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SOME ASPECTS OF L-AMINO ACID OXIDASE CATALYSIS

by

Lawrence Harvey Levine

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Physical Chemistry

Approved:

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Head of Major Department

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Dean of Graduate College

Iowa State University
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Ames, Iowa

1963

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INTRODUCTION

The science of enzymology has come a long way since the first enzyme was recognized in 1833 (1). An increase in the knowledge of the structural organic chemistry of biological substances made possible the study of enzyme specificity. Serious purification of enzymes did not begin until around 1920 but now, however, there have been almost one hundred enzymes crystallized and over five hundred have been purified partially.

The characteristic function of enzymes is the catalysis of biological reactions. Any study of this catalytic function must be based on quantitative measurements of the rates of the catalysed reactions. From the effect on the rate of variation in the experimental conditions, inferences may be made about the mechanism of the enzyme action.

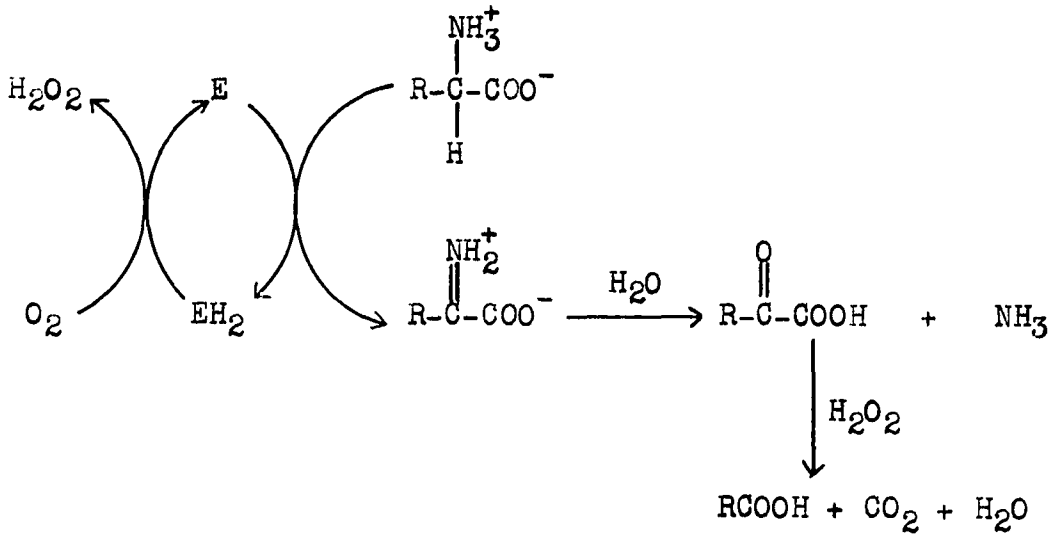
The availability of enzymes in the pure state has made possible quantitative investigations by physical methods. The development of enzyme kinetics, which received its initial impetus from the classical work of Michaelis has progressed in recent years and is presently being dynamically pursued with the objective of understanding the mechanism of enzyme catalysis.

In spite of the advances made since Brown first postulated the existence of the enzyme-substrate complex in 1902 (2) and Stern identified it as the red intermediate in the

catalase-peroxide reaction in 1936 (3) the nature of the enzyme-substrate complex is still not understood. The purpose of this investigation was to uncover some of the properties of this complex which might then aid in our understanding of the mechanism of enzyme catalysis.

The enzyme studied in this investigation was L-amino acid oxidase from snake venom. This enzyme contains flavin adenine dinucleotide (FAD) as the prosthetic group (4).

L-amino acid oxidase catalyses the oxidation of certain amino acids to the corresponding imino acids which subsequently undergo spontaneous hydrolysis to the ∝-keto acids. The ∝-keto acids thus formed may then be decarboxylated by the peroxide formed during the reoxidation of the enzyme by oxygen or other suitable oxidant.



In this investigation the enzyme-substrate complex was studied by kinetic treatment of the high substrate concentration inhibition and by the deuterium isotope effect.

ISOTOPE EFFECT

Discussion

After the existence of deuterium had been confirmed experimentally, it was predicted (5, 6) that hydrogen and deuterium should react at different rates because of the difference in their zero point energies. This prediction has been amply verified, and the isotope effect has been found to be of great value in the study of mechanisms of chemical reactions.

Theory

Three factors contribute to the generally lower reactivity of bonds to deuterium as compared to the corresponding bonds to hydrogen. These are the difference in free energy, the effect of the difference in mass on the velocity of passage over the potential energy barrier, and the possibility of non-classical penetration of the energy barrier (7, 8). The major factor which contributes to the free energy difference is the difference in zero point energy between a bond to deuterium and a corresponding bond to hydrogen.

The potential energy curves (Figure 1) for a bond to hydrogen and the corresponding bond to deuterium are essentially identical (9). The force constant for the stretching vibration is related to the frequency by Hooke's Law

$$\nu = (1/2\pi c)\sqrt{k/\mu}$$

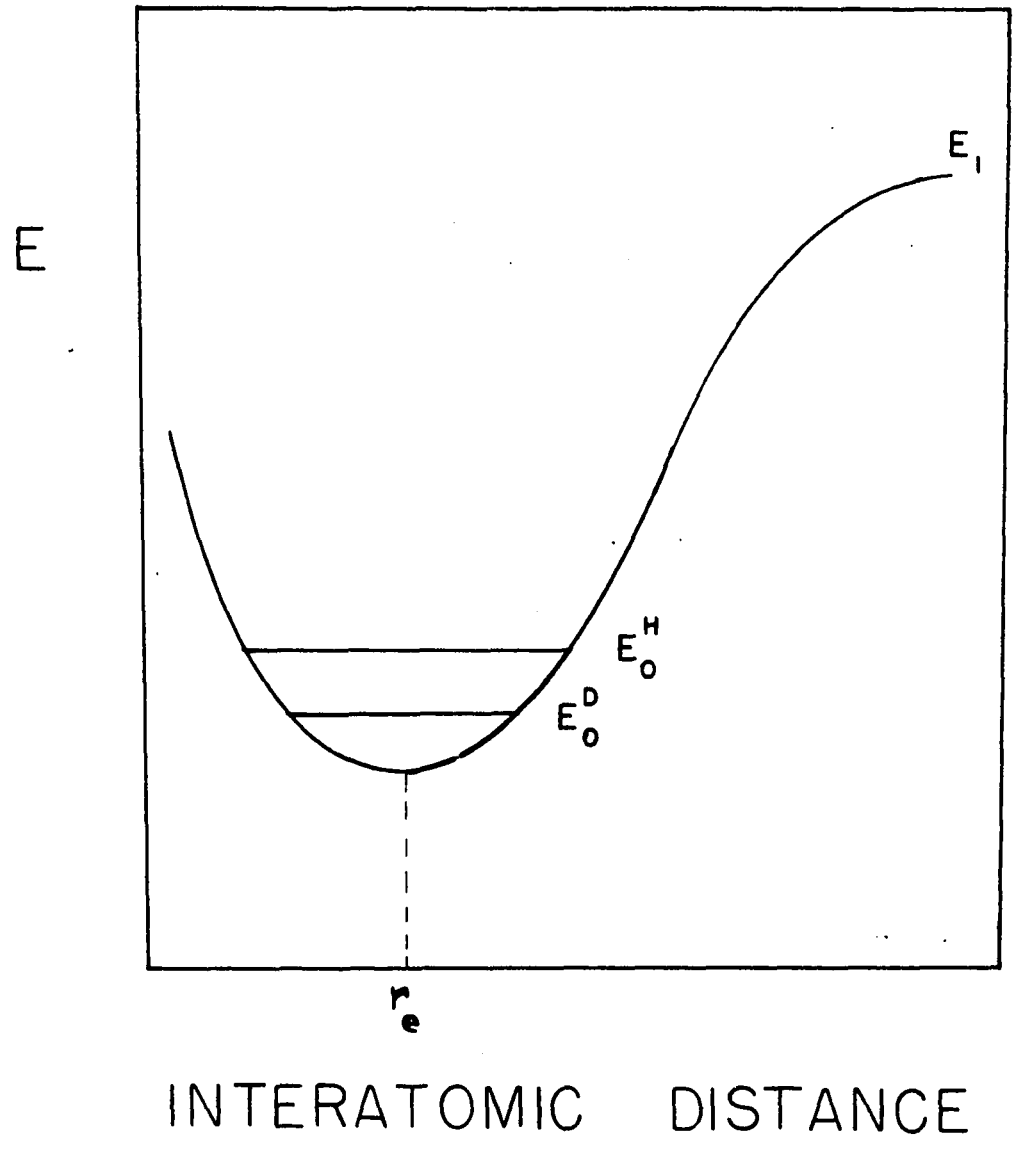


Figure 1. Morse curve relating potential energy and interatomic distance

where k is the force constant and μ is the reduced mass, which is approximately equal to 1 and 2 for most hydrogen and deuterium containing bonds, respectively.

The lowest level for any bond corresponds to 1/2 $h\nu$, where h is Planck's constant. This lowest level is known as the zero point energy, and corresponds to the vibrational energy of the bonds of a molecule at absolute zero. Room temperature is close enough to absolute zero so that most ($\sim$ 99%) of the bonds are in this vibrational level (9). There is a difference in zero point energy for a bond to hydrogen and the corresponding bond to deuterium which arises from the effect of the difference in mass on the stretching frequencies. This difference in zero point energy is of the order of 1.2-1.5 kcal./mole (9).

As a consequence of the difference in zero point energy the deuterium containing bond will have a higher enthalpy of reaction than the corresponding hydrogen containing bond. See Figure 2.

To simplify the calculation it is assumed that the non-reacting bonds are not affected during reaction and only the stretching frequency of the bond undergoing reaction need be considered. The resulting equation is then

$$k_H/k_D = e^{(h\nu_H - h\nu_D)/2kT}$$

At 25 C^o this equals 7.4.

But examination of experimental data reveals a spectrum

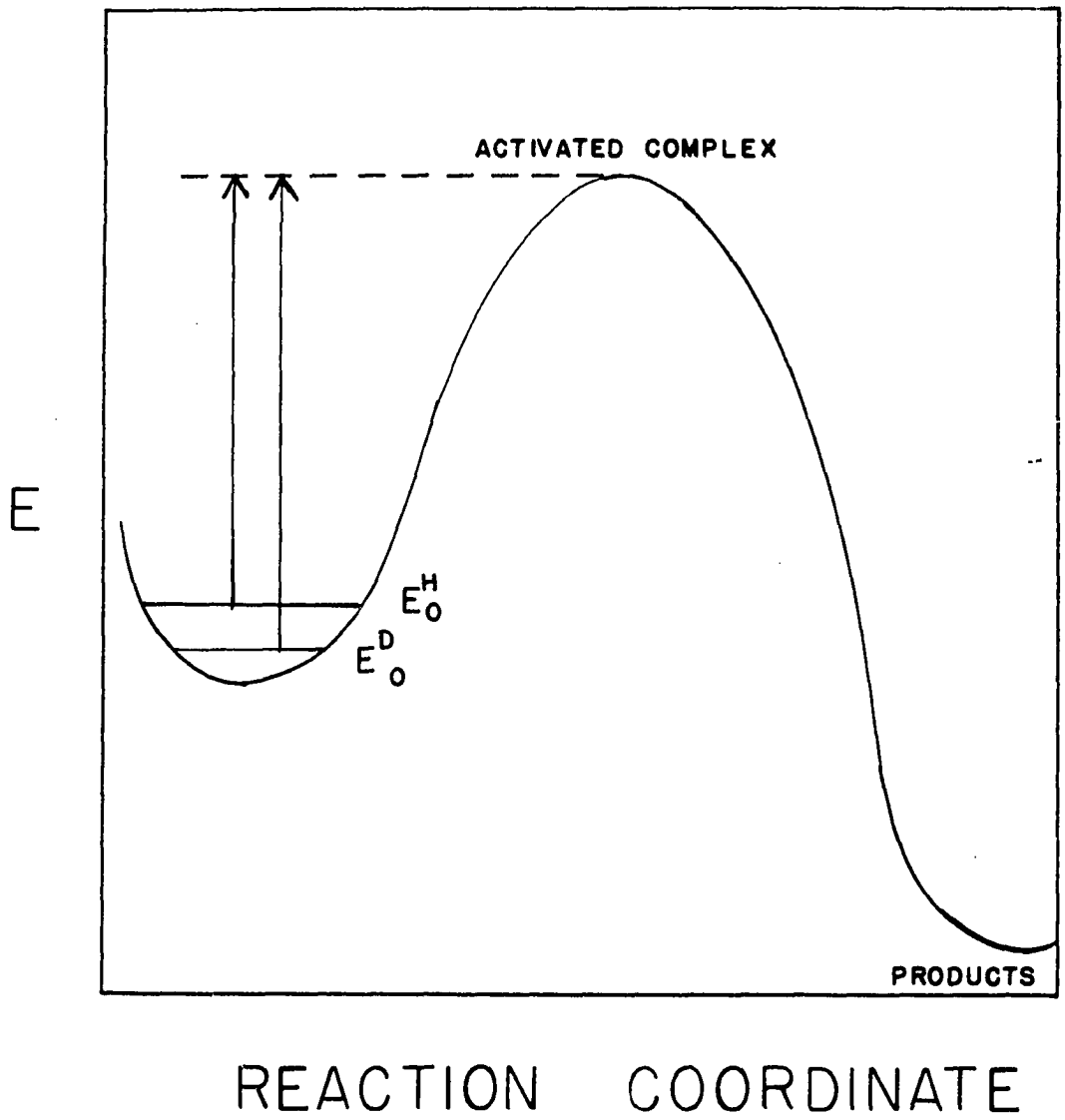


Figure 2. Schematic diagram of the potential energy along the reaction coordinate

of values of the isotope effect. For example, the ratio k_H/k_D for the bromination of acetone (10) is 7.7, for the bromination of nitro methane (11) is 6.5, and for the bromination of nitro ethane (12) is 10. The ratio for the oxidation of isopropanol with chromic acid (13) varies with experimental conditions from 6.9 to 8.4. The ratio for the permanganate oxidation of benzaldehyde in neutral solution (14) is 7, for the permanganate oxidation of formate ion (15) is 7.4 and for the oxidation of desoxybenzoin by selenious acid (16) is about 6; the chromic acid oxidation of phenyl-t-butyl carbinol has an isotope effect (17) of 14 and permanganate oxidation of phenyl-trifluoro-methyl carbinol (18) of 16. The attack of methyl radicals from diacetyl peroxide on toluene (19) shows an isotope effect of 9. The reaction of lithium cyclohexamine with αd-ethyl benzene (20) shows an isotope effect of 12 and the bromination of ethyl cyclopentane-2-carboxylate (21) a value of 10.

In addition to these large isotope effects there are also reactions with small effects. For example, the oxidation of ethanol with bromine (22) shows an effect of 4; the cyclization of N-chloromethyl amyl amine-4-d (23) shows an effect of 3.5. The elimination of trimethylamine from n-butyl-trimethylammonium ion gives a similar effect (24), and the elimination reaction with alkali from $C_6H_5CO_2CH_2X$ shows an effect which varies from 3.0 to 8.0 with change in X (25). The ratio

for the Cannizzaro reaction is 1.8 (26) and that for the oxidation of isopropanol by triphenyl methyl (27) is 1.8-2.6. The reaction of water with the diphenylborane-pyridene complex (28) gives an effect of 1.5. The adsorption of hydrogen on platinum black (29) has an isotope effect of 1.5.

A general explanation for the wide range of values is that the original calculation is an oversimplification. It is concerned with only one vibration; it does not take into account the entire reactant or transition state molecule; it does not take into account the rate of translation across the potential barrier; it does not take into account effects on the transmission coefficient; and it does not take into account the non-classical tunneling of hydrogen through the potential barrier.

The assumption was made that the only vibration of importance is the stretching of the hydrogen bond. This is not a proper vibration since the normal vibrations of a molecule will include stretching and bending motions of all the atoms.

A further refinement (30) takes into account the difference in zero point energy for the activated complex which partially cancels the difference in zero-point energy of the reactant, thereby decreasing the contribution of the zero-point energy to the activation energy and resulting in a lower isotope effect.

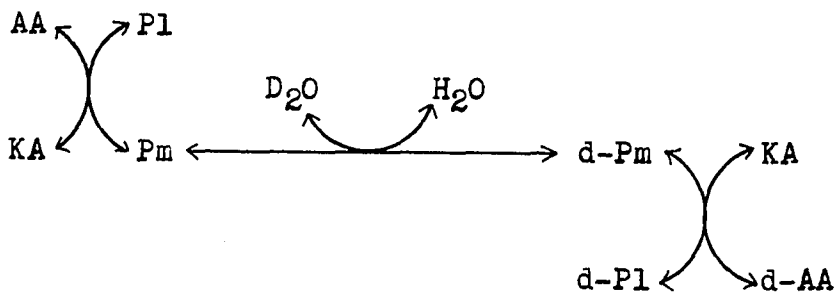
Synthesis of Deutero-leucine

In the investigations of the mechanisms of aspartase and fumarase (31, 32, 33, 34, 35) it was demonstrated that their substrates could be deuterated by carrying the reaction out in D_2O solvent.

Tomiya and Oshima (36) were able to synthesize all possible deuterium derivatives of L-aspartic acid by combining various enzymic and chemical reactions. They prepared ∝-deutero-L-aspartic acid by treating L-aspartic acid with aspartate-glutamate transaminase in deuterium oxide with pyridoxal phosphate added.

Metzler et al. (37) have shown that the transamination reaction can be catalysed non-enzymatically by pyridoxal phosphate in the presence of certain metal ions. The proposed mechanism is given in Figure 3.

When this reaction proceeds in D_2O solvent the pyridoxamine (VI) readily exchanges its amino nitrogens. This results in incorporation of the deuterium into the alpha position of the amino acid upon completion of the reverse reaction.



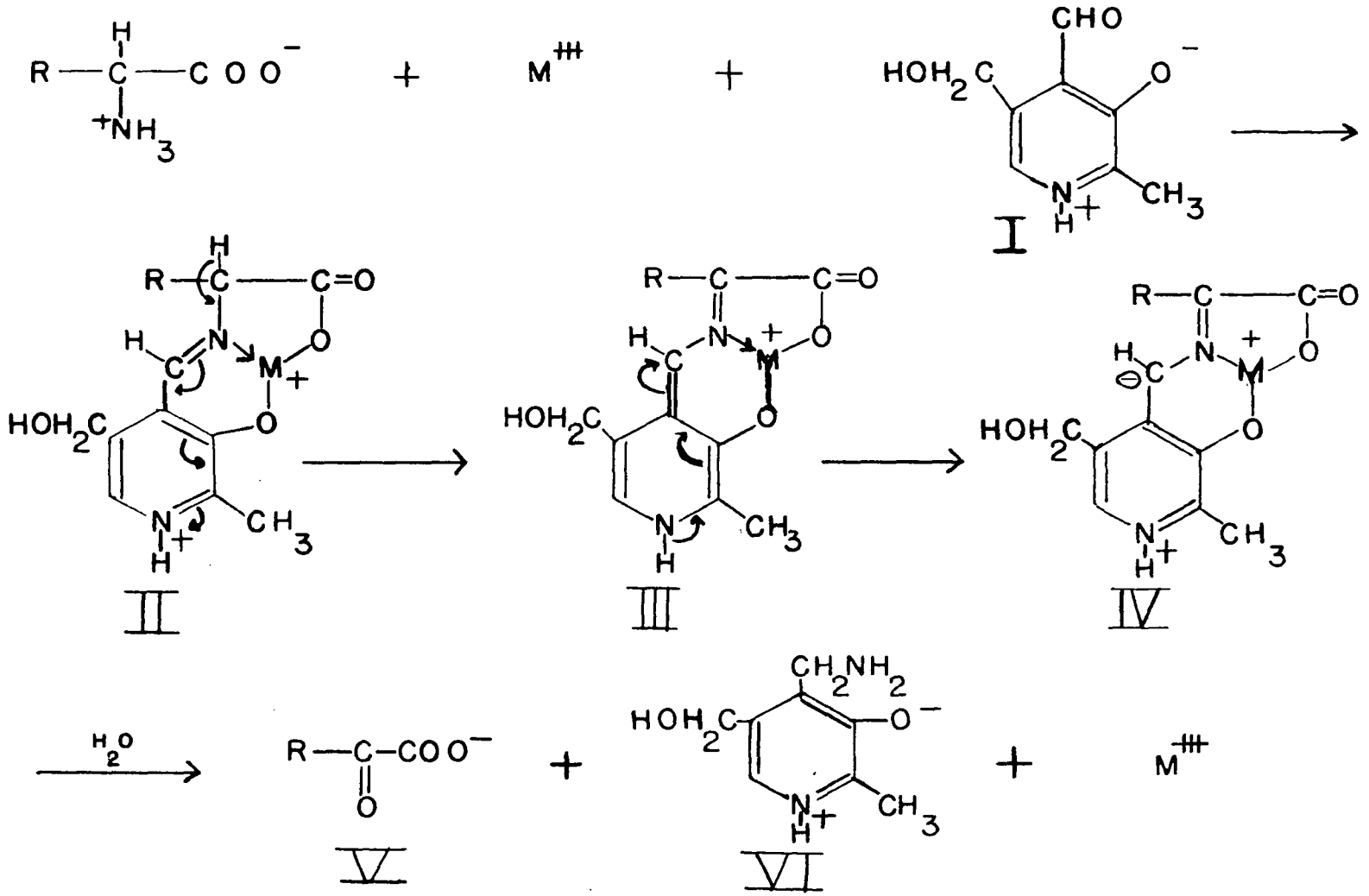


Figure 3. Mechanism of pyridoxal phosphate catalysed transamination

where,

AA = amino acid

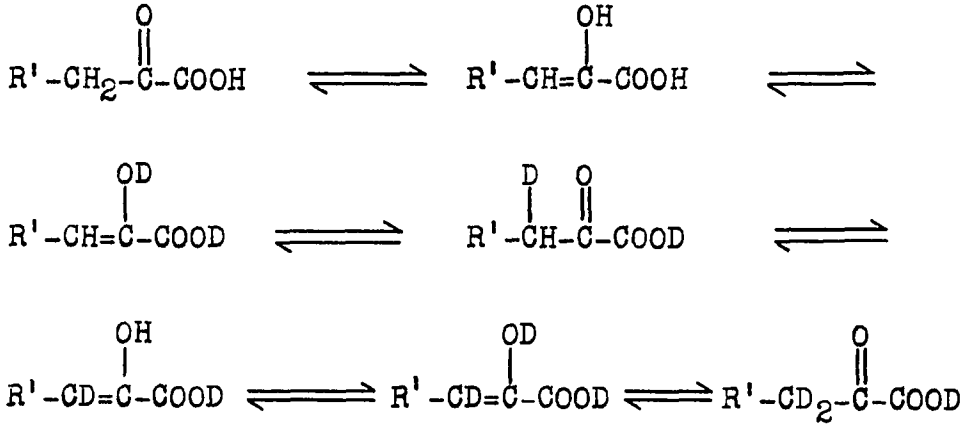
KA = keto acid

Pl = pyridoxal hydrochloride

Pm = pyridoxamine hydrochloride

d- = deuterated

The deuterium may also be incorporated into the beta position of the amino acid by the following sequence of events.



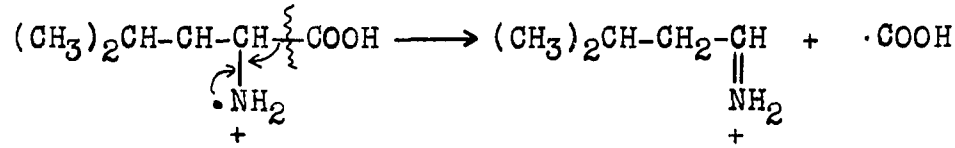
Mass Spectrographic Analysis

The ionizing beam of electrons in the mass spectrometer causes ionization and fragmentation of the amino acid in several ways, resulting in various ion fragments which are then collected according to the ratio of mass/charge. The following fragments should be observed for leucine (38).

Mass 131

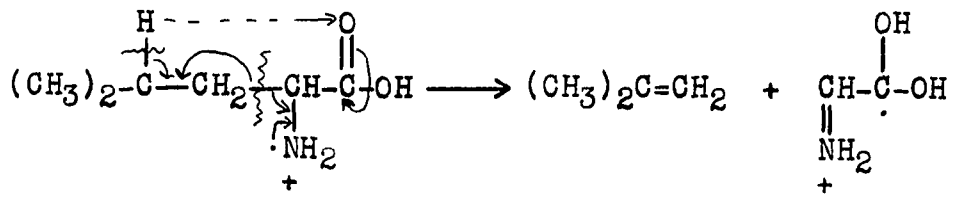
This is the parent ion peak. This peak will be shifted up one unit for each deuterium incorporated into the molecule.

Mass 86



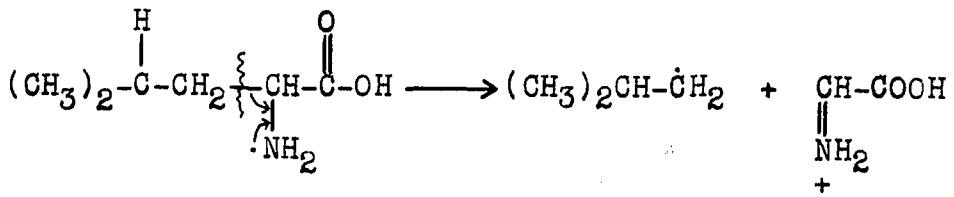
Every deuterium at positions other than the carboxyl will add one unit.

Mass 75



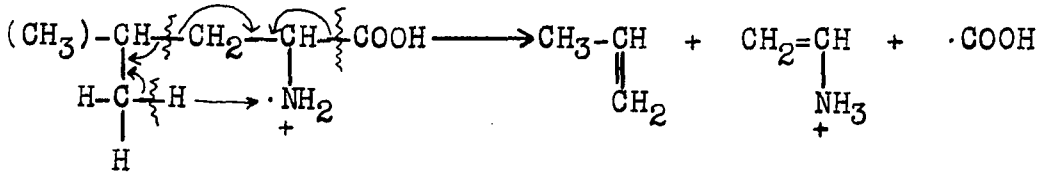
A shift in this peak measures the alpha deuterium content.

Mass 74



Any mass 74 fragment measures the extent of leucine undeuterated in the alpha position.

Mass 44



A shift in this peak measures the extent of incorporation of deuterium into the alpha and beta positions.

Experimental

Materials

Enzyme L-amino acid oxidase was purchased from Worthington Biochemical Corp. in the form of a lyophilized yellow solid. It was prepared according to the procedure of Wellner and Meister (39). The enzyme was kept in a freezer when not in use. Solutions were made by dissolving the enzyme preparation along with potassium chloride in 0.113 M Cacodylic acid buffer, pH 7.0. The solutions were kept refrigerated when not in use and were used within several days after preparation.

Amino acid L(-)leucine, purchased from Pfanstiehl Laboratories, Inc., was dissolved in water and a drop of toluene was added as a bacteriocide. This solution was also kept refrigerated when not in use.

Buffer Cacodylic acid, purchased from Mann Research Laboratories, Inc., was weighed out and dissolved in a fraction of the total volume of water desired. Hydrochloric acid or potassium hydroxide was then added with stirring to the desired pH. The volume was then brought almost to the final volume and the pH again adjusted. Now the final volume was attained and the pH recorded.

Pyridoxal hydrochloride Pyridoxal hydrochloride was

purchased from Sigma Chemical Co. (Lot P52B-66).

Aluminum ammonium sulfate Aluminum ammonium sulfate was purchased from Baker Chemical Co.

D_2O D_2O, purchased from General Dynamics Corp. (Batch number XX), was claimed to be greater than 99.7% pure.

Water Distilled water was glass redistilled from alkaline potassium permanganate solution.

Synthesis of deutero-leucine

A reaction mixture containing 6.6 grams of L-leucine, 500 mg. of pyridoxal hydrochloride, 250 mg. of alum and 200 grams of D_2O were mixed in a sealed vessel and placed in a 100° oven for 29 hours.

The solvent was partially evaporated and the product was recrystallized once from D_2O and then several times from water. The crystals still had some yellow-brown color so they were recrystallized using Darco G-60 decolorizing carbon and filtered through Whatman number 5 filter paper. Yield was 3.7 grams.

Activity assay

The oxidation of amino acid was carried out in a Warburg apparatus. The activity was followed manometrically by the drop in pressure due to the uptake of oxygen at constant volume.

Two ml. of enzyme solution of concentration sufficient to give a convenient reaction rate (5.0 mg. enzyme + 100 mg. KCl)/100 ml. of buffer was added to the main compartment of a Warburg flask (Figure 4).

L-leucine (26.2 mg./10 ml. water) or deutero-DL-leucine (52.4 mg./10 ml. water) was added to the side arm. The volume of leucine added was varied, but the total volume was kept constant by adding water such that the volume of amino acid plus the volume of water equaled 0.5 ml.

In the center well was placed 0.2 ml. of 10% potassium hydroxide solution and a strip of filter paper 40 x 12 mm. to absorb any carbon dioxide given off by the decarboxylation of the alpha-keto acid.

It was assumed that the ammonia given off by the hydrolysis of the imino acid was completely soluble in the aqueous solution.

The reaction vessel was then attached to an open end manometer, placed on the Warburg shakers and temperature equilibrated for ten minutes. The amino acid was then tipped into the main compartment at time zero. Twelve such reaction flasks were run simultaneously along with a blank which served as a thermo-barometer and in the deutero-amino acid assays, a reference leucine flask was added. The amino acid concentration ranged from 4×10^{-4} to 30×10^{-4} M.

The reaction flasks were constantly shaken to facilitate

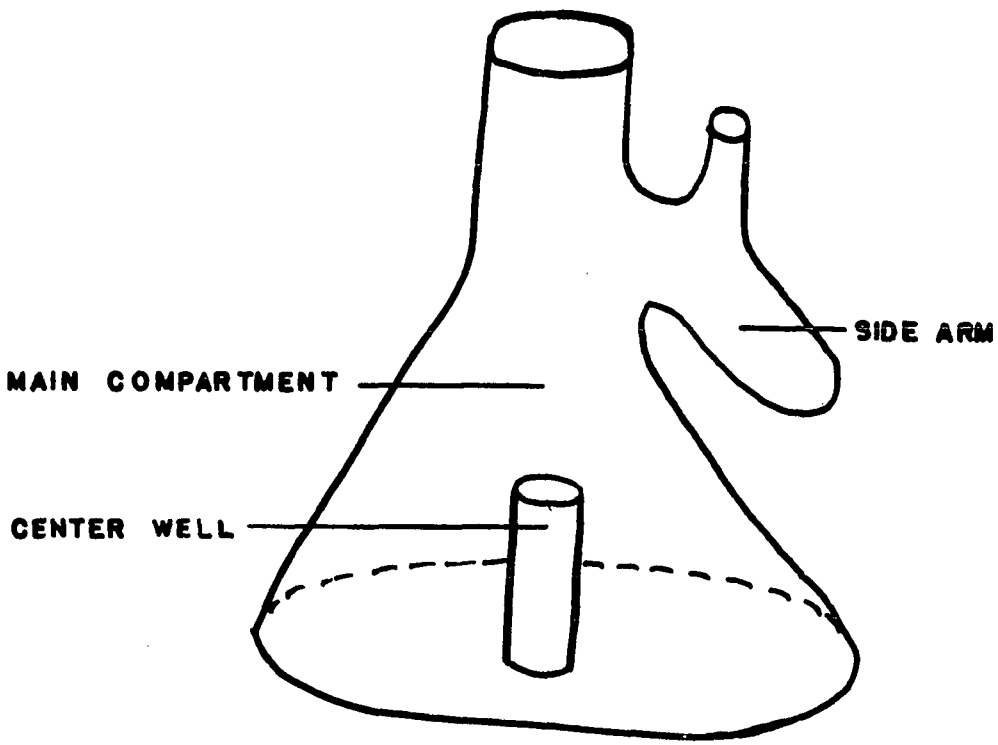


Figure 4. Warburg flask

the gas exchange at the liquid vapor interface. The shakers were stopped when readings were taken, usually every two minutes, but varying from one to five minutes.

Isotope Results

Mass spectrograph

The results of the mass spectra of leucine and deutero-leucine are given in Table 1.

The shift from 131 to 134 meant that the compound was predominantly tri-deuterated. This was confirmed by the shift from 86 to 89. The relative amounts were as follows:

d_0	less than 1%
d_1	1%
d_2	6%
d_3	92%

The shift from 74 to 75 which involves the alpha carbon shows that only 3% of the leucine remained undeuterated in the alpha position.

Hence, the isotopic leucine is 97% deuterated in the alpha position and 92% α, β, β-tri deutero-leucine.

Table 1. Mass spectra of leucine and deutero-leucine

Mass	Leucine	Deutero-leucine
39	5.6	3.3
40	0.8	2.1
41	12.3	7.5
42	6.2	3.4
43	19.0	16.8
44	51.9	4.9
45	1.6	6.8
46	3.0	7.9
47	--	47.5
73	1.0	--
74	86.0	3.0
75	13.0	85.0
76	--	12.0
77	--	--
84	--	--
85	--	--
86	99.1	0.4
87	--	0.9
88	0.7	5.7
89	0.2	92.0
90	--	--
131	100	--
132	--	--
133	--	--
134	--	100

Rate determinations

The rates found for leucine are tabulated in Table 2 and for deutero-leucine in Table 3. The Lineweaver-Burke plot for this data is shown in Figure 5. The lines drawn are calculated to best fit the data in the sense of least squares. The Michaelis constant, K_M, given by the quotient slope/

Table 2. Rates of L-leucine oxidation

$1/S \times 10^{-2}$ M	$1/v \times 10^{3}$ [$\mu l O_2$/30 min.]$^{-1}$									
2.5	6.3	6.1	6.3							
3.9	7.0	6.2	6.5							
5.0	6.0	6.8	5.8	5.9	7.1	6.7	6.5	7.8	8.3	7.2	7.2
5.95	9.7	6.8	6.9							
6.95	8.5	7.7	6.9							
7.8	7.3	6.7	7.4	7.8	8.1	8.8	8.3	8.1	7.9	7.6	8.2
8.32	10.6	9.8	8.9							
8.92	9.9	9.9	9.6							
9.6	9.3	10.2	8.7							
10.0	7.8	9.6	8.1	8.7	10.1	9.3	11.1	10.3		
10.4	9.3	11.6	10.0							
11.35	10.8	8.8	11.1							
11.9	8.4	8.1	9.2	10.6	10.0	13.2	11.2			
12.5	11.4	8.8	10.8							
13.9	9.6	10.3	12.8	11.2	11.9	11.1	14.3	11.6		
15.6	11.4	11.9	14.3	14.1	11.9	13.5	14.7	14.1		
16.65	13.3	12.2	14.7	12.5	13.9	13.3	15.4			
17.85	10.2	13.3	13.5	13.9	13.3	11.2	13.2	14.7		
19.2	11.4	14.1	15.6	14.9	12.5	16.7	15.9	14.9		
20.8	11.0	15.6	12.8	15.4	14.7	17.2	18.9	18.2		
22.7	14.1	16.4	14.5	16.4	9.6	17.5	16.4	18.9		
25.0	16.1	16.9	16.4	19.2	16.9	14.3	21.3			

intercept, and the maximum velocity, V, given by the reciprocal intercept on the ordinate are tabulated in Table 4.

Conclusions

An isotope effect, $k_H/k_D = 1.6$, has been found for the oxidation of deutero-leucine by L-amino acid oxidase. Therefore, the rate limiting step in the reaction must be the breaking of a hydrogen containing bond. Singer and Kearney (40) have previously reported that the rate limiting step is

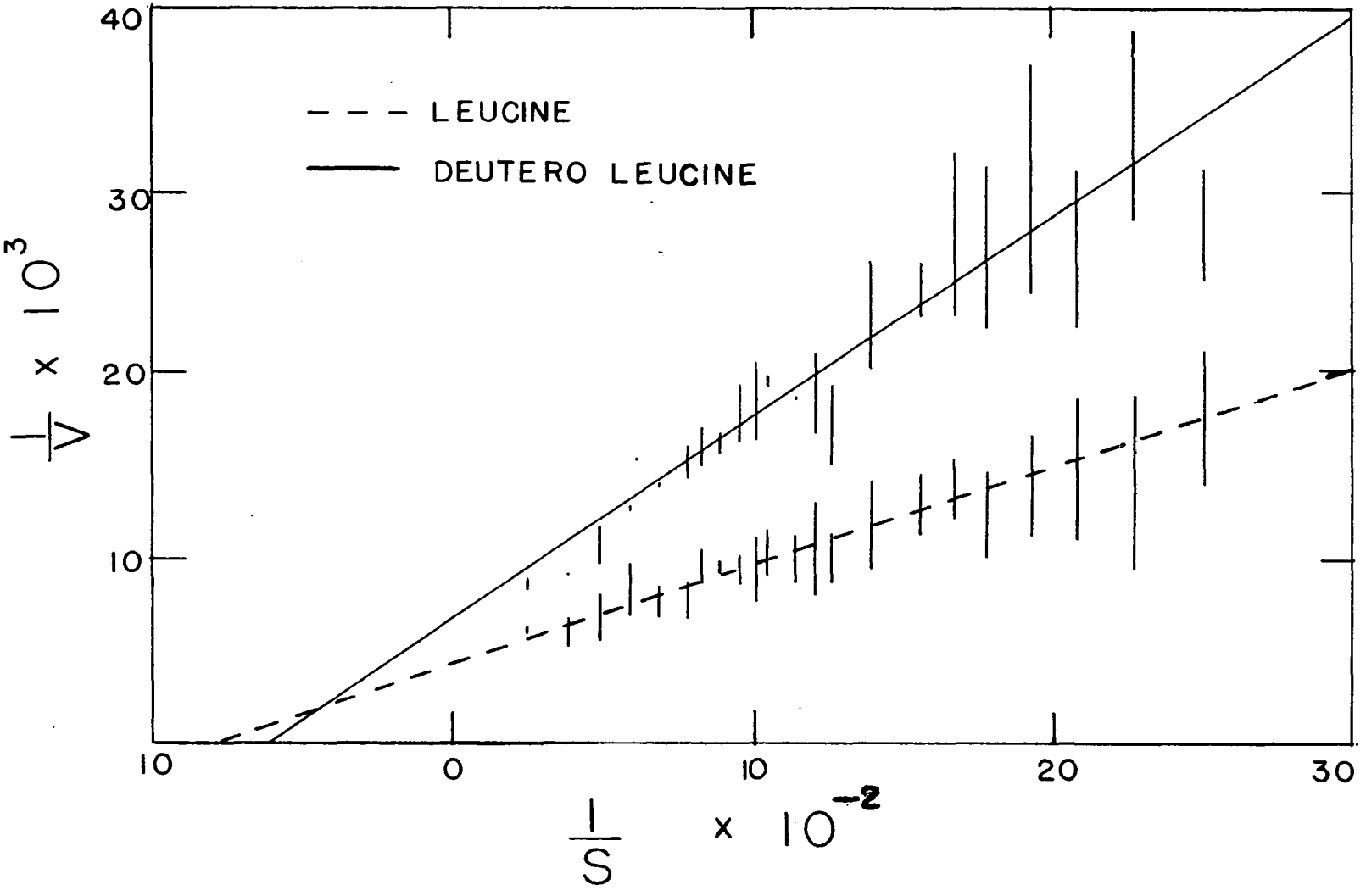
Table 3. Rates of deutero-leucine oxidation

$1/S \times 10^{-2}$ M	$1/v \times 10^{3}$ [$\mu l\ O_2$/30 min.]$^{-1}$						
2.5	8.5	8.8					
3.9	9.2						
5.0	11.2	11.2	11.4	9.9	12.5	12.3	17.2
5.95	13.0	13.0					
6.95	14.1	14.1					
7.8	15.4	16.4	15.6	14.5	16.4	16.1	14.9
8.32	17.2	15.2					
8.92	16.9	15.9					
9.6	19.6	16.4					
10.0	17.2	16.9	16.4	20.8	17.2		
10.4	20.0	19.6					
11.35	18.9	18.9					
11.9	19.6	16.9	19.6	21.3	18.5		
12.5	19.6	15.2					
13.9	24.4	22.2	20.4	26.3	23.8		
15.6	23.3	23.3	26.3				
16.65	23.3	32.3					
17.85	22.7	23.8	31.3	27.8			
19.2	24.4	37.0	26.3				
20.8	31.3	22.7	34.5	30.3			
22.7	38.5	28.6	32.3	38.5			
25.0	25.6	27.8	31.3				

Table 4. Michaelis constant and maximum velocity

	leucine	deutero-leucine
$K_M \times 10^4$	12.4 ± 1.1M	16.0 ± 2.0M
V	234 ± 19 $\mu l O_2$/30 min.	147 ± 17 $\mu l O_2$/30 min.

Figure 5. Lineweaver-Burke plots for leucine and deutero leucine


40
30
20
10
10
0
10
20
30
LEUCINE
DEUTERO LEUCINE
1/V x 10^3
1/S x 10^-2

the oxidation of the enzyme by oxygen. They base this conclusion on the observation that the absorption spectrum of the enzyme in the presence of substrate resembles the spectrum of the reduced enzyme.

If the oxidation of the enzyme is indeed the rate limiting step, then the rate is determined by the breaking of one of the H* containing bonds in the reduced enzyme which exists in the tautomeric forms (41) shown in Figure 6.

The existence of an isotope effect implies that the hydrogen exchange with solvent must be slow relative to the reaction being measured. In this case the exchange of the H*s, a phenolic and an amino hydrogen, would have to be slow. Although possible, this is improbable and it is more likely that the rate limiting step is the oxidation of the amino acid which removes the non-exchangable alpha-hydrogen.

An alternative explanation would be a two step reaction occurring on the enzyme-substrate complex. The first step would be the transfer of a hydrogen atom from the amino acid to the flavin group of the enzyme resulting in a flavin semiquinone and an amino acid radical. The second step would be the oxidation of the complex by oxygen. In this step one hydrogen atom would be removed from the amino acid radical. This last step would be rate limiting consistent with both the isotope effect which implies the rate limiting step to be the breaking of a bond to a non-exchangable hydrogen and with

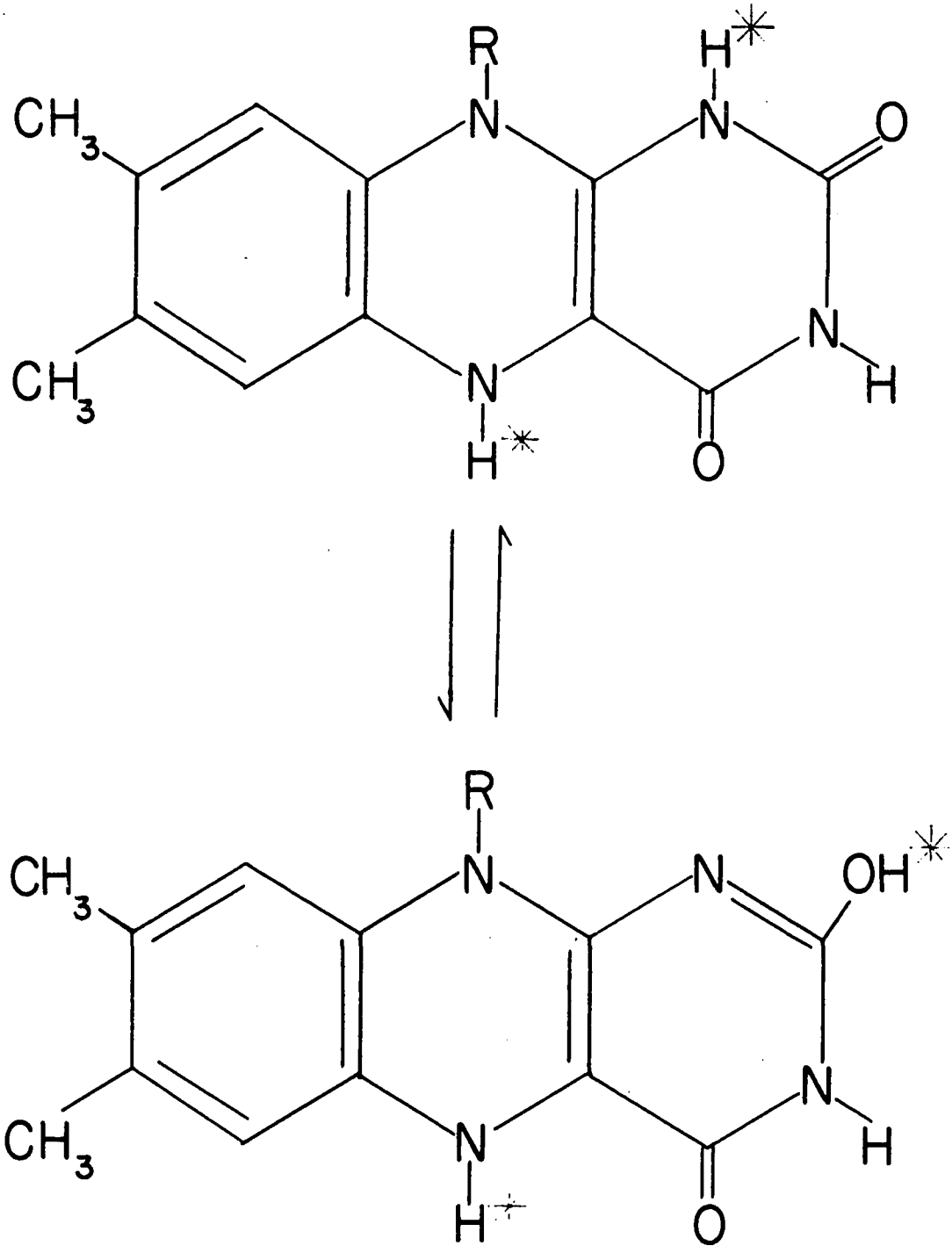


Figure 6. Tautomeric forms of riboflavin

the results of Singer and Kearney (40) in which the rate limiting step was the oxidation of the enzyme by oxygen.

It would be of interest to know the effect of the isotope on the binding of the substrate to the enzyme. The results of this investigation can be used to calculate that effect when one more kinetic parameter is determined.

A steady state treatment of the simple mechanism

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} \text{products}$$

yields

$$K_M = (k_2 + k_3)/k_1$$

$$V = k_3 E_T \qquad \text{or} \qquad k_3 = V/E_T$$

Hence, if either k_1 or k_2 is known, the dissociation constant,

$$k_s = k_2/k_1$$

for the enzyme-substrate complex can be calculated from the known value of k_3.

$$K_s = K_M - k_3/k_1 = k_2 K_M/(k_2 + k_3)$$

HIGH SUBSTRATE INHIBITION

Discussion

Zeller and Maritz (42) observed a characteristic inhibition of L-amino acid oxidase by high substrate concentration. Zeller (43) suggested that this inhibition was due to the combination of two amino acid molecules with the enzyme to yield an inactive complex. Singer and Kearney (4) postulated a competition between amino acid and oxygen as the reason for the inhibition. A similar mechanism was proposed by Dixon and Webb (44) using methylene blue as the oxidant.

Zeller and Maritz used leucine concentrations as high as 0.05 molar, Singer and Kearney used 0.01 molar and Dixon and Webb used 0.0125 molar leucine. Wellner and Meister (45) studied the inhibition at leucine concentrations up to 0.1 molar. They found that the mechanisms suggested by the earlier workers were not consistent with the experimental data at the higher leucine concentrations. They proposed the following mechanism for the oxidation of amino acid by L-amino acid oxidase.

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA \xrightarrow{k_3} EH_2 + I$$

$$EH_2 + O \underset{k_5}{\overset{k_4}{\rightleftharpoons}} EH_2O \xrightarrow{k_6} E + OH_2$$

$$EH_2 + A \underset{k_8}{\overset{k_7}{\rightleftharpoons}} EH_2A \xrightarrow{k_9} EH_4 + I$$

$$EH_4 + O \underset{k_{11}}{\overset{k_{10}}{\rightleftharpoons}} EH_4O \xrightarrow{k_{12}} EH_2 + OH_2$$

where,

A = amino acid

I = imino acid

E = oxidized enzyme

EH_2 = half reduced enzyme

EH_4 = completely reduced enzyme

O = oxidant, in this case oxygen.

This mechanism allows for the complexes EA, EH_2A, EH_4O and EH_2O but makes no mention of the complexes EH_4A or EO. Knowledge of the possibility of formation of these two complexes would be useful in explaining the nature of the enzyme-substrate complex.

This knowledge can be obtained kinetically by postulating the existence of the complexes EH_4A and EO.

$$EH_4 + A \underset{k_{14}}{\overset{k_{13}}{\rightleftharpoons}} EH_4A$$

$$E + O \underset{k_{16}}{\overset{k_{15}}{\rightleftharpoons}} EO$$

The velocity of the reaction as measured by the oxygen uptake is given by the following expression:

$$v = -dO/dt = k_4(EH_2)(O) - k_5(EH_2O) + k_{10}(EH_4)(O) - k_{11}(EH_4O) + k_{15}(E)(O) - k_{16}(EO)$$

A material balance yields

$$E_T = E_o + EA + EH_2 + EH_2O + EH_2A + EH_4 + EH_4O + EH_4A + EO$$

Using a steady state treatment and setting the rate of change of the intermediate concentrations equal to zero yields

$$dE_o/dt = k_2(EA) - k_1(E)(A) + k_6(EH_2O) - k_{15}(E)(O) + k_{16}(EO) = 0$$

$$d(EA)/dt = k_1(E)(A) - (k_2 + k_3)(EA) = 0$$

$$d(EH_2)/dt = k_3(EA) - k_4(EH_2)(O) + k_5(EH_2O) - k_7(EH_2)(A) + k_8(EH_2A) + k_{12}(EH_4O) = 0$$

$$d(EH_2O)/dt = k_4(EH_2)(O) - (k_5 + k_6)(EH_2O) = 0$$

$$d(EH_2A)/dt = k_7(EH_2)(A) - (k_8 + k_9)(EH_2A) = 0$$

$$d(EH_4)/dt = k_9(EH_2A) - k_{10}(EH_4)(O) + k_{11}(EH_4O) - k_{13}(EH_4)(A) + k_{14}(EH_4A) = 0$$

$$d(EH_4O)/dt = k_{10}(EH_4)(O) - (k_{11} + k_{12})(EH_4O)$$

$$d(EH_4A)/dt = k_{13}(EH_4)(A) - k_{14}(EH_4A) = 0$$

$$d(EO)/dt = k_{15}(E)(O) - k_{16}(EO) = 0$$

Solving 11 simultaneous equations in 10 unknowns yields

$$\frac{E_T}{V} = \frac{c_1(O)}{c_2(O) + c_3(A)} \left\{ \frac{(c_3 + c_6)(A) + c_5}{c_1 k_{12}(O)} + c_3(k_{11} + k_{12})(A) \left[\frac{k_{14} + k_{13}(A)}{k_{10}k_{12}k_{14}c_1(O)^2} \right] + \frac{k_6}{k_1(A)} + \frac{k_6 k_{15}(O)}{k_1 k_{16}(A)} + 1 + \frac{c_4}{c_1 k_{12}} \right\}$$

where,

$$c_1 = k_4(k_2 + k_3)(k_8 + k_9)$$

$$c_2 = k_3k_4k_6(k_8 + k_9)$$

$$c_3 = k_3k_7k_9(k_5 + k_6)$$

$$c_4 = k_4k_{12}(k_8 + k_9)(k_2 - k_6)$$

$$c_5 = k_3k_{12}(k_5 + k_6)(k_8 + k_9)$$

$$c_6 = k_3k_7k_{12}(k_5 + k_6)$$

If the two complexes in question do occur, then k_{13} and k_{15} will have finite values. If the complexes do not occur k_{13} and k_{15} will equal zero. These two cases lead to the following functional relationships.

If complexes occur:

$$\text{constant (O):} \quad E_T/V = \frac{a + b/(A) + c(A) + d(A)^2}{e + f(A)}$$

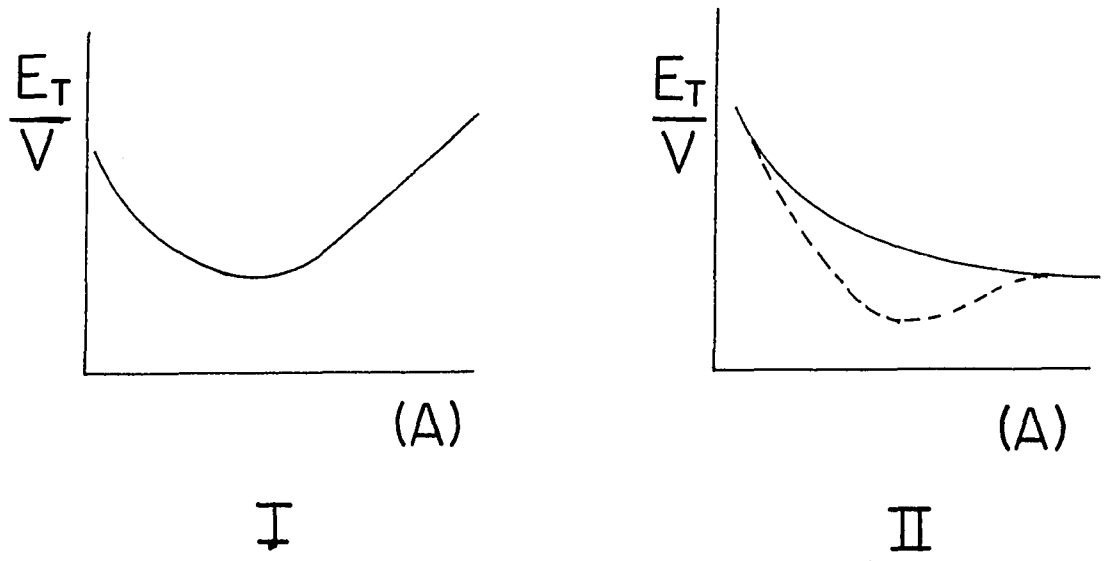
$$\text{constant (A):} \quad E_T/V = \frac{g + h/(O) + i(O) + j(O)^2}{k + l(O)}$$

If complexes don't occur:

$$\text{constant (O):} \quad E_T/V = \frac{a + m/(A) + c(A)}{e + f(A)}$$

$$\text{constant (A):} \quad E_T/V = \frac{g + n/(O) + i(O)}{k + l(O)}$$

Thus a plot of E_T/V against (A) at constant (O) should yield one of the following graphs:



If the complexes form, the curve should approach a straight line with finite slope as (A) $\longrightarrow \infty$ (curve I). If no complex forms, the curve should approach a straight line with zero slope (curve II). At lower (A), however, the shape of the curve depends on the relative magnitude of the kinetic parameters which are mostly unknown. The curve will either decrease asymptotically to a zero slope (solid line) or go through a minimum and then approach a line with zero slope (dashed line).

Experimental

Materials

Enzyme Agkistrodon piscivorus piscivorus (Cottonmouth mocassin) venom, purchased from Ross Allen's Reptile Institute, Silver Springs, Florida, in the form of yellow crystal-

line solid was dissolved in potassium chloride solution and used as such. Singer and Kearney (4) have shown that the crude material gives the same results as the more highly purified preparations. The solutions were kept refrigerated when not in use.

Amino acid L(-)leucine, purchased from Pfanstiehl Laboratories, Inc., or L-methionine, purchased from Nutritional Biochemicals Corp., was dissolved in water and one drop of toluene was added as a bacteriocide. These solutions were also kept refrigerated when not in use.

Buffer Cacodylic acid, same as that used in the isotope experiments, was used here.

Water The water used was the same as that used in the isotope experiments.

Activity assay

The activity assays were done manometrically in much the same way as those for the isotope experiments. Some changes were made, however, to accomodate the high substrate concentration.

0.5 ml. of venom solution (95 mg. of venom + 90 mg. of KCl)/25 ml. of buffer was added to the side arm of a Warburg flask. To the main compartment was added a varying volume of L-leucine (1836 mg. of L-leucine + 14.3 ml. of buffer)/ 100 ml. of solution . To keep the total volume and buffer

concentrations constant, a solution of buffer was added so that the volume of amino acid plus water equaled 3.50 ml. To the center well was added 0.25 ml. of 10% potassium hydroxide solution.

The assay was carried out in the same way as it was for the isotope experiments.

Results

Rate determinations

The rates found for high leucine concentration are tabulated in Table 5 and those for methionine in Table 6. The results are plotted in Figures 7 and 8. The lines drawn are calculated to be the best fit in the sense of least squares. The lines had the slopes shown in Table 7.

Table 5. High substrate inhibition-reciprocal velocity at various leucine concentrations

S x 10^3 M	1/v x 10^3 (μl O_2/30 min.)$^{-1}$
123	4.1 4.1 4.3 3.4 4.0 3.7 3.8 4.0 3.7 3.8 3.9
114	3.9 4.7 3.7 4.8 5.0 3.7 3.8 3.6 3.6 3.7 4.1
109	3.9 4.2 4.7
105	4.4 4.6 3.9 4.3 4.3 3.7 3.8 3.9 3.6 4.0 4.1
101	4.4 4.9 5.2
93	4.4 4.5 5.1
88	4.1 4.3 4.4 3.7 3.8 4.0 3.4 3.5 3.8 3.2 3.4 3.9
78	4.4 4.5 4.7

Table 6. High substrate inhibition-reciprocal velocity at various methionine concentrations

S x 10^3 M	1/v x 10^3 (μl O_2/30 min.)$^{-1}$					
200	5.4	5.2	5.1	5.0	5.3	5.3
196	5.1	5.2	5.3	5.0	5.2	5.4
186	5.1	4.8	5.1	4.8	4.8	4.9
182	4.9	5.1	5.1	5.0	5.0	5.2
171	4.5	4.8	4.9	4.7	4.7	4.9
168	4.9	5.0	6.1	5.1	5.1	5.4
143	4.6	4.9	5.3	4.6	4.6	4.8
140	4.8	4.8	5.6	5.4	5.5	5.5

Table 7. Slopes of high substrate inhibition plots

Amino acid	m x 10^3
Leucine	-4.1 ± 4.3
Methionine	2.1 ± 2.2

Conclusions

The reciprocal velocity against amino acid concentration plots yielded lines with small slopes (see Figures 7 and 8). In both cases the line with zero slope fell within the standard deviation of the experimental line.

Before these experimental results could be assigned to zero slope or to finite slope, an estimate had to be made of the magnitude of the finite slope.

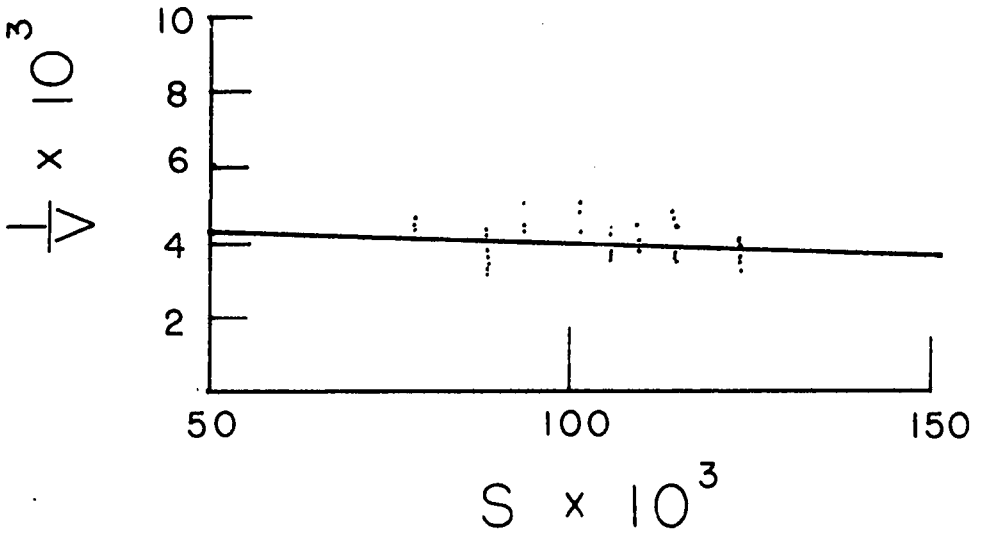


Figure 7. Rate of oxidation at high leucine concentration

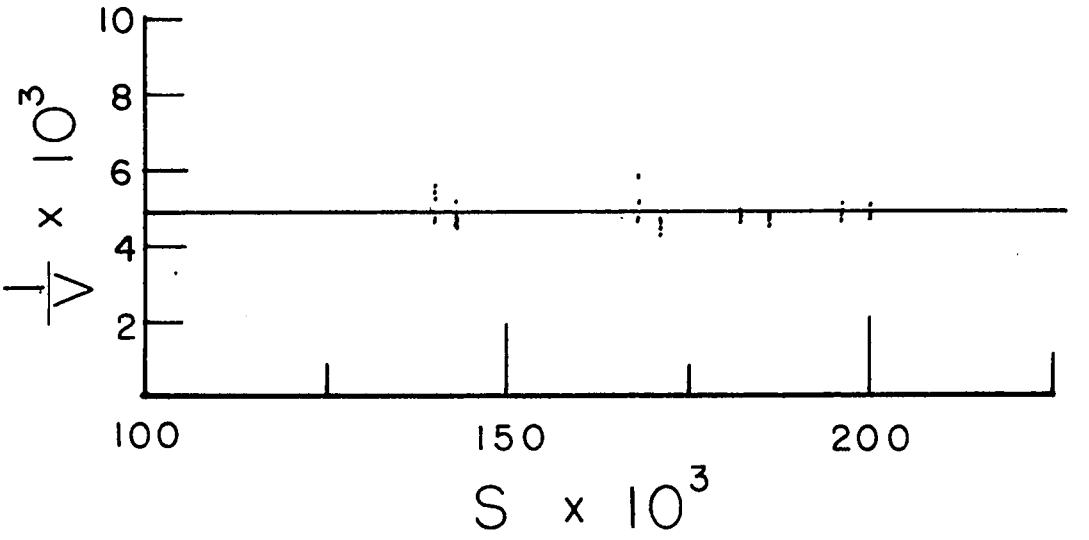


Figure 8. Rate of oxidation at high methionine concentration

Examination of the equation for the reciprocal velocity (see page 28) yielded a slope

$$m = \frac{k_{13}(k_{11} + k_{12})}{k_{10}k_{12}k_{14}(O)E_t}$$

for a plot of reciprocal velocity against amino acid concentration. Wellner and Meister (45) found that $(k_{11} + k_{12})/k_{10}k_{12}$ had a value of 8.6×10^{-4} min. M. for leucine and 8.5×10^{-4} min. M. for methionine. Thus at an oxygen concentration of 1.25×10^{-3} M and an enzyme concentration of 10^{-5} M, the calculated slope equals

$$10^4 \frac{k_{13}}{k_{14}} \text{ (30 min./mole}^2\text{)}$$

where k_{13}/k_{14} may be called the stability constant of the EH_4A complex. It can be seen that the stability constant is magnified by 10^4 in the slope and in order for the slope to be equal to its upper limit, 10^{-3}, the stability constant would have to be equal to 10^{-7}.

As a comparison, the stability constant for the EA complex estimated from the reciprocal of the Michaelis constant is 10^3 (46). Hence, the EA complex is at least 10^{10} times as stable as the EH_4A complex. Thus the reduced enzyme does not complex with the amino acid.

The only apparent differences between the oxidized and reduced flavin are the charge distribution and the presence of the hydrogen atoms in the reduced flavin. Hence, the enzyme-

substrate complex may involve a charge transfer or a hydrogen bonded complex. This is consistent with the findings of Yagi and Ozawa (47) for a model system for D-amino acid oxidase.

SUMMARY

The kinetics of L-amino acid oxidase from snake venom were studied with the purpose of obtaining information about the mechanism of its action and about the nature of the enzyme-substrate complex.

α, β, β-trideuterio leucine was synthesized by a non-enzymic transamination reaction with pyridoxal hydrochloride and was subjected to the action of L-amino acid oxidase. The results were compared with regular leucine yielding an isotope effect of 1.6. This led to the conclusion that the rate limiting step involved the breaking of the alpha-carbon to hydrogen bond of leucine. Proposals were made about the mechanism of the oxidation.

The nature of the enzyme-substrate complex was investigated by studying the kinetics of the high substrate inhibition characteristic of this enzyme. It was found that the completely reduced enzyme could not complex with the amino acid. Since the only apparent difference between the oxidized and reduced enzymes appears to be the charge distribution and the presence of the extra hydrogens on the reduced flavin group, it was concluded that the enzyme-substrate complex may involve a charge transfer or a hydrogen bonded complex.

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