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A D-AMINO ACID OXIDASE FROM *CHLORELLA VULGARIS*

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Summary

A procedure has been developed for the partial purification from *Chlorella vulgaris* of an enzyme which catalyzes the formation of HCN from D-histidine when supplemented with peroxidase or a metal with redox properties. Some properties of the enzyme are described. Evidence is presented that the catalytic activity for HCN formation is associated with a capacity for catalyzing the oxidation of a wide variety of D-amino acids. With D-leucine, the best substrate for O_2 consumption, 1 mol of ammonia is formed for half a mol of O_2 consumed in the presence of catalase. An inactive apoenzyme can be obtained by acid ammonium sulfate precipitation, and reactivated by added FAD. On the basis of these criteria, the *Chlorella* enzyme can be classified as a D-amino acid oxidase (EC 1.4.3.3). Kidney D-amino acid oxidase and snake venom L-amino acid oxidase, which likewise form HCN from histidine on supplementation with peroxidase, have been compared with the *Chlorella* D-amino acid oxidase. The capacity of these enzymes for causing HCN formation from histidine is about proportional to their ability to catalyze the oxidation of histidine.

Introduction

We have described the generation of HCN from histidine as a general reaction catalyzed by amino acid oxidases (EC 1.4.3.2 and EC 1.4.3.3) supplemented with peroxidase or metals such as Mn^{2+} or a particulate fraction from *Chlorella vulgaris* [1,2]. The present paper describes some of the properties of a soluble protein from *Chlorella*, which may be classified as a D-amino acid oxidase, and which is a component of the HCN-generating system in *Chlorella* extracts.

Materials and Methods

Horse-radish peroxidase, purity grade I, and catalase (beef liver crystal suspension, 20 mg/ml) were purchased from Boehringer, Mannheim, and FAD

and FMN from Sigma, St. Louis. D-amino acid oxidase (pig kidney, crystal suspension, approx. 15 units per mg) and L-amino acid oxidase (*Crotalus terr. terr.*, crystal suspension, 7 units per mg) were purchased from Boehringer, Mannheim.

C. vulgaris cells were grown as described in the previous paper [1]. *Chlorella* cell suspensions (250 μl cells per ml) were disrupted with a Ribi cell fractionator RF-1 (Ivan Sorvall, Inc) at a N_2 pressure of 12 000 lb/inch ^{2} (840 kg per cm^2), temperature 4—7°C, with a flow rate of 5 ml per min. The Ribi extract was centrifuged for 10 min at 12 000 × *g* in the SS-34 head of a Sorvall refrigerated centrifuge, and the precipitate was discarded.

Protein was determined in most experiments by the method of Lowry et al. [3], and only in a few initial experiments estimated from the ratio of absorbance at 280 and 260 nm [4]. The oxygen uptake was determined manometrically or with a Gilson Oxygraph, Model IC-OXY, equipped with a Clark electrode. The O_2 content of a solution saturated with air was taken as 0.286 μmol O_2 per ml at 20°C [5].

Enzyme assays

The activity of the enzyme was measured in two ways: (a) Activity in the HCN-forming reaction after supplementation with peroxidase; the formed HCN was determined. (b) Activity in the oxidative deamination reaction; the O_2 uptake was determined.

(a) HCN-forming reaction. The reaction was carried out in Warburg vessels with a center trough containing alkali to collect the HCN, as previously described [1]. The reaction mixture contained: 200 μmol potassium phosphate buffer, pH 7.0, 50 μmol KCl, 30 μmol D-histidine, 100 μg peroxidase, and *Chlorella* D-amino acid oxidase in a total volume of 3 ml. The vessels were gassed with O_2 for 2 min. After 30 min shaking at 21°C, 0.1 ml 2 M H_2SO_4 was tipped from a sidearm into the main compartment to stop the enzyme reaction.

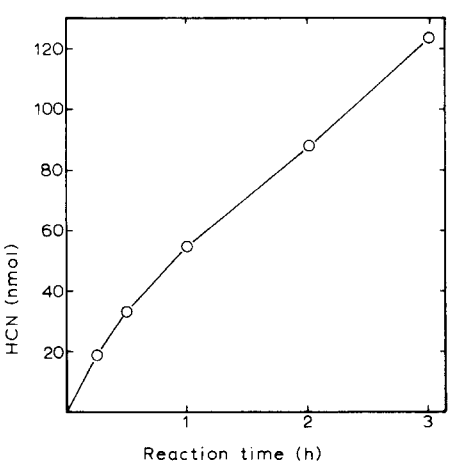

Fig. 1. Time course of HCN generation. Assay as under Materials and Methods. D-Amino acid oxidase from *Chlorella* (1.27 mg protein) was used. The enzyme reaction was stopped at the indicated times (15—180 min) and HCN was collected for another 2.5 h.

The collection of HCN was continued for another 2.5 h. Any changes from this standard assay procedure are given in the legends to the figures and tables.

The choice of the 30 min reaction period was based on a study of the time course of HCN generation, as shown in Fig. 1. These results indicated that the reaction proceeded almost linearly for 30 min, but that the rate of HCN generation declined with longer time intervals. One unit of enzyme was defined as that amount which generates 1 nmol HCN in 30 min.

(b) Oxidative deamination reaction. The activity of the *Chlorella* enzyme in catalizing the oxidative deamination reaction was measured by following the O_2 uptake with the Gilson Oxygraph. The reaction mixtures contained: 200 μmol potassium phosphate buffer, pH 7.0, 30 μmol D-leucine, and the enzyme in a total volume of 1.85 ml. The solutions were saturated with air and the reaction temperature was 20°C. One unit of enzyme was defined as the amount which consumes 1 nmol O_2 in 30 min.

When the L-amino acid oxidase from *Cr. terr. terr.* or the D-amino acid oxidase from pig kidney was used, the assay conditions were the same except that potassium phosphate buffer, pH 7.0, was replaced by buffer, pH 7.5, in both assays.

Ammonia determination

Ammonia was determined by mixing aliquots of the reaction mixture from the Warburg vessels with 1 ml 0.2 M phosphate buffer, pH 7.0, 50 μmol KCl, sufficient 1 M NaOH to neutralize the H_2SO_4 added to stop the reaction, and water to give a total volume of 3 ml. Then 3 ml saturated K_2CO_3 were added. A stream of argon was bubbled rapidly through the solutions contained in three consecutive flasks: the first containing 1 M H_2SO_4, the second containing the unknown solution, and the final receiver flask containing 3 ml 16.5 mM HCl. The ammonia collection was complete in 60 min. The ammonia was then determined with Nessler's reagent [6].

Results and Discussion

Purification of D-amino acid oxidase from Chlorella

Ribi extract (550 ml) of *C. vulgaris* was stored frozen at −20°C for at least 24 h and then thawed and centrifuged for 2 h at 38 000 × *g*. Potassium phosphate buffer, pH 7.0, was added to the supernatant to give a final concentration of 0.02 M, and sodium benzoate [7] to give a final concentration of 0.1%. The pH was brought to 5.5 with 1 M acetic acid. The solution was placed in a waterbath kept at 45°C and was maintained for 5 min at 40—42°C (heating to 55°C in the presence of sodium benzoate resulted in a total loss of activity). The precipitate formed on heating was removed by centrifugation for 10 min at 12 000 × *g*. The supernatant was cooled in an ice cold bath and solid $(NH_4)_2SO_4$ was added with stirring. The bulk of enzyme activity was in the protein fraction which precipitated between 30 and 60% saturation. This precipitate was dissolved in 0.2 M potassium phosphate buffer, pH 7.0, and dialyzed against 1 l 0.05 M potassium phosphate buffer, pH 7.0, containing 0.2 M L-histidine, and afterwards two times against 1 l of the same buffer without L-histidine. After clarification by centrifugation, the dialyzed protein solution was sub-

TABLE I

PARTIAL PURIFICATION OF D-AMINO ACID OXIDASE

Step	Volume (ml)	Total protein (mg)	Enzyme activity (units)		Specific activity (units/mg)		Ratio O_2 uptake (with leucine) / HCN (from histidine)	Yield (%)
			HCN (from histidine)	O_2 uptake (with leucine)	HCN (from histidine)	O_2 uptake (with leucine)		
Ribi extract	550	8520	18 700	—	2	—	—	100
Ribi extract, heated	500	4100	21 500	—	5	—	—	115
Ammonium sulfate 30—60% saturation	40	1160	14 400	26 600	1 2	23	1.85	77
DEAE-Sephadex column	21	34	4 540	8 320	134	244	1.83	24

jected to chromatography on a column of DEAE-Sephadex. The column (2 × 35 cm) was equilibrated with 0.05 M potassium phosphate buffer, pH 7.0, and elution was carried out with a linear gradient formed from equal volumes of 0.05 and 0.25 M potassium phosphate buffer, pH 7.0. The peak of the activity was eluted between 0.13 and 0.14 M phosphate. At this stage a specific activity of about 90—140 units/mg was consistently obtained. Typical results of this partial purification procedure are summarized in Table I. Subsequent use of a CM-Sephadex column led to an additional 3—4-fold increase in specific activity, but the yield was low and variable and the results are not shown.

During the development of the purification procedure, the HCN formation from D-histidine was used as an assay system. Later, application of a D-amino acid oxidase assay with D-leucine as a substrate indicated that the two reactions were closely associated. This is shown in Table I. It was not practical to apply the oxidase assay directly to the first extract, but after ammonium sulfate precipitation, the ratio of the two activities remained constant. Moreover, the elution profiles from the DEAE—Sephadex column of the two activities were identical (Fig. 2).

For the examination of pH profile and for the study of some of the effects of inhibitors, the enzyme was used after the fractionation with ammonium sulfate. Otherwise, purification was carried through the DEAE-Sephadex chromatography, but the specific activity varied considerably, because side fractions as well as the peak fraction were used.

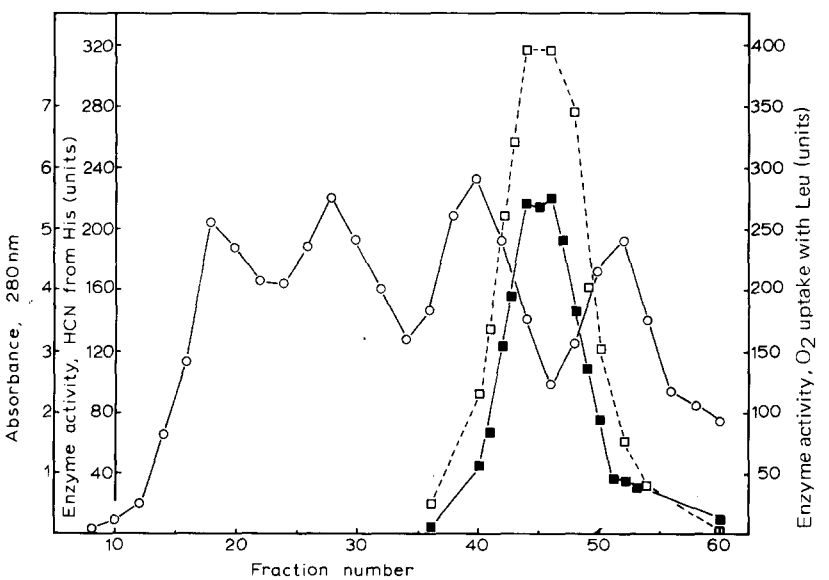

Fig. 2. DEAE-Sephadex chromatography of crude D-amino acid oxidase from *Chlorella*. A column (2 × 35 cm) was packed with DEAE-Sephadex A-50 and equilibrated with 0.05 M phosphate buffer, pH 7.0. Enzyme solution was applied in the same buffer and elution performed with a linear gradient of phosphate buffer, pH 7.0 (0.05—0.25 M). Fractions of 7 ml were collected and protein was measured by absorbance at 280 nm. Enzyme activity was determined by assays a and b as described under Materials and Methods. ○———○, absorbance, 280 nm; ■———■, enzym activity: HCN from D-histidine; □———□, enzyme activity: O_2 uptake with D-leucine.

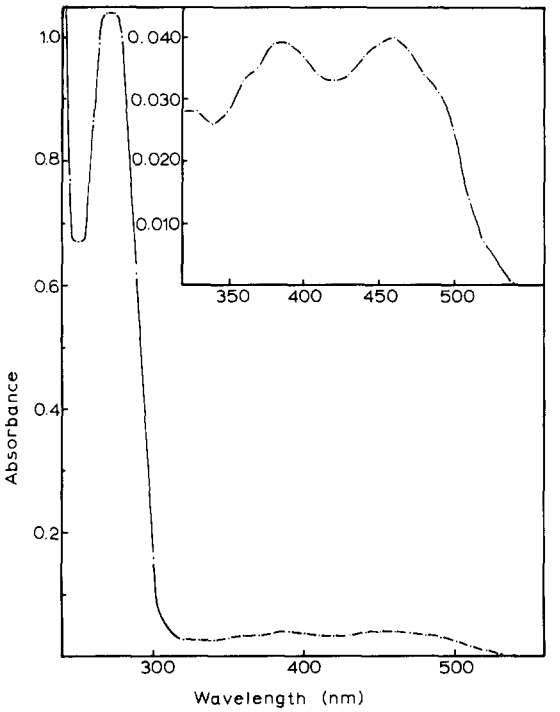

Fig. 3. Absorption spectrum of the partially purified D-amino acid oxidase from *Chlorella*. The spectrum was measured with a Zeiss spectrophotometer PMQII. The enzyme was partially purified (two consecutive DEAE-Sephadex columns), 0.7 mg protein per ml of 0.13 M phosphate buffer, pH 7.0.

Absorption spectrum of the enzyme

The partially purified D-amino acid oxidase from *Chlorella* had a typical flavin absorption spectrum (Fig. 3), with maxima at 460 and 385 nm and minima at 418 and 335 nm [7]. The ratio of absorbance at 275 to 460 nm was 26. For the crystalline D-amino acid oxidase from pig kidney a ratio of 9.5—10 has been reported [8].

Resolution and reconstitution of the enzyme

Treatment with acid ammonium sulfate has often been used to separate flavin from protein [9]. Application of this procedure led to the preparation of inactive apoenzyme which could be activated with FAD. The apoenzyme was prepared as follows: 2.5 ml of enzyme solution (2.4 mg protein per ml) in 0.1 M potassium phosphate buffer, pH 7.0, were mixed with 15 ml saturated ammonium sulfate solution, and 5.5 ml 0.1 M HCl were added dropwise with stirring. All solutions were kept in ice. After centrifugation (10 min at 12 000 × *g*) the pellet was dissolved in 2.5 ml 0.2 M potassium phosphate buffer, pH 7.0 The solution had a protein concentration of 2.1 mg per ml.

Reconstitution could be achieved with FAD (73%) while only minor activity was detected when FMN was added (5%) indicating that the D-amino acid oxidase from *Chlorella* has FAD as a prosthetic group (Table II). In control experiments with 50 nmol FAD or 50 nmol FMN in the same reaction mixture but without apoenzyme, 0.06 and 0.04 nmol HCN, respectively, were found.

TABLE II

RECONSTITUTION EXPERIMENT WITH D-HISTDINE OXIDASE APOENZYME

The reaction mixture contained 200 μmol phosphate buffer, pH 7.0, 50 μmol KCl, 30 μmol D-histidine, 100 μmg peroxidase, holoenzyme (0.24 mg) or apoenzym (0.21 mg) and FAD or FMN as indicated. The reaction period was 3 h. When FAD or FMN was added, the Warburg vessels were wrapped with aluminium foil to keep them completely dark.

Preparation	Addition		HCN (nmol)
	Compound	Amount (nmol)	
Holoenzyme	none		30.6
Apoenzyme	none		0.5
Apoenzyme	FAD	50	22.4
Apoenzyme	FAD	1	20.4
Apoenzyme	FMN	50	1.5
Apoenzyme	FMN	1	1.5

The experiments reported here show that FAD is required for enzyme activity. All D-amino acid oxidase so far characterized have FAD as a prosthetic group.

Specificity of D-amino acid oxidase

Like other amino acid oxidases, the *Chlorella* enzyme catalyzed oxygen uptake with a wide variety of amino acids, as show in Table III. The best

TABLE III

SPECIFICITY OF *CHLORELLA* D-AMINO ACID OXIDASE: FOR OXIDATION OF AMINO ACIDS

Assay b as under Materials and Methods, with D-amino acid oxidase (1 mg protein) and 30 μmol of the indicated amino acids except 10 μmol of tyrosine. The O_2 uptake for D-leucine was 0.163 μmol per 30 min.

Amino acid	O_2 uptake (relative activity)
D-Alanine	28
D-Arginine	8
D-Aspartic acid	8
D-Asparagine	39
D-Glutamic acid	37
D-Glutamine	57
Glycine	18
D-Histidine	36
D-Isoleucine	20
D-Leucine	(100) *
D-Lysine	13
D-Methionine	92
D-Phenylalanine	22
DL-Proline	15
D-Serine	18
D-Threonine	18
D-Tryptophan	6
D-Tyrosine	54
D-Valine	24

* Activity arbitrarily assigned a value of 100, other values given as percentage of rate with most active substrate.

TABLE IV

SPECIFICITY OF *CHLORELLA* D-AMINO ACID OXIDASE: HCN FORMATION

Assay a as under Materials and Methods, with D-amino acid oxidase (1 mg protein) and 30 μmol of the indicated amino acids. The reaction was run for 3 h. The HCN formed from D-histidine was 0.212 μmol.

Amino acid	HCN formation (relative activity)
D-Histidine	(100) *
D-Tyrosine	6
D-Phenylalanine	2
D-Tryptophan	0

* Activity arbitrarily assigned a value of 100, other values given as percentage of rate with most active substrate.

substrate was D-leucine, followed by D-methionine. The oxygen uptake value for D-histidine was about 36% of that for D-leucine. The ammonia production was measured with D-leucine and D-histidine (see Table VII). With respect to its relatively high effectiveness with leucine, methionine and histidine, the *Chlorella* D-amino acid oxidase rather resembles the D-amino acid oxidases from *Trigonopsis variabilis* [10] and octopus liver [11].

As reported elsewhere, D-histidine is the best substrate for the HCN-forming reaction [1]. Table IV shows the relative amounts of HCN obtained with partially purified *Chlorella* enzyme and the aromatic amino acids. The failure of tryptophan to give HCN reflects the low rate of tryptophan oxidation.

Some characteristics of the HCN-forming reaction with the Chlorella enzyme

pH Optimum. The effect of pH on the rate of HCN generation was determined over a pH range of 4.5—9.5 (pH 4.5—6.0 sodium acetate buffer, pH 6.0—8.0 potassium phosphate buffer, pH 8.0—9.5 sodium borate buffer). A rather symmetrical curve was obtained with a maximum at pH 7, declining to zero at pH 5 and 9. The pH profile for the O_2-consuming reaction (without peroxidase) had a much broader peak extending into alkaline pH values. Phosphate buffer of relatively high concentration (0.067 M) gave considerably better HCN yields at pH 7.0 than HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) or lower concentrations of phosphate.

Activity as a function of D-amino acid oxidase and peroxidase concentration. With excess peroxidase the HCN generation increased linearly with the amount of D-amino acid oxidase added, over a 50-fold range (Fig. 4A, curve a). Curve b of the same figure shows the results with a limiting amount of peroxidase.

With a given amount of D-amino acid oxidase, the HCN formed increased with increasing peroxidase concentrations to give a hyperbolic saturation curve (Fig. 4B).

Activity as a function of D-histidine and oxygen concentration. When the activity was measured as a function of D-histidine concentration the usual hyperbolic saturation curve was obtained and a K_m value of $3.2 \cdot 10^{-3}$ M was calculated. L-Histidine was not a substrate for the *Chlorella* D-amino acid oxidase.

No HCN was obtained in the absence of O_2. The yield of HCN increased

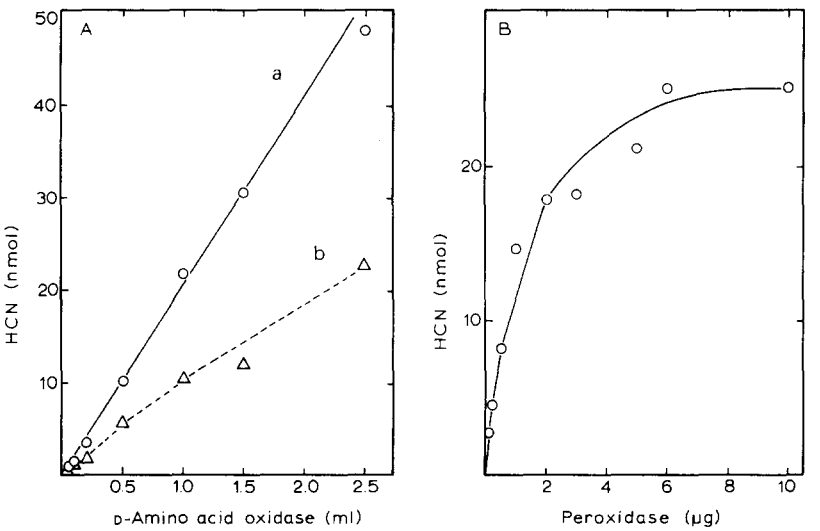

Fig. 4. HCN generation as a function of D-amino acid oxidase concentration and peroxidase. (A) The reaction mixture contained 200 μmol phosphate buffer, 50 μmol KCl, 30 μmol D-histidine, 100 μg (curve a) and 0.5 μg (curve b) peroxidase, and D-amino acid oxidase as indicated (1.5 mg protein per ml). (B) The reaction mixture contained 200 μmol phosphate buffer, pH 7.0, 50 μmol KCl, 30 μmol D-histidine, D-amino acid oxidase (0.50 mg protein), and various concentrations of peroxidase.

TABLE V

EFFECT OF VARIOUS INHIBITORS

The reaction mixture contained 200 μmol phosphate buffer, pH 7.0, 50 μmol KCl, 30 μmol D-histidine, 100 μg peroxidase in part A, or 5 μg peroxidase in part B, D-amino acid oxidase (1.3 or 2.0 mg protein) and the inhibitors as indicated.

Substance	Concentration (M)	Inhibition (%)
Part A		
L-Histidine	5×10^{-2}	71
	2×10^{-2}	51
	0.5×10^{-2}	22
Sodium benzoate	10^{-2}	94.5
	10^{-3}	62.6
p-Chloromercuribenzoate	10^{-3}	96.4
	10^{-4}	68.0
o-Phenanthroline	10^{-2}	0
Nordihydroguajaretic acid	10^{-4}	99.5
Guajacol	10^{-2}	53.2
Part B		
NaN_3	10^{-2}	100.0
	10^{-3}	70.8
NaF	10^{-2}	28.0
NADH	10^{-2}	95.8
	10^{-3}	68.8
NADPH	5×10^{-3}	80.6
	10^{-3}	63.0

linearly with increasing O_2 concentrations up to 100% (20% O_2 : 6 nmol HCN, 50% O_2 : 15.5 nmol HCN, 100% O_2 : 30 nmol HCN, values obtained under normal assay conditions).

Effect of inhibitors. L-Histidine had an inhibitory effect on the HCN generation from D-histidine as shown in Table V. Inhibition was also observed with sodium benzoate (a typical inhibitor of D-amino acid oxidase) and with *p*-chloromercuribenzoate, nordihydroguaretic acid, NaN_3, NaF, NADH and NADPH. NaN_3 and NaF are common inhibitors of peroxidase.

Heat stability. Heating for 10 min at 65°C resulted in a complete loss of activity of the D-amino acid oxidase. At 60°C 93%, at 55°C 76%, at 50°C 57% and at 45°C 35% of the activity were lost.

Comparison of three amino acid oxidases. Two commercially available crystalline amino acid oxidases and the *Chlorella* enzyme were compared with respect to the HCN formation from D- or L-histidine. The values were 9.0 μmol HCN/mg protein per 30 min for crystalline L-amino acid oxidase from *C. terr. terr.*, 0.46 μmol HCN/mg protein per 30 min for crystalline D-amino acid oxidase from pig kidney and 0.12 μmol/mg protein per 30 min for the partially purified *Chlorella* D-amino acid oxidase. The relative activity for the O_2 consumption with histidine was approximately in the same proportion. The L-amino acid oxidase from *C. terr. terr.* is a much more effective HCN producer than the D-amino acid oxidase from pig kidney or *Chlorella*.

Effect of catalase

The effect of catalase on the HCN-forming reaction is shown in Table VI. A 10-fold excess (by weight) of catalase over peroxidase was required to observe 23% inhibition. The inhibition by catalase could be overcome by adding more peroxidase. It is clear that catalase is not a very effective inhibitor. The partially purified *Chlorella* D-amino acid oxidase was not completely free of catalase activity, but the amount present was insufficient to interfere with HCN production.

Effect of peroxidase on O_2 consumption and NH_4^+ formation

The D-amino acid oxidase of *Chlorella* catalyzed the expected oxidative deamination of D-leucine. In the presence of added catalase, about 2 mol of ammonia were formed for each mol of O_2 consumed [11]. There was no HCN production from D-leucine, and addition of peroxidase had no appreciable

TABLE VI

EFFECT OF CATALASE

Assay a as under Materials and Methods, with 1 μg peroxidase and catalase as indicated and D-amino acid oxidase (0.75 mg protein).

Additions	HCN (nmol)
None	19.4
200 μg catalase	0.6
40 μg catalase	5.0
20 μg catalase	10.6
10 μg catalase	15.0

TABLE VII

EFFECT OF PEROXIDASE ON O_2 UPTAKE AND NH_4^+ FORMATION

The reaction mixtures contained 200 μmol phosphate buffer, pH 7.0, 50 μmol KCl, 30 μmol D-leucine or D-histidine, 20 μg catalase, 200 μg peroxidase when indicated, and *Chlorella* D-amino acids oxidase (2.1 mg protein) in the experiment with D-leucine and 4.2 mg protein in the experiments with histidine. The Warburg vessels (two side-arms) were gassed with O_2 and the reaction was started by tipping the amino acid. The O_2 uptake was determined manometrically over a period of 90 min. The reaction was stopped by tipping H_2SO_4, HCN was collected for another 3 h and NH_4^+ was determined on aliquots of the reaction mixtures.

Additions	O_2 uptake (μmol)	NH_4^+ formation (μmol)	HCN formation (μmol)
D-Leucine	1.1	2.3	<0.001
D-Leucine, peroxidase	1.0	2.3	<0.001
D-Histidine	1.6	3.3	0.004
D-Histidine, peroxidase	2.5	1.6	0.506

effect on either O_2 consumption or NH_4^+ production with this substrate, as shown in Table VII. With D-histidine as a substrate in the absence of peroxidase, the reaction was also that of a simple oxidative deamination, with O_2 consumption equivalent to half the NH_4^+ production and virtually no HCN formation. Addition of peroxidase, with histidine, caused a marked increase in O_2 consumption, and a decline in the ammonia production. Thus, under conditions where HCN is formed, peroxidase causes a decline in NH_4^+ production. This is further indication (see ref. 1) that the nitrogen of HCN probably has its origin in the α-amino group of histidine. Further evidence is required, of course, to substantiate this tentative conclusion. It may be relevant, in this connection, however, that amino acid oxidases have been shown to activate the β-position of the α-amino acid substrate [8].

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References

1 Pistorius, E.K., Gewitz, H.-S., Voss, H. and Vennesland, B. (1977) Biochem. Biophys. Acta 481, 384—394
2 Pistorius, E.K., Gewitz, H.-S., Voss, H. and Vennesland, B. (1976) Veröffentlichungen der Universität Innsbruck Vol. 108, pp. 115—116
3 Bailey, J.L. (1967) Techniques in Protein Chemistry, 2nd edn., p. 340, Elsevier, Amsterdam
4 Layne, E. (1957) in Methods in Enzymology (Colowick, S.P. and Kaplan, N.O., eds.), Vol. 3, pp. 447—454, Academic Press Inc., New York
5 Hodgman, C.D. (1954—1955) in Handbook of Chemistry and Physics, 36th ed., p. 1610, Chemical Rubber, Cleveland, Ohio
6 Wriston, J.C. (1970) in Methods in Enzymology (Tabor, H. and Tabor, C.W., eds.), Vol. 17A, p. 733 Academic Press, New York
7 Yagi, K. (1970) in Methods in Enzymology (Tabor, H. and Tabor, C.W., eds.), Vol. 17B, pp. 608—623, Academic Press, New York

8 Bright, H.J. and Porter, D.J.T. (1975) in The Enzymes (Boyer, P.D., ed.), 3rd edn., Vol. XII, pp. 421—505 Academic Press, New York
9 Warburg, O. and Christian, W. (1938) Biochem. Z. 298, 150—165
10 Berg, C.P. and Rodden, F.A. (1976) Anal. Biochem. 71, 214—222
11 Meister, A. (1965) in Biochemistry of the Amino Acids 2nd edn., Vol. 1, pp. 294—319, Academic Press, New York