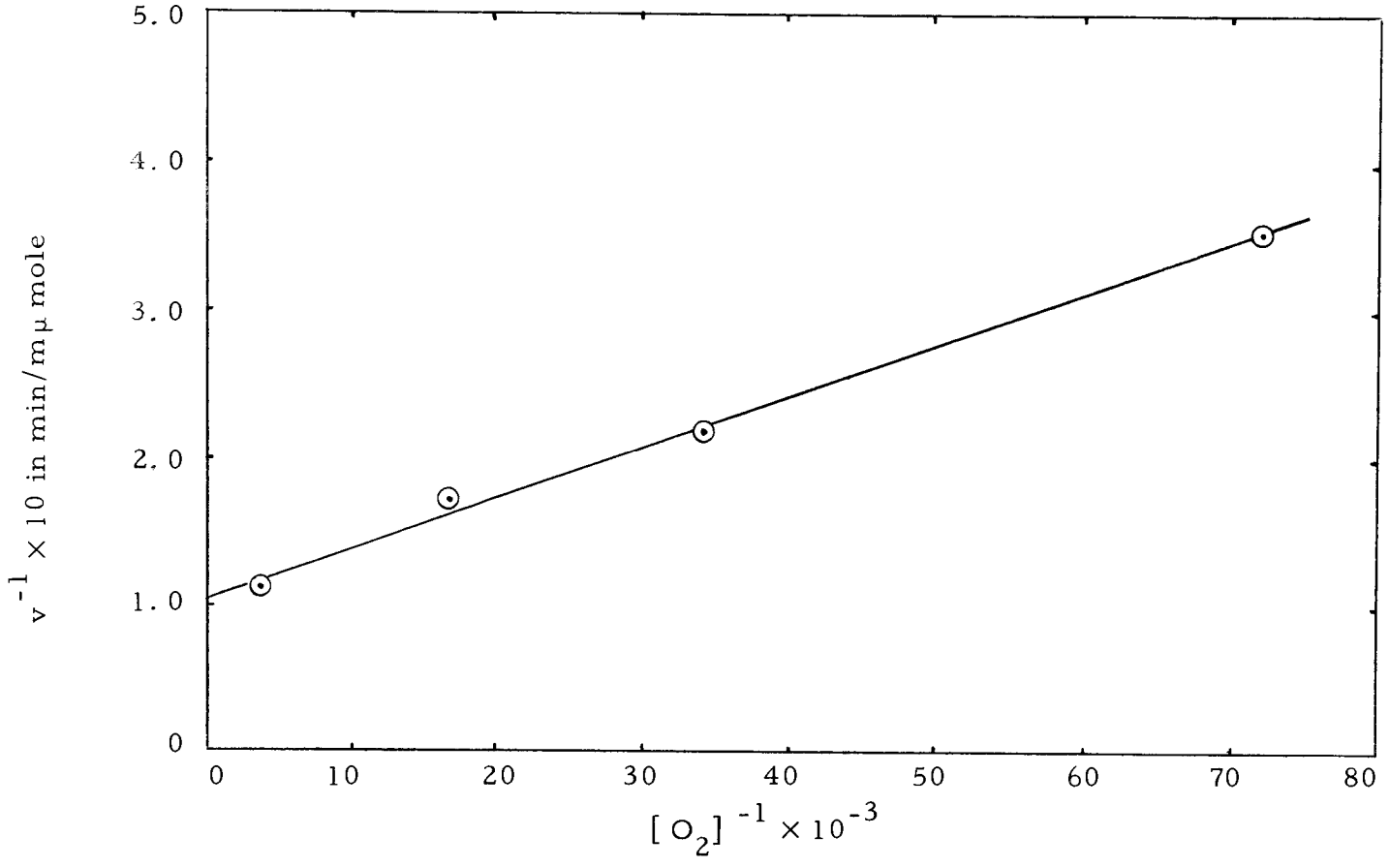


Figure 8. Lineweaver-Burk plot of the influence of oxygen tension on the rate of benzylamine oxidation by plasma amine oxidase (PAO) at pH 7.1. Benzylamine, 3300 mμ moles, and 0.138 mg of PAO were incubated in a total volume of 1.1 ml.

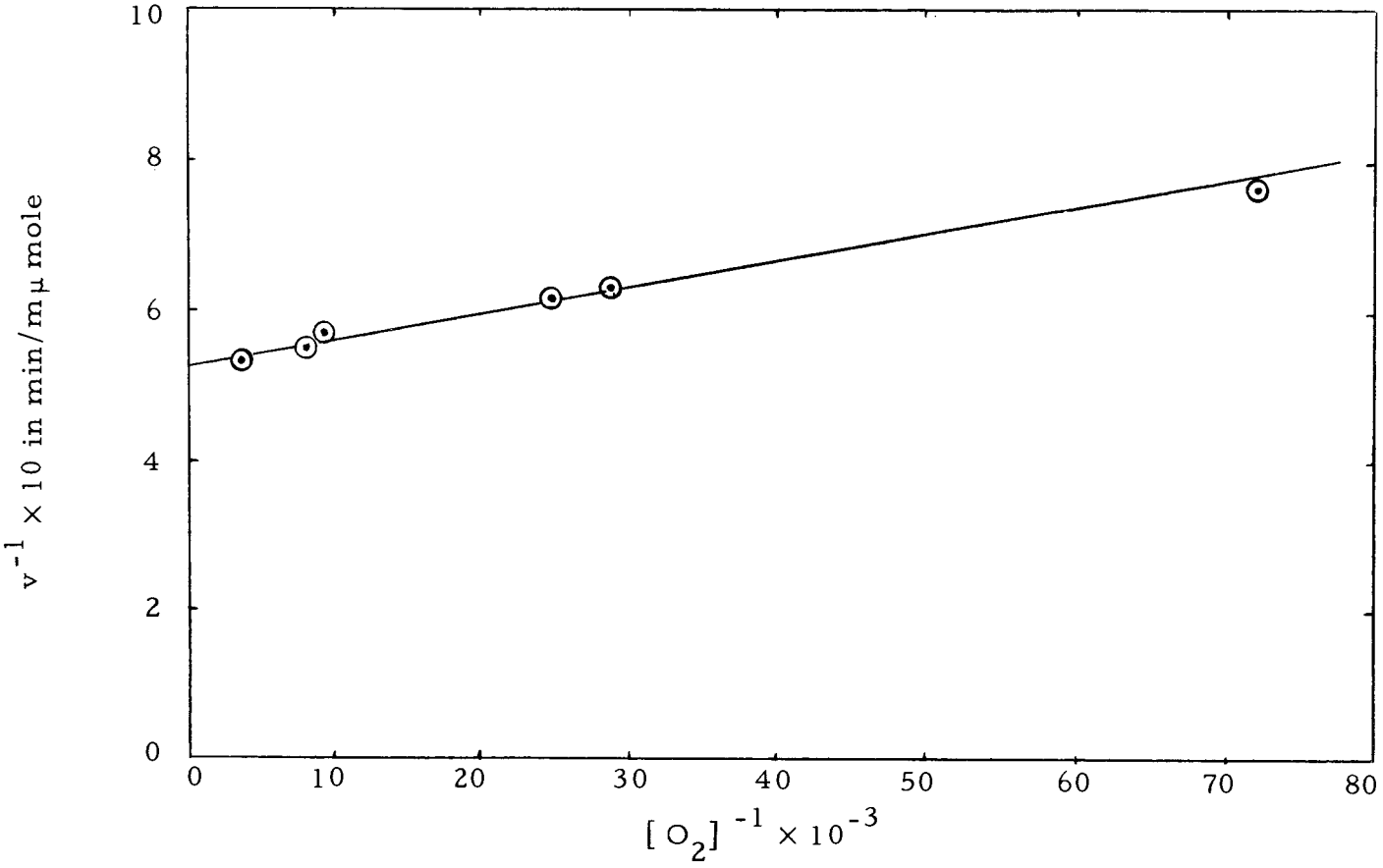


Figure 9. Lineweaver-Burk plot of the influence of oxygen tension on the rate of benzylamine oxidation by plasma amine oxidase (PAO) at pH 7.1. Benzylamine, 330 mμ moles, and 0.138 mg of PAO were incubated in a total volume of 1.1 ml.

Table 1. Summary of O_2 Km values

Enzyme	Substrate	Substrate concentration	O_2 Km	V_{max}*
PAO	benzylamine	3.3×10^{-3} M	3.3×10^{-5} M	9.5
	benzylamine	3.3×10^{-4} M	6.7×10^{-6} M	1.9
DAO	tryptamine	1.0×10^{-3} M	8.2×10^{-5} M	33.9
	tryptamine	2.0×10^{-4} M	4.1×10^{-5} M	16.3
ppDAO	tryptamine	2.0×10^{-4} M	6.6×10^{-6} M	3.4
	putrescine	2.1×10^{-4} M	5.3×10^{-4} M	220.8

*V_{max} in mμ m/min/reaction mixture

the reciprocal plot line through the other points. It was assumed that the equilibration time (8-12 hours) was too short to establish equilibrium at so low [O_2] in the solution. For example, four mμ moles of PAO produced 6.4 mμmoles of product. To fit the least squares line only 1.32 mμ moles of product should have been produced. However, if it is assumed that one mole of product can be produced per mole of enzyme in the absence of oxygen, then 5.32 mμ moles (4 + 1.32) should have been produced, and the values would not be expected to fit the line. At some of the low oxygen tensions, there was found to be more product formed than there was oxygen initially in the solution. For example, at 1.07% O_2, there would be about 15 mμ moles of oxygen in the 1.1 ml. of solution. With PAO and 3300 mμ moles of benzylamine, 28 mμ moles of product were formed in ten minutes. Since this is almost twice the amount of available oxygen initially present in the solution, there was the possibility that the reaction rate was limited by the rate of diffusion of oxygen into the solution. Umbreit, Burris, and Stauffer (1964), working at much higher oxygen consumptions in manometric techniques comment on the importance of shaking the solution. Although the points measured at low O_2 tensions were in good correlation (correlation coefficients were in all cases greater than 0.97) with those points where there was enough oxygen initially in the solution for the reaction rate to remain constant during the entire incubation

period, an experiment was conducted to determine if vigorous agitation would significantly change the amount of product formed. The experiment was performed with 0.138 mg of PAO at 1.07% O_2 tension using 3300 mμ moles of benzylamine. After a ten hour equilibration period, a reaction was run as usual with occasional agitation. Another reaction was run as usual except that the reaction mixture was carefully stirred 30 seconds out of every minute during the ten minute incubation period. There was observed to be no significant difference in the amount of product formed in the two reactions. These results support the conclusion that the rate of O_2 diffusion into the reaction mixture did not limit the reaction rate of any of the reactions which were used in obtaining data for the calculation of O_2 Km values.

Enzyme Purity

The proportion of protein which constituted active enzyme in the amine oxidase preparations was determined for the calculations of the molar ratio of aldehyde formed to enzyme present in the O_2-free reaction mixtures. The percent activities of the enzyme preparations are shown in Table 2. The specific activities of DAO and PAO are based upon assays in this laboratory with highly purified preparations. The specific activity of PAO (500 units/mg) using the spectrophotometric assay is in good agreement with that of

Table 2. Percentage of active protein in the enzyme preparations used in this study.

Enzyme	Assay method	Substrate	Specific activity*	Specific activity* of pure enzyme	% Activity
PAO	Spectrophotometric	Benzylamine	316	500	63
	Radiotracer	Benzylamine	65	132	49
DAO	Radiotracer	Tryptamine	300	295	100
ppDAO	Radiotracer	Tryptamine	28	295	9.5

*specific activity units - spectrophotometric, OD × 1000/mg-min
radiotracer, mμ mole/mg-min at 25 °C

Yamada and Yasunobu (1962b) indicating a purity of 95% or more. The specific activity value of PAO by the radiotracer assay method was calculated from the turnover number determined by the spectrophotometric assay procedure. The specific activity of DAO listed in the table was determined in this laboratory.

The difference found in the percent activities using the spectrophotometric and radiotracer assays on PAO may reflect an influence by several factors. For example, the radiotracer assay was conducted in the system described on pages 11 to 16. The system was equilibrated for 10.5 hours at 1 to 2° C with air and then assayed. Some inactivation of the PAO during the course of the equilibration may occur. The DAO and ppDAO preparations were also assayed after equilibration as described above for PAO. The radiotracer assay is used as an indication of the percent activity of all three enzymes for the O_2-free N_2 studies.

Molar Ratio Under Anaerobic Conditions

In this series of experiments, an attempt was made to find the number of moles of aldehyde produced per mole of enzyme under an atmosphere of O_2-free N_2. PAO (1.38 mg) was used for each assay and was found to be 49 percent active. The amount used was therefore, equal to 0.68 mg of pure enzyme. The substrate was benzylamine 7-^{14}C at a concentration of 3300 mμ moles containing 2.3×10^6

dpm per 1.0 ml. of buffer. The equilibration time using O_2-free N_2 was 36 hours. Duplicate assays were performed with ten and 20 minute incubation periods to determine the effect of an increase in incubation time. Blanks were run under identical conditions since there was a slight decrease in the blank count as the O_2 tension decreased in the system. The results are summarized in Table 3. An assay in the presence of 100 μg of catalase showed no significant increase in product formation. Since PAO is reported to have a molecular weight of 170,000 (Achee, 1968), the calculated amount of active enzyme used in the experiments was 3.98×10^{-9} moles. The average counts of the ten and 20 minute incubations is 1760 ± 220 cpm (±σ). The total amount of aldehyde formed is 3.97×10^{-9} moles. The molar ratio or turnover number under anaerobic conditions is therefore,

$$\frac{3.97 \times 10^{-9}}{3.98 \times 10^{-9}} = 0.998 \pm 0.12\ (\sigma)$$

Using DAO the data were not as close to a ratio of one as with PAO, nor were the duplicate points as good. Part of the variation was due to the fact that the experiments involving 20 minute incubation periods were run much later than the experiments with ten minute periods, and a new substrate was used. There was also the possibility that more of the enzyme had been inactivated by handling when the 20 minute incubations were run because all of the Km

studies were performed in between these two sets of experiments--a period of six to eight weeks. DAO, at a concentration of 0.05 mg per 0.1 ml., was used, and assuming a molecular weight of 96,000 (Mann, 1961), there were 5.21×10^{-10} moles of enzyme. The tryptamine concentration was 100 mμ mole containing 2.62×10^{6} dpm per 1.0 ml. The molar ratio for the ten minute incubation experiments was determined to be 0.88 ± 0.18 and the 20 minute incubation experiments had a ratio of 0.63 ± 0.10. These data are also summarized in Table 3.

The ppDAO was run in an O_2-free N_2 atmosphere in the presence of tryptamine and putrescine. The tryptamine concentration was the same as that used for DAO. The amount of enzyme was 0.113 mg of which 9.5% was active in the system. This is 1.41×10^{-10} moles of active enzyme and it produced 1.11×10^{-10} moles of aldehyde to give a molar ratio of 0.79. With 192 mμmoles containing 2.57×10^{6} DPM of putrescine, the ppDAO formed 1.89×10^{-10} moles of product for a ratio of 1.34. For the ppDAO ratios, no standard deviations could be given as they are single runs.

These results indicate that PAO and DAO in an anaerobic atmosphere will produce approximately one mole of aldehyde per mole of enzyme irrespective of time of incubation or presence of catalase. This could indicate that the enzyme can produce one mole of product per mole of enzyme in the absence of oxygen, but that oxygen is

Table 3. Stoichiometry of the formation of the aldehyde product by amine oxidases in the presence of excess amine substrate and the absence of molecular oxygen

Enzyme	Moles active enzyme	Incubation time in min	Substrate	CPM-blank[#]	Average cpm cpm ± σ	Moles product	Molar ratio
PAO	3.98×10^{-9}	10	Benzylamine	1,871	1760 ± 219	3.96×10^{-9}	0.99 ± .12
		10		2,062			
		20		1,514			
		20		1,593			
DAO	5.21×10^{-10}	10	Tryptamine	6,120	7742 ± 1658	4.61×10^{-10}	0.88 ± .18
		10		9,436			
		20		5,820*	5060 ± 760	3.28×10^{-10}	0.63 ± .10
		20		4,300*			
ppDAO	1.41×10^{-10}	10	Tryptamine	1,988		1.11×10^{-10}	0.79
		10	Putrescine	1,884		1.89×10^{-10}	1.34

* new substrate

\# blanks--Benzylamine 7-^{14}C, 1,776 cpm and 1,810 cpm (Av. 1,790 cpm); Tryptamine 2-^{14}C, 5,630 cpm (2,750 cpm for new substrate); Putrescine 1,4-^{14}C, 1,110 cpm

needed in a later step to return the enzyme to a form that can again catalyze the oxidative deamination of another amine molecule. The possibility cannot be excluded (from these data) that the enzyme-substrate complex that is formed under anaerobic conditions (Mann, 1961; Yamada and Yasunobu, 1962b) does not form the aldehyde, but the boiling of the solution to inactivate the enzyme creates a toluene-extractable product from the complex.

The data support the speculative mechanism of McEwen, Cullen, and Sober (1966) shown in Figure 2 which shows an initial pathway to the formation of the aldehyde from the amine in the absence of oxygen.

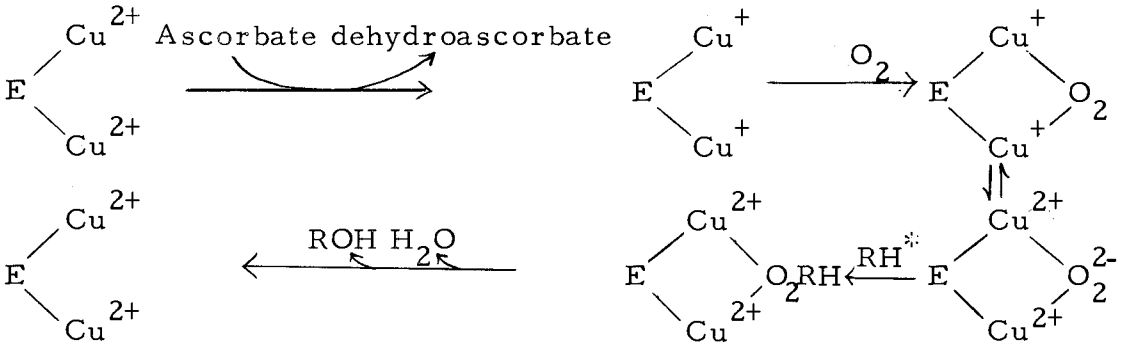
DISCUSSION

While there have been no previously published O_2 Km values for amine oxidases, the O_2 Km values for several other copper-containing proteins has been determined. Frieden, Osaki, and Kobayashi (1965) in a report on copper proteins and oxygen published several O_2 Km values which included:

Cytochrome Oxidase	4×10^{-6} M
Ascorbate Oxidase	2.2×10^{-4} M
Ceruloplasmin	7.9×10^{-6} M
Uricase	2×10^{-6} M
Tyrosinase	5.5×10^{-5} M

The O_2 Km values found in this research vary from 6.7×10^{-6} M for PAO with benzylamine (3.3×10^{-4} M) to 5.3×10^{-4} M for DAO with putrescine (2.1×10^{-4} M). These are in the same order of magnitude as the ones listed above. However the amine oxidase O_2 Km's change with substrate concentration so that an O_2 Km value cannot be stated without indicating the amine concentration used. Goldstein, Joh, and Garvey (1968) working with dopamine β-hydroxylase, another copper-containing enzyme, came to a similar conclusion in finding that the apparent O_2 Km changed with change in the dopamine concentration. They found that the apparent O_2 Km decreased with increase in substrate concentration. The opposite was found to be

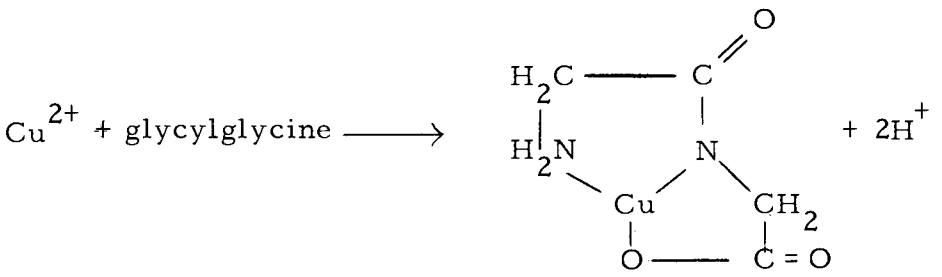
true with DAO and PAO; an increase in substrate concentration resulted in an increase in the apparent O_2 Km value. This was considered reasonable since the velocity (including V_{max}) increased with an increase in substrate concentration creating an added need for oxygen. In the copper-containing enzymes,the oxygen is considered to be bound to the copper, and some mechanisms have been proposed. Goldstein, Joh, and Garvey (1968), proposed the following reaction scheme for dopamine β-hydroxylase:



*RH = dopamine

Other proposed schemes are similar to the one shown above. Unfortunately in these schemes the valence of copper changes, and no valence change has been observed in the amine oxidases when they are reacted with substrate in the absence of O_2 (Hill and Mann, 1962; Mondovi et al., 1967, Yamada and Yasunobu, 1962b; and Yamada et al., 1963). ESR work by itself does not appear to give enough evidence

to support conclusively the statements that copper does not change valence state in the amine oxidases, since model systems of Cu(II) complexes have shown different signals depending on the ligands involved (Hemmerich, 1966). Much is yet to be learned on the ESR signals of Cu(II) complexes in the amine oxidases. Hemmerick (1966) has studied the Cu(II) affinities of protein functional groups. With Cu(II)-protein complexes, he found a highly stable five-member chelate ring was formed which involved deprotonation of a peptide nitrogen as in the example:



Buffoni (1968) reduced pig PAO enzyme with sodium borohydride both in the presence and absence of substrate under anaerobic conditions. She was able to inactivate the enzyme in both cases, and the data suggested that an imine bond was formed between the aldehyde group of pyridoxal and the substrate, and that the pyridoxal phosphate was linked to the enzyme in the form of a Schiff base These data must be observed with caution, however, because her anaerobic conditions consisted of evacuation of the air and reacting in a vacuum. The possibility of oxygen still being present and allowing the reaction to

proceed further than under true anaerobic conditions cannot be ruled out.

A model has been proposed by Hamilton (1968) for amine oxidase with no valence change of the metal ion. However he found his model to be the most effective with Mn(III), and Co(II) and he admits that Cu(II) which is found in the amine oxidases is ineffective in the model.

The observed discrepancies between the apparent O_2 Km's of DAO and ppDAO at the same tryptamine concentration is not easily explained (DAO Km = 4.1×10^{-5}, ppDAO Km = 6.6×10^{-6} M). The ppDAO was only 9.5% active and the nature of the remaining protein is not known. Since at some of the points assayed, the amount of oxygen in the solution was very small, the presence of catalase as an impurity, with its very high turnover number, could possibly make more oxygen available for the ppDAO as well as preventing any possible H_2O_2 inhibition, thus giving an apparent O_2 Km lower than that for the purified DAO.

The molar ratio studies support the model of McEwen, Cullen, and Sober (1966) shown in Figure 2 in that the data suggest that one mole of aldehyde is produced per mole of enzyme under anaerobic conditions. The molar ratio studies were in an O_2-free N_2 system in which an activated copper column described in the materials section reduced the concentration of O_2 to less than 4×10^{-5}% O_2 at 760 mm pressure. Therefore, at 25°C, the oxygen concentration

in the enzyme-substrate solution would be less than 5.2×10^{-10} M, assuming that there are no leaks permitting diffusion of O_2 into the system. Since the flow rate was 40 to 70 ml/min, there was a slight positive pressure in the system during equilibration, and catalase did not increase velocity, it was assumed that no significant amount of oxygen leaked into the system. This O_2 concentration is equal to 5.7×10^{-13} moles of O_2 in the 1.1 ml. reaction volume. Since the number of moles of active enzyme was in all cases greater than 10^{-10} moles, the concentration of O_2 was low enough to consider the system anaerobic, therefore one can tentatively conclude that the reaction proceeded to form a mole of aldehyde per mole of enzyme in the absence of O_2. As a check on the value obtained at this very low O_2 tension, some points should be run below the point determined from the least squares line, for each enzyme at a given substrate concentration, to give one mole of product in ten minutes incubation period. For example, the least squares line for PAO reacted with 3300 mμ mole of benzylamine was $v^{-1} = 3.44 \times 10^{-6} [O_2]^{-1} + 0.105$. From this line, the oxygen tension necessary to allow one mole of product to be formed per mole of enzyme in ten minutes is 0.1%. The amount of product formed at 0.1% should give a ratio of two moles of product per mole of enzyme and the values of O_2 tension below 0.1% should show a progression toward a limit of one mole of product per mole of enzyme instead of zero, thus substantiating the idea that one mole

of product is formed per mole of enzyme under anaerobic conditions. Work is in progress in this laboratory to determine the velocities at oxygen tensions below 0.1%.

The data presented here suggest a mechanism in which the substrate (amine) reacts with the enzyme to form a product (aldehyde) and a reduced enzyme. The reduced enzyme then reacts with O_2 to oxidize the enzyme, and the oxidized enzyme reacts with H_2O to form the active enzyme:

$$E + S_{amine} \rightleftharpoons ES_{amine} \longrightarrow E_{reduced} + P_{aldehyde}$$

$$E_{reduced} + S_{O_2} \rightleftharpoons E_{reduced}S_{O_2} \longrightarrow E_{oxidized} + H_2O_2$$

$$E_{oxidized} + H_2O \longrightarrow E + NH_3$$

This mechanism is consistent with the data supporting one mole of aldehyde per mole of enzyme in the absence of oxygen, and the data showing that the apparent O_2 Km increases with, and is, therefore, dependent on an increase in amine concentration.

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