

Kinetics and Other Characteristics of Diamine Oxidase of Pea Seedlings

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1. Spectrophotometric titration with phenylhydrazine has given evidence that diamine oxidase of pea seedlings contains one mol of reactive carbonyl groups per mol of enzyme.

2. The effect of variation in concentration of five amine substrates and oxygen, on the steady-state kinetic behaviour, have been studied in detail. The kinetics are found to conform to a Dalziel rate equation without ϕ_{12} term, and thus the enzymatic reactions are proposed to follow a ping-pong mechanism. Investigation of the pH dependence of the enzyme has given strong indication that for good binding the amine group in the substrate, which participates in the oxidation, must be uncharged, while the other amine group ought to be positively charged.

3. Electron paramagnetic resonance spectra showed that the protein-bound copper is not significantly reduced in the presence of substrate under anaerobic conditions, but a distinct shift in the signal gives evidence for a change in the way that the copper is bound.

Diamine oxidase of pea seedlings, like kidney diamine oxidase and plasma amine oxidase (benzylamine oxidase), contains copper¹⁻⁴ and a functional carbonyl group, which in the case of the last two enzymes is almost certainly pyridoxal phosphate.⁵⁻⁶ However, attempts to demonstrate pyridoxal phosphate in pea seedling diamine oxidase have been unsuccessful.⁷

The presence and requirement of Cu²⁺ in the enzyme is well established,¹⁻⁴ but its function is not understood. Cupric ions are required to activate copper-free oxidase of pea seedlings,⁴ bovine plasma,⁸ and pig kidney.⁹ Electron paramagnetic resonance studies have provided strong evidence that no change in valence state of copper occurs in benzylamine oxidase of blood plasma during catalysis.¹⁰ The situation

in the case of pig kidney diamine oxidase is more obscure, and evidence for reduction of copper has been reported.¹¹ The present investigation gives evidence for no reduction of copper in diamine oxidase of pea seedlings in the presence of substrate under anaerobic conditions.

The catalytic mechanism for the enzyme has not been well clarified. For some other amine oxidases strong evidence has been given for a ping-pong mechanism.¹²⁻¹⁴ The present study attempts to clarify some characteristic kinetic behaviour of pea seedling diamine oxidase, and to see whether a mechanism similar to that found with other amine oxidases can be applied to this enzyme with its much higher specific activity.

MATERIALS AND METHODS

Diamine oxidase was prepared from pea seedling as described by Hill and Mann,⁴ followed by column chromatography on Sephadex G-200. The enzyme thus obtained was 80–90 % pure according to specific activity tests, and was used for most of the experiments. For determination of the amino acid composition, the enzyme was further purified by analytical gel electrophoresis at pH 7.8. Preparations thus obtained had a specific activity of 85 U based on protein estimations with the Folin-Ciocalteu method as modified by Lowry *et al.*¹⁵ and oxygen consumption measurements with a Clark electrode combined to an Eschweiler Combi-analysator U. The electrophoretically purified enzyme was used as a reference for almost pure enzyme. The specific activity was well above that reported by Hill and Mann⁴ for an almost pure enzyme preparation. However, they used somewhat different

estimation procedures which may account for the discrepancy.

Other chemicals used in this investigation were of highest available purity. ^{14}C -Putrescine was obtained from Amersham, and catalase (2 times crystallized from bovine liver) was obtained from Sigma.

Titration with phenylhydrazine. Titrimetric experiments with phenylhydrazine at pH 7.2 in 0.2 M phosphate buffer were carried out according to methods previously described.¹⁶ The concentration of the inhibitor hydrazine was such that 2–6 portions were required to reach the equivalence point. The enzyme selected for these experiments was more than 85 % pure according to specific activity determinations. In contrast to what has been found with other hydrazines,^{4,17} the equilibration between phenylhydrazine and enzyme was very rapid. However, to ensure that equilibrium was attained, the reaction was allowed to proceed for 2 min after each addition of phenylhydrazine before measurements were made. 15 μl of this enzyme suspension were then assayed in the presence of 4 mM putrescine.

Steady-state kinetic methods. The enzymatic activity of diamine oxidase of pea seedlings was determined from the initial oxygen consumption at various concentrations of oxygen and amine substrate. The 2.5 ml reaction vessel contained enzyme, substrate and 50 μg catalase

in 0.2 M potassium phosphate buffer. Above pH 8.0, 0.15 M potassium phosphate and 0.05 M potassium borate was used as the buffer. Estimations of initial activity were not possible at very low oxygen concentration. However, as the product inhibition is very low with all the substrates examined, except tryptamine, the kinetics at various oxygen concentrations could be determined by following oxygen consumption continuously until all the oxygen was consumed. The same procedure was used with tryptamine as substrate, but initial activity estimations were made down to an oxygen concentration of 5 μM , so that the product formed never exceeded 10 μM and the product inhibition could be neglected.

Transient-state kinetic methods. Transient-state kinetic experiments were performed in the Aminco-Morrow stopped-flow apparatus described by Lindström *et al.*¹⁰ Anaerobic conditions were attained by the pumping-flushing technique described by Carrico *et al.*¹⁸

Electron paramagnetic resonance spectra of diamine oxidase. EPR spectra of diamine oxidase (about 80 % pure) were recorded at pH 8.0 in a Varian E-3 spectrometer at 77 K and 9.2 GHz. Spectra of the substrate-reduced form of the enzyme were determined in presence of 4 mM putrescine. The substrate was added as a solution directly into the EPR tube in an amount that diluted the enzyme by 20 %, and made the enzyme completely reduced and the solution anaerobic within a few seconds.

Table 1. Amino acid composition found in acid hydrolysate from electrophoretically purified diamine oxidase of pea seedling. Values are expressed as mol of amino acid per mol of methionine found in the hydrolysate.

Aspartic acid	16.8
Threonine ^a	9.2
Serine ^a	26.5
Glutamic acid	24.3
Proline	Not determined because of interference
Glycine	22.1
Alanine	11.2
Half-cystine	—
Valine	7.6
Methionine	1.0
Isoleucine	7.0
Leucine	8.2
Tyrosine	4.3
Phenylalanine	4.9
Histidine	4.7
Lysine	6.6
Tryptophan	—
Arginine	4.5
Ornithine	5.8

^a Compensated for degradation during 24 h hydrolysis in 6 M HCl.

RESULTS

General properties. The general properties of the enzyme preparations used in the present investigation agree in all essential features with those reported by Hill and Mann.^{2,4,20} The amino acid composition of the electrophoretically purified enzyme (Table 1) shows an unusually high content of ornithine, and as no half-cystine was observed, sulfur bridges seem to be absent in the enzyme.

Active site titration with phenylhydrazine. Pea seedling diamine oxidase like amine oxidase from pig kidney⁹ and blood plasma,¹⁹ shows a broad and fairly weak absorption in the visible region. On addition of phenylhydrazine the enzyme solution turns yellow due to the formation of an absorption band centered around 435 nm. Equilibrium is rapid and essentially completed within 15 s after each addition of phenylhydrazine at an enzyme concentration of 15 μM . No further change in absorption could be observed after addition of a threefold excess of phenylhydrazine, but the enzyme still showed

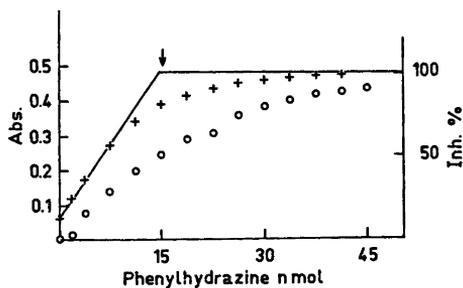


Fig. 1. Spectrophotometric titration of pea seedling diamine oxidase with phenylhydrazine. (+) shows the titration of 15.0 nmol enzyme in 1 ml 0.1 M phosphate buffer, pH 7.0, with 5 μ l or 10 μ l portions of 375 μ M phenylhydrazine, measured at 435 nm; (O) shows the inhibition found simultaneously. Absorbancies and inhibitions have been corrected for dilution effects, and refer to a volume of 1 ml. Arrow indicates point of equimolar amounts of enzyme and inhibitor.

a low activity if measured in the presence of 4 mM putrescine (Fig. 1). The residual activity may be explained by the competition between substrate and hydrazine, as has been found with other hydrazines.¹⁷ From Fig. 1, the number of active sites per molecule of enzyme is estimated graphically to be close to one, assuming a molecular weight of 96 000.⁴ In an attempt to provide evidence for the presence of pyridoxal phosphate, indicated by the binding of hydrazines, the enzyme was reacted with ¹⁴C-putrescine in an anaerobic solution, and reduced with NaBH₄, as described by Buffoni.⁵ The treatment led to a significant incorporation of radioactivity into the protein, but no product which would indicate pyridoxal phosphate in the native enzyme was identified from paper electrophoresis at pH 3.6 after acid hydrolysis.

Electron paramagnetic resonance spectra of the enzyme. Fig. 2A shows the EPR spectrum of an 80 % pure preparation of pea seedling diamine oxidase as such, and Fig. 2B shows the spectrum recorded after reduction of the enzyme with an excess of substrate. The main features of the spectra indicate that the EPR signal arises largely from protein-bound Cu²⁺. However, the contribution from an overlapping signal is possible on the basis of comparison with the EPR spectra reported for pig kidney diamine oxidase¹¹ and benzylamine oxidase.¹⁰ This might be related to the presence of small amounts of

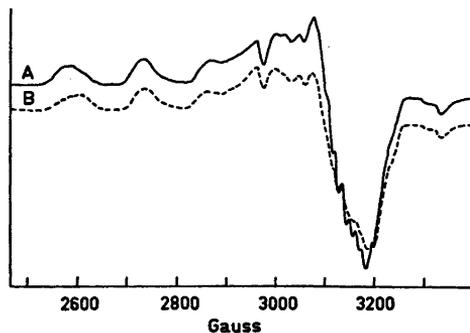


Fig. 2. Electron-paramagnetic-resonance spectra of 80 % pure diamine oxidase at 77 K and 9.2 GHz. Spectra were recorded at pH 8.0 in 0.4 ml aerobic 0.2 M phosphate buffer (A) and in an anaerobic solution after addition of 80 μ l of a 24 mM solution of putrescine (B).

Mn²⁺, which has been reported as an impurity in pea seedling diamine oxidase preparations which are not homogeneous.²⁰ Integration of the spectra in Fig. 2 showed that there is no reduction of copper in the presence of a large excess of substrate, but the minor shifts observed for the two low-field hyperfine lines indicate a significant change in the binding situation of copper.

Kinetic experiments. Several amine oxidases have been reported to conform to a ping-pong mechanism.^{12,14} As for pea seedling diamine oxidase, the ping-pong mechanism has been proposed from kinetic experiments with tryptamine by Yamasaki *et al.*¹⁷ Attempts to verify their results showed that the strong product inhibition observed with tryptamine as substrate could substantially affect the results. The investigations described in this paper are therefore necessary for a more careful evaluation of the mechanism.

Preliminary experiments with different substrates showed that the apparent K_m for O₂ was always very low and was dependent on the nature and the concentration of the amine substrate. With poor substrates such as benzylamine the apparent affinity for oxygen was too high for a determination of the apparent K_m for O₂ (< 1 μ M). Detailed kinetic studies of steady-state rate behaviour of the enzyme were carried out using the substrates: putrescine, cadaverine, histamine, tryptamine, and lysine. Each substrate was found to give linear and

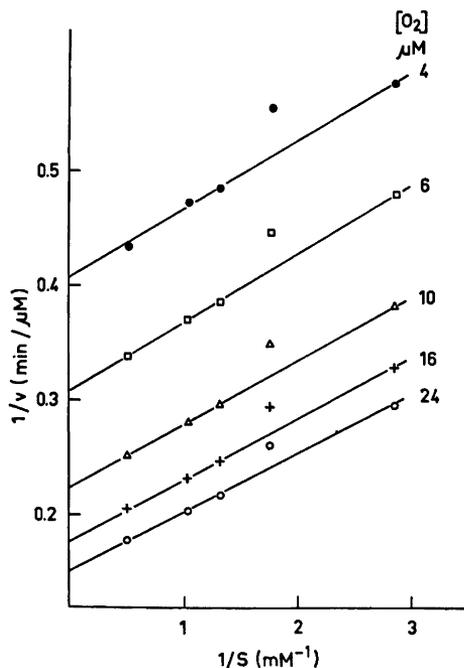


Fig. 3. Lineweaver-Burke plots of the effect of putrescine concentration on the rate of oxidation at different fixed concentrations of oxygen. Reaction solutions contained the diamine oxidase, catalase, and the substrates at 25 °C in 0.2 M phosphate buffer, pH 7.0.

parallel reciprocal plots when the oxygen concentration was varied at different constant substrate concentrations or *vice versa*, as is exemplified with putrescine (Figs. 3 and 4) and histamine (Figs. 5 and 6) at pH's 7.0 and 7.2, respectively. These data are consistent with the rate equation:

$$C_e/v = \phi_0 + \phi_1/\phi_2 + \phi_2/S \quad (1)$$

As can be seen from Fig. 7 estimates of ϕ_1 obtained for the different substrates, except histamine, exhibit no significant variation with pH over the range 6–9. In reality the small variation observed may be ascribed to limitations in the function of the pO_2 apparatus at oxygen concentrations below 3 μM . Figs. 8 and 9 show that both ϕ_0 and ϕ_2 are strongly dependent on pH and vary in magnitude for the different substrates. Slopes of the straight lines obtained in plots of $\log \phi_2$ vs pH (Fig. 8) are essentially independent of the substrate, but the magnitude

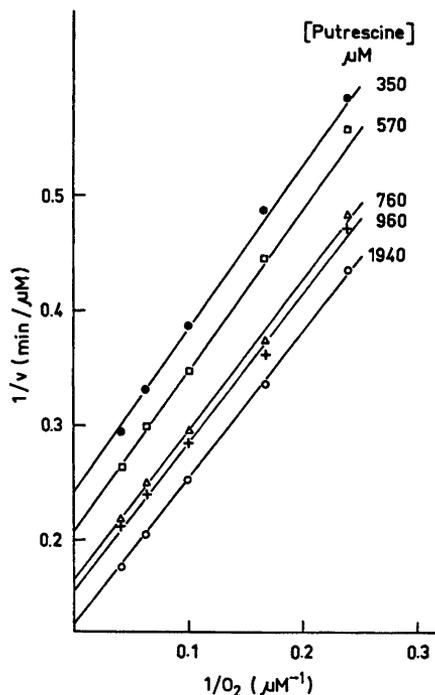


Fig. 4. Lineweaver-Burke plots of the effect of oxygen concentration on the rate of oxidation at various fixed concentrations of putrescine. Conditions as in Fig. 3.

of ϕ_2 is obviously quite dependent on the nature of the substrate. ϕ_0 shows a minimum between pH 7.5 and 8.3 for all the substrates except histamine. Thus histamine deviates markedly in the behaviour of both ϕ_0 and ϕ_1 compared with the other substrates. In order to exclude the possibility that histamine reacts with a second active site in the enzyme or with some other enzyme present in the preparation, the influence of histamine on oxygen consumption was determined in the presence of cadaverine. The Lineweaver-Burke plots in Fig. 10 show that there is a strict competition between the two substrates for the same site in the enzyme.

Anaerobic-transient-state-kinetic experiments. If diamine oxidase of pea seedling is reduced by substrate under anaerobic conditions, the absorption band centered around 500 nm is replaced by bands with maxima at 466, 437.5, and 350 nm.⁴ The formation of these bands and the disappearance of the 550 nm band was found to be very fast in the presence of 2 mM

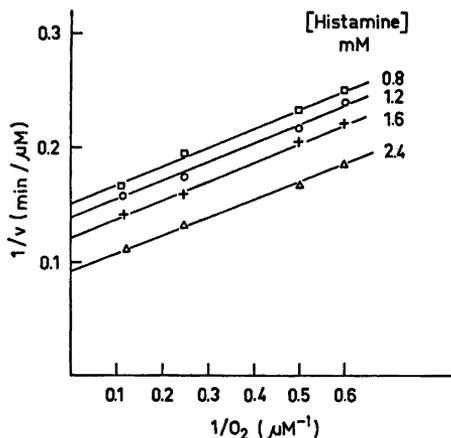


Fig. 5. Lineweaver-Burke plots of the effect of oxygen concentration on the rate of oxidation at various fixed concentrations of histamine. Conditions as in Fig. 3 except that pH was 7.2.

histamine. At 25 °C and pH 7, most of the absorption changes were within the dead-time (5 ms) of the instrument, but from what was registered all the chromophore changes seemed to be simultaneous.

DISCUSSION

Pea seedling diamine oxidase, pig kidney diamine oxidase and benzylamine oxidase are closely related in many respects. All these enzymes catalyze the oxidation of various amines

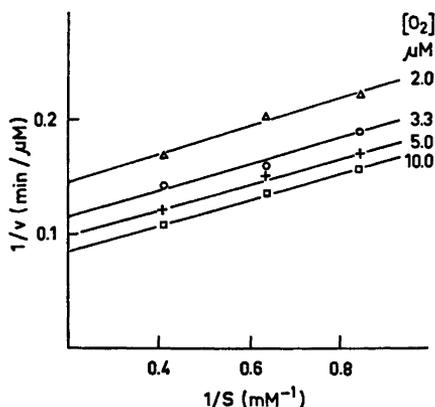


Fig. 6. Lineweaver-Burke plots of the effect of histamine concentration on the rate of oxidation at various fixed concentrations of oxygen. Conditions as in Fig. 5.

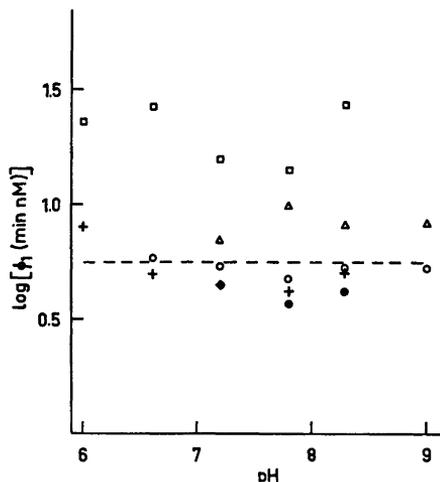
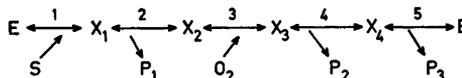


Fig. 7. Logarithmic plots of ϕ_1 for various substrates vs. pH. For conditions see text. The symbols used are for histamine (□), lysine (Δ), tryptamine (●), cadaverine (+), and putrescine (○).

by molecular oxygen with the formation of the corresponding aldehydes, ammonia, and hydrogen peroxide. In this paper evidence is given that diamine oxidase of pea seedlings, like two other amine oxidases, conform to the rate equation (1), which for the other enzymes, has been taken as evidence for a ping-pong mechanism.^{13,14} The results in this paper are in accordance with the mechanism in Scheme 1, suggested for benzylamine oxidase by Taylor *et al.*¹⁴ The kinetic investigation cannot, however, provide any information as to the order in which the different products are released. If the kinetics are estimated under circumstances where no product inhibition is observed, steps 2, 4, and 5 can be regarded as irreversible, and thus: $\phi_0 = 1/k_2 + 1/k_4 + 1/k_5$, $\phi_1 = (k_{-3} + k_4)/k_3k_4$, and $\phi_2 = (k_{-1} + k_2)/k_1k_2$. An examination of the different ϕ_1 terms (Figs. 7, 8, and 9) shows that,



Scheme 1. The ping-pong mechanism suggested by Taylor *et al.*¹⁴ for reactions catalyzed by benzylamine oxidase. S stands for amine substrate and P_i for the formed products. X_i denotes intermediately formed enzymatic species.

contrary to what is found with benzylamine oxidase,¹⁰ the maximum activity under physiological conditions is largely independent of the oxygen concentration (ϕ_1).

For benzylamine oxidase a mechanism has been proposed, where the unprotonated amine is the real substrate.^{21,22} A similar situation seems very likely for diamine oxidase of pea seedlings, as $\log(\phi_2)$ decreases almost linearly with pH. All the substrates give slopes close to -1 (-0.6 to -0.9), which would be expected if only the substrates with uncharged amine groups can be oxidized by the enzyme, as the amine groups that participate in the oxidation always have pK_a 's above 9.5. If this assumption is taken into account, the linear decreases of $\log(\phi_2)$ vs. pH would largely reflect the increasing substrate concentration with increasing pH. The magnitude of ϕ_2 , and most likely the binding of the substrate to the enzyme is, however, strongly dependent on the structure and the ionization of the substrate. All sub-

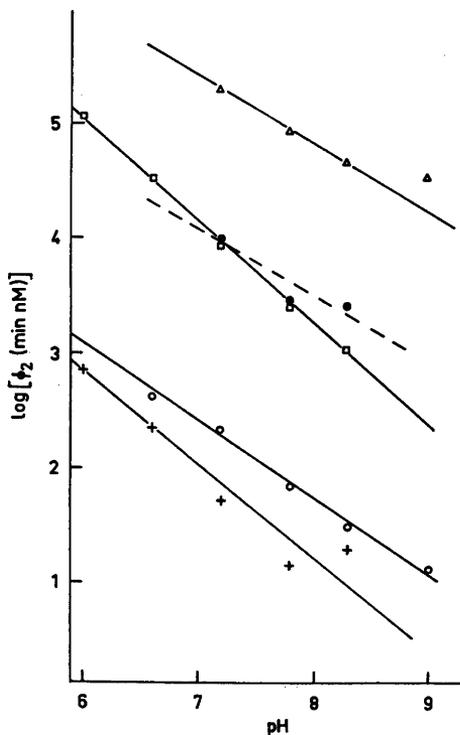


Fig. 8. Logarithmic plots of ϕ_2 for various substrates vs. pH. Symbols and conditions as Fig. 7.

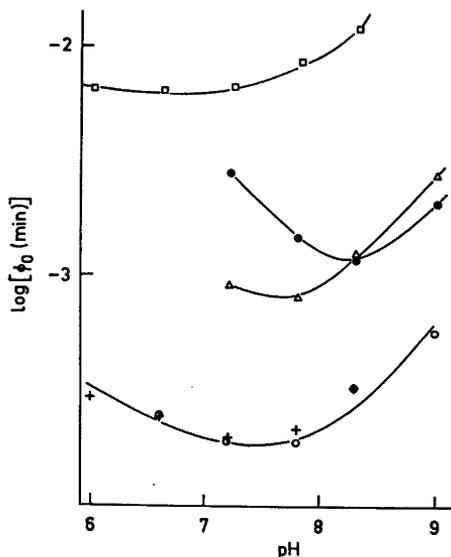
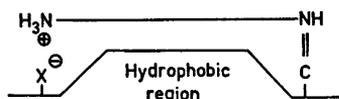


Fig. 9. Logarithmic plots of ϕ_0 for various substrates vs. pH. Symbols and conditions as in Fig. 7.

strates of pea seedling diamine oxidase have one primary amine group which participates in the oxidation procedure, one hydrophobic region in the middle, and one specific region at the other end of the molecule. According to the nature of the specific region, the substrates can be divided into three main groups, which are characterized by different ϕ_2 values. Low ϕ_2 values are found in the first group, which contains substrates with a positively charged primary amine in the specific part of the substrate. Substrates in the second group are mainly uncharged in the specific part and are characterized by intermediate ϕ_2 values. High ϕ_2 values are found in the third group, which contains substrates with both a positively and negatively charged group in the specific part of the substrate.

The mode of enzyme-substrate interaction may, according to the discussion above, be described as in Scheme 2, proposed for pig kidney diamine oxidase.²² The negatively charged group of the enzyme proposed in Scheme 2 is most likely a carboxyl group. Preliminary experiments in this laboratory have shown, that modification of carboxyl groups with carbo-diimide causes strong inhibition of pea seedling diamine oxidase activity. This inhibition may



Scheme 2. The binding situation proposed for enzyme-substrate interaction with pig kidney diamine oxidase by Barsley *et al.*²² (X) stands for a negatively charged group in the enzyme.

be due to modification of the negatively charged group proposed in Scheme 2.

From Figs. 7 and 9 it is clear that the behaviour of ϕ_0 and ϕ_1 differs markedly between histamine and all the other substrates used. The difference in ϕ_0 can be explained by the low pK_a for the imidazole moiety in histamine, which causes deprotonization mainly between pH 6 and 7, thus resulting in a poorer binding situation at high pH. The cause of the much higher ϕ_1 obtained with histamine is more difficult to explain but might reflect a negative co-operation between the imidazole moiety of the substrate and a group of the enzyme which participates in the binding of oxygen.

A valence change of copper could not be detected after reaction of diamine oxidase and substrate in an anaerobic solution. This does not exclude the possibility that reduction of copper may be an obligatory step in the catalytic mechanism, as has been pointed out by Mondovi *et al.*¹¹ A rapid redox equilibration between certain enzymatic species, favouring

those containing copper in the oxidized state, would result in less than stoichiometric reduction of the electron paramagnetic resonance signal by substrate under anaerobic conditions. If such a situation exists in the case of diamine oxidase of pea seedlings, it may be concluded that the anaerobic equilibrium concentration of enzyme species containing copper in the reduced state is less than 5% of the total enzyme concentration. The present investigation does not, however, give any indication of a valence change of copper during catalysis.

From this investigation it is clear that diamine oxidase of pea seedlings is closely related to other amine oxidases in many respects. Thus, all amine oxidases investigated show ping-pong mechanism. However, contrary to benzylamine oxidase,¹⁰ the rate determining steps in catalysis with pea seedling diamine oxidase are independent of the oxygen concentration under physiological conditions. The function of copper must be quite similar for all the enzymes, even if it is doubtful that the cofactor is pyridoxal phosphate in pea seedling diamine oxidase.

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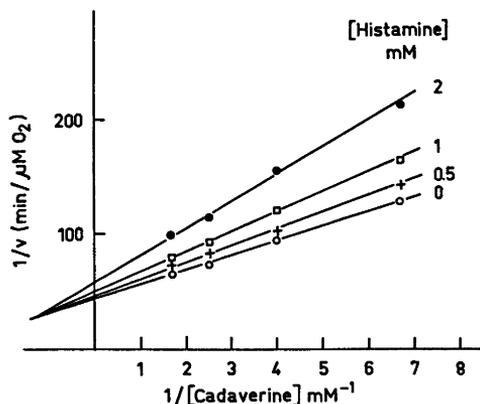


Fig. 10. Lineweaver-Burke plots of the effect of cadaverine concentrations on the rate of oxidation at various fixed concentrations of histamine. Conditions as in Fig. 3 except that pH was 7.2.

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