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CYANIDE FORMATION FROM HISTIDINE IN *CHLORELLA*

A GENERAL REACTION OF AROMATIC AMINO ACIDS CATALYZED BY AMINO ACID OXIDASE SYSTEMS

ELFRIEDE K. PISTORIUS, HANS-SIEGFRIED GEWITZ, HELGA VOSS and BIRGIT VENNESLAND

Forschungsstelle Vennesland der Max-Planck-Gesellschaft, Harnackstrasse 23, Dahlem, 1000 Berlin 33 (Germany)

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Summary

The formation of HCN from D-histidine in *Chlorella vulgaris* extracts is shown to be due to the combined action of a soluble protein and a particulate component. Either horse-radish peroxidase (EC 1.11.1.7) or a metal ion with redox properties can be substituted for the particulate component. Ions of manganese and vanadium are especially effective, as are *o*-phenanthroline complexes of iron. Cobalt ions are less active.

The D-amino acid oxidase (EC 1.4.3.3) from kidney and the L-amino acid oxidase (EC 1.4.3.2) from snake venom likewise cause HCN production from histidine when supplemented with the particulate preparation from *Chlorella* or with peroxidase or with a redox metal ion. The stereospecificity of the amino acid oxidase determines which of the two stereoisomers of histidine is active as an HCN precursor.

Though histidine is the best substrate for HCN production, other naturally occurring aromatic amino acids (viz. tyrosine, phenylalanine and tryptophan) can also serve as HCN precursors with these enzyme systems. The relative effectiveness of each substrate varies with the amino acid oxidase enzyme and with the supplement. With respect to this latter property, the particulate preparation from *Chlorella* behaves more like a metal ion than like peroxidase.

Introduction

The formation, by *Chlorella vulgaris* Beijerinck, of an inactive HCN complex of nitrate reductase (EC 1.6.6.1) was previously reported [1]. Evidence was also presented that histidine could function as an HCN precursor in these algae [2]. In vivo, oxygen and light-stimulated HCN production [2,3], and this was

also the case in vitro, when histidine was added to French press extracts of the cells [2]. Under these latter circumstances, the D-isomer of histidine was about ten times more effective as an HCN precursor than the L-isomer.

The present study describes a *Chlorella* enzyme system which can form HCN from D-histidine in the dark. This system is composed of a soluble D-amino acid oxidase, described in more detail elsewhere [4], and of a particulate component which can be replaced by peroxidase or by a metal ion such as Mn^{2+}. We will also show that the formation of HCN, especially from histidine, but also from other aromatic amino acids, is a general reaction catalyzed by both D-amino acid-oxygen oxidoreductases (deaminating) EC 1.4.3.3, and L-amino acid-oxygen oxido-reductases (deaminating) EC 1.4.3.2, when they are supplemented with peroxidase or suitable metal ions.

Materials and Methods

Reagents and biological materials

Amino acids were obtained from Hoffmann-La Roche, Baden; E. Merck, Darmstadt, or Sigma, St. Louis. Horse-radish peroxidase, purity grade I, crystalline beef liver catalase (for analytical use), 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. Dried venom type I (0.3 unit per mg) from *Crotalus adamantus* was purchased from Sigma, St. Louis.

The growth of *Chlorella vulgaris* on a nitrate/mineral salts medium and the preparation of French press extracts was as previously described [3] with the following modifications: 400 µl cells were inoculated into 500 ml medium and grown for 48 h to reach a cell density of about 6 µl cells per ml. The harvested cells were washed with water and broken in a French pressure cell. Cells high in chlorophyll contained more D-amino acid oxidase for a given volume than cells low in chlorophyll.

The *Chlorella* extracts were stored frozen at −20° C. For the experiment in Table I, the "supernatant" fraction was prepared from a thawed extract by cen-

TABLE I

EFFECT OF *CHLORELLA* EXTRACT AND PEROXIDASE ON HCN FORMATION FROM HISTIDINE

The 3.0-ml reaction mixtures contained 200 µmol phosphate buffer, pH 7.0, 50 µmol KCl and when indicated, 100 µg peroxidase, 30 µmol L- or D-histidine, and 0.5 ml fresh total extract of *Chlorella* or 0.5 ml supernatant from such an extract. In the experiment of line 1, the samples were illuminated with red light (44.5 µEinsteins per min). All other experiments were run in the dark.

Preparation	HCN (nmol)		
	with no addition	with L-histidine	with D-histidine
1. Total extract, red light	1.3	3.8	40.0
2. Total extract	0.0	0.2	1.4
3. Total extract plus peroxidase	0.1	0.0	80.5
4. Supernatant	0.0	0.1	0.2
5. Supernatant plus peroxidase	0.0	0.0	80.1

trifugation for 40 min at 48 000 × *g*. Solid $(NH_4)_2SO_4$ was added to the supernatant to obtain a precipitate between 30 and 60% saturation at 0° C. This precipitate was dissolved in 0.02 M phosphate buffer, pH 7.0, and dialyzed overnight against the same buffer. The final volume was adjusted to one-fifth that of the original extract. The protein content was approx. 50 mg per ml.

The pellet obtained by centrifugation of the frozen and thawed French press extract was washed with five volumes of 0.01 M phosphate buffer, pH 7.0, sedimented by centrifugation, and suspended in the same buffer to give the original volume. This suspension of washed particles from *Chlorella* contained 2.7 mg chlorophyll per ml. The washed particles from leaves of *Spinacea oleracea* or *Tetragonia expansa* were prepared as previously described [5], and suspended in water to give a chlorophyll concentration of 2.7 mg per ml.

Extracts of *Neurospora crassa* were prepared from the mycelium of a wild-type strain obtained from the Institut für Gärungsgewerbe, Berlin. The mycelia were grown on Fries basal medium, induced with nitrate and disrupted to give a crude extract, as described by Garrett and Greenbaum [6]. We are indebted to K. Jetschmann and W. Völker for the preparation of this material. Phosphate buffers were prepared from potassium salts.

Experimental procedure

Reaction mixtures were incubated in the main compartment of Warburg vessels containing a center trough for alkali (0.2 ml 0.1 M NaOH). The vessels were gassed with O_2 and incubated with shaking at 21° C for 3 h in the dark, unless otherwise indicated. Cyanide was determined chemically on aliquots of the alkali by the method of Guiltbault and Kramer [7]. This entire procedure has been described and evaluated elsewhere [8]. At times, the method of Epstein [9] was also used for HCN determination. The results obtained by the two methods were in good agreement. Oxygen consumption was measured manometrically or with an O_2 electrode as described elsewhere [4].

Results

Chlorella

We have reported previously that light and O_2 are necessary for HCN formation with French press extract of *Chlorella* and added histidine [2]. The experiment summarized in Table I shows this light requirement. Both L- and D-histidine gave HCN in the light, though the yield from D-histidine was about ten times higher than that from L-histidine (line 1). There was relatively little HCN formed form D-histidine and algal extract in the dark (line 2), but when horseradish peroxidase was added to the algal extract (line 3), HCN was formed from D-histidine in even larger amounts than were obtained on illumination. This dark reaction occurred only with the D-isomer of histidine. Peroxidase alone was ineffective (not shown). The *Chlorella* component necessary for the reaction was present in the supernatant, after insoluble material had been removed by centrifugation (line 5). This *Chlorella* component was not dialyzable, and could be inactivated by heat (10 min at 65° C). It was present in the 30—60% $(NH_4)_2SO_4$ fraction prepared from the supernatant. The effect of supple-

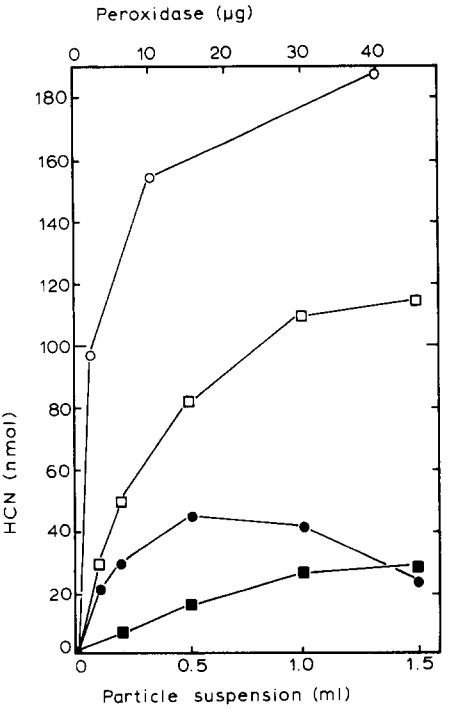

Fig. 1. Effect of added peroxidase and of *Chlorella* and leaf particle preparations on HCN formation. The 3-ml reaction mixtures contained 200 µmol phosphate buffer, pH 7.0, 50 µmol KCl, 30 µmol D-histidine and 0.2 ml D-amino acid oxidase fraction from *Chlorella* extracts (30—60% $(NH_4)_2SO_4$). In addition, different amounts of peroxidase or of washed particle suspensions were added as indicated: ○———○, peroxidase; □———□, *Chlorella* particles; ●———●, New Zealand spinach particles; ■———■, spinach particles.

TABLE II

SPECIFICITY OF D-AMINO ACID OXIDASE FROM *CHLORELLA* AND D-AMINO ACID OXIDASE FROM KIDNEY

For the *Chlorella* enzyme, the 3.0-ml reaction mixtures contained 200 µmol phosphate buffer, pH 7.0, 50 µmol KCl, 100 µg peroxidase, 1.1 mg partially purified enzyme and 30 µmol of the indicated amino acid or 300 µmol glycine. For the kidney enzyme, the 3.0-ml reaction mixtures contained 200 µmol phosphate buffer, pH 7.5, 100 µmol $(NH_4)_2SO_4$, 10 µg peroxidase, 50 µg D-amino acid oxidase and 30 µmol of the indicated amino acid or 90 µmol glycine. Other amino acids tested included DL-proline, DL-threonine, glycine, and the D-isomer of alanine, arginine, aspartic acid, asparagine, cysteine, glutamic acid, glutamine, isoleucine, leucine, lysine, serine, and valine. In no instance was more than 0.1 nmol HCN obtained, usually less.

Amino acid tested	HCN (nmol)	
	Chlorella enzyme	Kidney enzyme
D-Histidine	23.6	115
D-Phenylalanine	0.8	44
D-Tyrosine	1.3	6
D-Tryptophan	0.1	12
D-Methionine	0.2	0
L-Histidine	0	0
L-Phenylalanine	0	0

menting this protein fraction with washed *Chlorella* particles or with peroxidase is shown in Fig. 1.

The first column of Table II shows the specificity of the HCN-forming reaction of *Chlorella* for various D-amino acids. Although D-histidine is the best substrate, smaller amounts of HCN are formed from phenylalanine and tyrosine. The HCN formation was dependent on O_2 (not shown). The soluble protein of *Chlorella* may be called a D-amino acid oxidase, since it catalyzes O_2 uptake with a variety of D-amino acids [4]. The ratio of O_2 taken up to HCN formed from D-histidine was about 3—4, under the most favorable circumstances.

Kidney D-amino acid oxidase

The second column of Table II shows that with the combination of peroxidase and crystalline kidney D-amino acid oxidase [10—12], HCN was obtained from D-histidine. Other aromatic D-amino acids likewise gave HCN. The amounts of HCN that were obtained varied somewhat with pH and O_2 tension, with rather different results for each of the aromatic amino acids (not shown). Near the alkaline pH optimum (pH 8.3) for the O_2 consumption catalyzed by the kidney enzyme, the amounts of O_2 consumed were far larger than the amount of HCN formed. At a pH of 7.5, however, with D-histidine, O_2 consumption and HCN production were of the same order of magnitude.

Snake venom

Snake venom is known to be a particularly active source of L-amino acid oxidase [13,14]. On addition of peroxidase to snake venom or to the purified L-amino acid oxidase from snake venom, relatively large amounts of HCN were obtained from L-histidine but not from D-histidine. The yield of HCN was proportional to the L-amino acid oxidase activity of the preparations. Thus, there was nothing in the crude venom which interfered with the HCN-forming reaction. For reasons of economy, crude venom was used for most of the experimentation, and the data actually presented here were obtained with unfractionated venom, but all of the major observations were duplicated at least once, with the purified enzyme. As in the case of the D-amino acid oxidase from kidney, snake venom supplemented with peroxidase gave HCN from all of the aromatic L-amino acids tested, with histidine giving decidedly more HCN than tryptophan, phenylalanine or tyrosine (Table III). Traces of HCN were obtained from some of the non-aromatic amino acids. The conditions used for the measurements of Table III were selected to optimize the HCN yield from histidine. Under different conditions (pH, buffer, etc.), the relative yields of HCN from the various amino acids can be altered somewhat quantitatively, but not sufficiently to alter the generalizations. There was no correlation whatever between the yield of HCN from a particular amino acid and the O_2 consumption observed with that amino acid. By increasing the amount of snake venom, and using 0.3 μmol histidine instead of the rate-saturating amount of 30 μmol, the reaction of the substrate was brought to completion. Under such circumstances, the yield of HCN was 20—25% of the amount of L-histidine added. That is, about one histidine molecule in every 4 or 5 was degraded to give HCN. Thus, the HCN-forming reaction sequence is not quantitatively large in relation

TABLE III

SPECIFICITY OF SNAKE VENOM

The 3.0-ml reaction mixtures contained 200 μmol of phosphate buffer, pH 7.5, 50 μmol KCl, 180 μg peroxidase, 0.2 mg snake venom powder (0.3 unit per mg) and 30 μmol of the indicated amino acid or 90 μmol glycine. Other amino acids tested included glycine, and the L-isomer of alanine, cysteine, glutamic acid, lysine, ornithine, proline, serine and threonine. In no instance was more than 0.1 nmol HCN obtained, usually less.

Amino acid tested	HCN (nmol)
L-Histidine	467
L-Tryptophan	34
L-Phenylalanine	32
L-Tyrosine	2
L-Arginine	0.4
L-Asparagine	0.4
L-Leucine	0.3
L-Methionine	0.3
L-Aspartic acid	0.2
L-Glutamine	0.2
L-Citrulline	0.2
L-Isoleucine	0.2
L-Valine	0.2
D-Histidine	0
D-Phenylalanine	0

to the normal oxidative deamination reaction, but it is nevertheless large enough to be more significant than a trivial side reaction.

Neurospora

Both L- and D-amino acid oxidases have been shown to be present in *N. crassa* [10.15]. The experiment in Table IV shows that HCN was formed from both isomers of histidine when a fresh extract of *Neurospora* mycelium was supplemented with peroxidase. The yield was higher with L-histidine than with D-histidine, in keeping with reports that the L-amino acid oxidase of *Neurospora* is more abundant than the D-amino acid oxidase [10,15].

The action of metal ions

A variety of metal ions could be substituted for peroxidase to cause HCN

TABLE IV

HCN FORMATION WITH *NEUROSPORA* EXTRACTS

The 3.0-ml reaction mixtures contained 200 μmol phosphate buffer, pH 7.0, 50 μmol KCl, 0.3 ml *Neurospora* extract (4.56 mg protein per ml) and when indicated 100 μg peroxidase and 30 μmol L-histidine or D-histidine.

Additions	HCN (nmol)
None	0.0
Peroxidase	4.1
L-Histidine	0.1
D-Histidine	0.0
L-Histidine, peroxidase	56.4
D-Histidine, peroxidase	23.6

TABLE V

THE EFFECT OF METAL IONS ON HCN FORMATION FROM HISTIDINE

The 3-ml reaction mixtures contained 50 μmol KCl, 200 μmol phosphate buffer, pH 7.5, for the experiments with snake venom and kidney enzyme and pH 7.0 for the experiments with *Chlorella* enzyme, 30 μmol L- or D-histidine, 1.0 μmol of the metal salts as indicated, and the designated enzyme. The Fe-*o*-phenanthroline solutions were prepared by mixing 0.1 ml 0.1 M $(NH_4)_2Fe(SO_4)_2$ or $FeCl_3$, 0.2 ml 0.1 M *o*-phenanthroline and 0.7 ml H_2O, and 0.1 ml was added to the reaction mixture where indicated.

Additions	HCN (nmol)		
	Chlorella enzyme (400 μg)	Kidney enzyme (50 μg)	Snake venom (50 μg)
None	0.1	—	—
$MnSO_4$	27.2	39.5	50.4
$VOSO_4$	31.4	35.2	38.8
$NaVO_3$	28.2	38.5	36.8
$(NH_4)_2Fe(SO_4)_2$	1.5	1.8	11.6
Fe^{2+}-*o*-phenanthroline	33.4	36.2	43.2
Fe^{3+}-*o*-phenanthroline	14.1	13.5	44.0
$CoSO_4$	10.8	11.7	12.8
$KCr(SO_4)_2$	0.5	1.2	2.1
$CuSO_4$	0.2	0.7	1.6
$(NH_4)_6Mo_7O_{24}$	0.2	1.3	1.9
$NiSO_4$	0	2.1	4.4
Na_2WO_4	0.7	2.3	1.8
$CaCl_2$	0.8	0.7	1.7
$MgCl_2$	0.6	1.1	1.9

formation from histidine in the presence of the various amino acid oxidases, as shown in Table V. Manganese and vanadium salts were particularly effective, as was the *o*-phenanthroline complex of iron, whether added in the ferrous or the ferric form. Ferrous ammonium sulfate had relatively less effect, perhaps because of solubility limitations. Co^{2+} was also active but less so than manganese, vanadium and the iron complexes. Trace effects were observed also with other cations. Neither sulfate nor ammonium influenced the reaction at the concentrations here employed (not shown).

The stimulatory effect of metal ions on HCN formation could be observed not only with histidine, but also with the other aromatic amino acids (not shown). In general, there was considerable quantitative variation between separate duplicate measurements in the experiments with metal ions.

The action of Mn^{2+} with histidine was examined further. Table VI shows the effect of Mn^{2+} concentration on HCN yield with the *Chlorella* enzyme. The maximum yield of HCN observed with Mn^{2+} was only about half as great as that obtained with saturating amounts of peroxidase. When suboptimal amounts of Mn^{2+} and peroxidase were added together, the stimulatory effects were additive, but if the peroxidase was present in saturating amounts, added Mn^{2+} generally inhibited HCN production somewhat, especially at the higher concentrations tested (not shown). With snake venom and with kidney D-amino acid oxidase, the Mn^{2+} effect resembled that described in Table VI for the *Chlorella* enzyme.

TABLE VI

EFFECT OF Mn^{2+} ON HCN GENERATION FROM HISTIDINE

The 3.0-ml reaction mixtures contained 200 μmol phosphate buffer, pH 7.0, 30 μmol D-histidine, 1.0 mg amino acid oxidase fraction from *Chlorella* (30—60% $(NH_4)_2SO_4$) and peroxidase or $MnSO_4$ as indicated.

Additions	HCN (nmol)
None	0.6
1000 nmol $MnSO_4$	25.6
100 nmol $MnSO_4$	33.6
20 nmol $MnSO_4$	22.8
10 nmol $MnSO_4$	7.5
1 nmol $MnSO_4$	1.4
100 μg peroxidase	56.0

The activation of HCN production by particulates

The original fresh *Chlorella* extract from which both washed particles and D-amino acid oxidase were prepared gave little HCN from D-histidine in the dark (Table I, line 2). Freezing and thawing of such extracts resulted in an activation of the dark HCN-forming reaction. When tests were carried out under the conditions of Table I (line 2), for example, 0.5 ml of fresh extract gave 2.0 nmol HCN from D-histidine. After it had been held at −25° C for 80 min, and thawed, the same extract gave 16.5 nmol HCN from D-histidine under identical conditions. Storage for 24 h at −25° C resulted in an additional increase to 28 nmol HCN formed, in a similar test. Further storage led to a decrease rather than an increase in HCN yield. Apparently, a certain amount of disruption of subcellular structure is necessary to unmask the activity of the insoluble fraction. The nature of the 'unmasking' is not clear; i.e. it may involve an activation of some sort or a removal of an inhibition.

Washed particulate preparations from leaves could also substitute for the action of peroxidase, to activate HCN formation from D-histidine. Fig. 1 shows the effects on HCN yield of increasing amounts of peroxidase, of a *Chlorella* particulate fraction, and of particulate fractions from spinach (*S. oleracea*) leaves and from leaves of New Zealand spinach (*T. expansa*). In all cases, the same amount of a crude D-amino acid oxidase preparation from *Chlorella* was present. The HCN-forming reaction tended to saturate with increasing amounts of peroxidase or of the various fractions. The maximum level reached was lower for *Chlorella* particulates than for peroxidase, and still lower for the leaf fractions.

Table VII shows the relative effectiveness of peroxidase, Mn^{2+} and *Chlorella* particles in stimulating HCN production from various amino acid substrates with snake venom and with kidney enzyme. Measurements were made both in air and in O_2. These show that the effect of substituting O_2 for air varied considerably with the different substrates. The major conclusion drawn from Table VII was that the behaviour of the particles was more similar to the behaviour of Mn^{2+} than to the behaviour of peroxidase, on the basis of the relative HCN yields. Note, for example, that in the case of phenylalanine, much less HCN was obtained with Mn^{2+} or particles than with peroxidase. In contrast, the yield of HCN from tryptophan and tyrosine in O_2 was actually higher with Mn^{2+} or particles than with peroxidase.

TABLE VII

HCN GENERATION FROM AROMATIC AMINO ACIDS: EFFECT OF *CHLORELLA* PARTICLES, PEROXIDASE AND Mn^{2+}

The 3.0-ml reaction mixtures contained 50 μmol KCl and 200 μmol phosphate buffer, pH 7.5. Other additions as indicated, with 30 μmol each of the various amino acids.

Additions	HCN (nmol)					
	Peroxidase (100 μg)		Mn^{2+} (1 μmol)		*Chlorella* particles (4 mg chlorophyll)	
	Air	O_2	Air	O_2	Air	O_2
Snake venom: 50 μg						
L-Histidine	120	114	54	66	30	35
L-Phenylalanine	16	33	1	2	2	5
L-Tryptophan	17	28	7	36	18	46
L-Tyrosine	2	5	4	9	4	6
D-Amino acid oxidase from kidney: 50 μg						
D-Histidine	84	118	25	42	37	52
D-Phenylalanine	57	55	2	4	6	9
D-Tryptophan	11	12	13	23	17	21
D-Tyrosine	2	6	12	22	15	18

Peroxidase activity could not be detected in the particles by standard tests, although we could easily measure added peroxidase activity at a level equivalent to the particle cofactor activity for HCN production. This activity of the particles was heat stable. The particles contain both Mn and Fe in bound form. It seems possible that the stimulatory effct of the particles is due to their bound metal(s), though further studies are required to identify the effective chemical components.

Discussion

There is evidence that HCN formation occurs in vivo in *C. vulgaris* [2,3], though these cells do not appear to accumulate cyanogenic glycosides [8]. Previous studies have also shown that histidine can function as an HCN precursor in extracts of these cells, D-histidine being considerably more effective than L-histidine [3]. The present data show that the production of HCN from D-histidine in *Chlorella* extracts in the dark is due to the combined action of a soluble protein and of a particulate fraction. The soluble protein has been identified as a D-amino acid-oxygen oxidoreductase, and its properties are described elsewhere in more detail [4]. The active component of the particulate fraction has not been identified with certainty, but is most probably a metal ion with redox properties. Either horse-radish peroxidase or Mn^{2+} or iron complexes can be substituted for the particulate fraction. As here employed, the latter preparation is of multiple origin and contains both Mn and Fe in bound insoluble form. Further work is required to determine the cellular location of this activity and to pinpoint its reactive group(s). Still open also are the questions of

how light stimulates HCN production in crude extracts, and of how HCN is formed from L-histidine. It has been reported that small amounts of L-amino acid oxidase activity are present in thylakoids from some algae [16]. Thus one might assume that HCN is formed independently from L-histidine, or that L-histidine is somehow racemized to D-histidine.

The evidence presented here shows that the capacity to produce HCN from histidine and from other aromatic amino acids is apparently a general property of amino acid oxidases, both D and L, provided they are suitably supplemented. Such a reaction might explain how L-amino acid oxidase could contribute to the toxicity of snake venom, a question which has previously been elusive [13]. Though L-amino acid oxidase alone has been shown to be non-toxic in mice [17], the combined presence of the amino acid oxidase with enzymes causing extensive tissue degradation might lead to HCN production. In addition, since amino acid oxidases have a wide distribution, the question is raised whether HCN production may be of general importance physiologically. Such a possibility has in fact previously been indicated by the demonstration of cyanide-utilizing enzyme reactions in cells not known to contain cyanogenic glycosides. The subject has recently been reviewed [18].

It is noteworthy that histidine is particularly favored as an HCN precursor, though it is often a relatively poor substrate for O_2 consumption, for example, with the kidney D-amino acid oxidase. An early study of Edelbacher and Grauer [19] is of interest in this connection. These authors described a cyanide-sensitive oxidation of D-histidine, catalyzed by liver extracts. The imidazole group was maintained, and the yield of ammonia was low. Their conclusion that there was a separate D-histidine oxidase in liver, distinct from D-amino acid oxidase, was apparently not accepted. The present results suggest that they may in fact have been dealing with a HCN-forming reaction catalyzed by D-amino acid oxidase plus a peroxidase-like supplement. The peroxidase-catalyzed HCN formation would be expected to be inhibited by HCN. In the procedure here employed, the HCN is removed continuously from the reaction mixture. Nevertheless, the HCN-producing reaction cannot be run very rapidly. With snake venom and peroxidase, the most active system studied, we have never obtained more than about 1 μmol of HCN in 3 h at 20°C regardless of how much of the enzymes were added.

The present experiments do not contribute information about the reaction mechanism for HCN formation, except perhaps for the fact that phenylalanine and tyrosine can likewise yield HCN. If we assume a similar reaction mechanism with all substrates, then the α-amino group of the amino acid represents the only possible source of the nitrogen in the HCN. As already suggested [2], we suspect a reaction mechanism rather similar to the reaction sequence for the synthesis of cyanogenic glycosides, as proposed by Conn and his associates [20] (see also Pistorius and Voss [4]).

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