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THE FORMATION OF HYDROGEN CYANIDE FROM HISTIDINE IN THE PRESENCE OF AMINO ACID OXIDASE AND PEROXIDASE

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Summary

Conditions were sought to increase the yield of HCN from L-histidine incubated with L-amino acid oxidase (L-amino acid:oxygen oxidoreductase (deaminating), EC 1.4.3.2) from snake venom, and horseradish peroxidase (donor:hydrogen-peroxide oxidoreductase, EC 1.11.1.7). Small amounts of histidine and high buffer concentrations favored high HCN yields, which reached a maximum of 72%.

Imidazole 4-aldehyde and imidazole 4-carboxylic acid were identified among the reaction products, together with CO_2, NH_3, H_2O_2 and imidazole acetic acid. The CO_2 formed was equal to the histidine oxidized, and to the sum of NH_3 plus HCN formed. The production of HCN was associated with an increased O_2 uptake, which was established from the beginning of the reaction, with no apparent lag and ranged from 1.2 to 1.6 μmol extra O_2 taken up/μmol HCN formed. The system was inhibited by catalase, but added superoxide dismutase caused a small stimulation of both HCN production and O_2 consumption, and a larger stimulation of H_2O_2 accumulation. Added hydroxylamine was cooxidized to nitrite in an amount equimolar with the HCN formed. This nitrite formation was inhibited by superoxide dismutase. The facts could be interpreted in terms of superoxide anion formation during the HCN-producing reaction.

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Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Hepps, *N*-2-hydroxyethylpiperazine-*N'*-3-propanesulfonic acid.

Cytochrome *c*, heme, or ferricyanide could be substituted for peroxidase, but were less effective. The initial rates of HCN formation from phenylalanine, tyrosine and tryptophan were higher, but the eventual yields of HCN from these amino acids were lower than those from histidine.

Introduction

The oxidation of amino acids by amino acids oxidase (L-amino acid:oxygen oxidoreductase (deaminating), EC 1.4.3.2) has been studied extensively [1—4]. In broad outline, and superficially, the enzyme-catalyzed reaction is thought to give the imino (or enamine) derivative of the amino acid (Eqn. 1), which is hydrolyzed rather rapidly but non-enzymically to the keto acid and ammonia (Eqn. 2). The reduced flavin of the enzyme reduces O_2 to H_2O_2. This H_2O_2 oxidizes the keto acid non-enzymically (Eqn. 3). In the presence of catalase, the H_2O_2 is converted to O_2 and H_2O (Eqn. 4), and Reaction 3 no longer takes place, with the consequence that the O_2 uptake is reduced by 50%, and no CO_2 is formed.

$$R \cdot CHNH_2 \cdot COOH + O_2 \rightarrow R \cdot CNH \cdot COOH + H_2O_2 \quad (1)$$

$$R \cdot CNH \cdot COOH + H_2O \rightarrow R \cdot CO \cdot COOH + NH_3 \quad (2)$$

$$R \cdot CO \cdot COOH + H_2O_2 \rightarrow R \cdot COOH + CO_2 + H_2O \quad (3)$$

$$2\ H_2O_2 \rightarrow O_2 + 2\ H_2O \quad (4)$$

When the substrate is an aromatic amino acid (histidine mainly, but also tryptophan, phenylalanine or tyrosine), the addition of horseradish peroxidase to the amino acid oxidase system has been shown to lead to extra O_2 consumption and the formation of considerable quantities of HCN [5,6]. The present studies with L-amino acid oxidase from snake venom and horseradish peroxidase (donor:hydrogen-peroxide oxidoreductase, EC 1.11.1.7) were carried out primarily with L-histidine to further characterize the HCN-producing reaction.

Materials and Methods

Materials

L-Amino acid oxidase (snake venom, *Crotalus terrificus terrificus*, crystal suspension in 3.2 M ammonium sulfate, 7 units/mg), horseradish peroxidase, purity grade I, catalase (bovine liver crystal suspension), L-glutamate dehydrogenase (solution in 50% (v/v) glycerol) were purchased from Boehringer, Mannheim; superoxide dismutase (erythrocuprin) from bovine erythrocytes (freeze-dried powder, about 10 000 units/mg) from Miles; Hepes and Hepps from Serva; dried venom type 1 L-amino acid oxidase (0.6 unit/mg from *Crotalus adamantus*), L-1′-methyl histidine and L-3′-methyl histidine and α-DL-methyl histidine dihydrochloride from Sigma, St. Louis; L-histidine, *o*-dianisidine, hydroxylamine, potassium ferricyanide and hydrogen peroxide solution were purchased from E. Merck, Darmstadt. Imidazole pyruvic acid

hydrochloride, 4-hydroxymethyl imidazole and imidazole 4-acetic acid hydrochloride were purchased from Calbiochem.

Imidazole pyruvic acid oxime was prepared from imidazole pyruvic acid and hydroxylamine [7]. Imidazole 4-acetonitrile (4-cyanomethylimidazole) was prepared from histidine as described by Tabor [8]. 4(5)-Hydroxymethyl imidazole hydrochloride was synthesized according to Totter and Darby [9], and compared with a commercial sample obtained from Calbiochem. Both gave identical infrared spectra. Imidazole 4-aldehyde and imidazole 4-carboxylic acid were prepared from 4(5)-hydroxymethyl imidazole as described by Turner and coworkers [10,11], by oxidation with nitric acid according to Pyman [12]. Imidazole 4-aldehyde has a strong absorption band with a maximum at 265.5 nm in 95% alcohol. The molar extinction coefficient was 13 260 in alcohol (Turner gives 11 700) and 14 356 in water. This band disappears on acidification, as described by Turner. It shifts to longer wavelengths in alkali (see later).

Chromatography

Chromatography of imidazole derivatives has been described frequently [8,13—16]. In the present studies, many different methods were employed, most often with samples incubated in phosphate buffer. After evaporation to dryness under reduced pressure at room temperature, the residue was extracted with 95% ethanol and aliquots were usually applied to Whatman No. 1 paper prepared for circular chromatography. Solvent systems used for development included mainly *n*-butanol/pyridine/H_2O (1 : 1 : 1) [16], methanol/chloroform/10% formic acid (3 : 3 : 1) [14], isopropanol/formic acid/H_2O (40 : 2 : 10) [15], and 100% ethanol/H_2O (77 : 23) [8]. For staining, the Pauly test was generally used [8], but iodine [8] or $FeCl_3$ in 95% ethanol [15] were also employed. The latter stain gives a blue spot with imidazole pyruvic acid and a faint pink spot with imidazole 4-aldehyde, but no color with hydroxymethyl imidazole, histidine, imidazole acetic acid or imidazole carboxylic acid.

Analytical methods

Determination of O_2 consumption, CO_2 formation and NH_3 production. The reactions were carried out in Warburg vessels containing a center trough connected to a side arm, and another side arm connected to the main compartment [17]. Base (0.2 ml of 0.2 N NaOH) was added to the center trough for the collection of CO_2 and HCN. After being filled, the vessels were gassed with O_2 and incubated with shaking at 30°C in the dark. The reaction was started by addition of L-amino acid oxidase from the side arm to the main compartment. The O_2 consumption was calculated from the observed manometric pressure change. When the O_2 consumption was complete, acid was added to the side arm connected to the main compartment. After reequilibration the CO_2 was released by addition of excess acid (0.2 ml 1 N H_2SO_4) from the side arm into the base in the center trough and acid (0.2 ml 4 N H_2SO_4) was also added from the other side arm to the contents of the main compartment. CO_2 production was calculated from the measured pressure increase. HCN contributes only negligibly to the pressure. Corrections were applied for blank values determined with all reagents, but without incubation. NH_3 was determined by

the glutamate dehydrogenase method [18]. At times, rates of O_2 consumption were also determined with a Gilson Oxygraph, Model IC-OXY, equipped with a Clark electrode.

Determination of HCN. HCN was determined chemically [19,20] on aliquots of the alkali in the center trough. Identical reaction mixtures in separate vessels were set up for the HCN determinations. In experiments of 3 or more h, the long incubation time ensured the complete transfer of HCN from the reaction mixture to base in the center well. In short-term incubations, where the reaction was not run to completion, separate vessels were also used for HCN determinations. The reaction was stopped by addition of 0.1 ml of 60% perchloric acid or 0.2 ml 4 N H_2SO_4 from the side arm to the main compartment, and the incubation was continued with shaking for another 3 h to ensure complete HCN collection. In a companion vessel to that used for HCN determination, the reaction was also stopped by acid addition, and suitable aliquots were taken for determination of other products.

Determination of H_2O_2, and cooxidation of NH_2OH to NO_2^-. H_2O_2 was determined on suitable aliquots, without delay, with *o*-dianisidine and peroxidase [21]. Elstner and coworkers have described the use of NH_2OH as a trapping agent for $O_2^{\cdot-}$ [22,23]. They have suggested that the reation proceeds as shown in Eqn. 5:

$$NH_2OH + 2\ O_2^{\cdot-} + H^+ \rightarrow NO_2^- + H_2O_2 + H_2O \qquad (5)$$

That is, one molecule of NO_2^- should be formed for two molecules of $O_2^{\cdot-}$ consumed. The reactions were carried out in separate Warburg flasks, one for each time interval, when HCN was also measured. Alternatively, when only NO_2^- was to be measured, the reactions were carried out in test tubes placed in a water bath maintained at 30°C, and O_2 gas was bubbled through the reaction mixture. For determination of NO_2^-, 0.5 ml aliquots of the reaction mixture were mixed with 0.5 ml of a 1% acid sulfanilamide solution, followed by addition of *N*-1-naphthylethylenediamine hydrochloride [24]. High concentrations of Hepes buffer interfered with the NO_2^- determination, whereas Hepps did not, and was therefore preferred for NO_2^- measurements.

Determination of imidazole 4-aldehyde. This substance was determined by measurement of its absorption band in alkali at 280.5 nm. It was necessary to remove all imidazole pyruvic acid from the solution. An aliquot of the acidified reaction mixture was first neutralized and then made to 5 mM H_2O_2. After 15 min at room temperature, all imidazole pyruvic acid had been oxidized to imidazole acetic acid, which shows no absorption in the region of the ultraviolet where the imidazole aldehyde can be measured. Residual H_2O_2 was removed by adding a few μg catalase, and an aliquot was diluted with 0.1 N NaOH for measurement of absorption curves. The molar extinction coefficient of imidazole 4-aldehyde in 0.1 N NaOH was $16.05 \cdot 10^3$. The alkaline absorption band disappears reversibly on acidification and irreversibly on reduction with sodium borohydride. It was convenient to determine the difference spectrum between a sample to which sodium borohydride had been added and a sample not so treated. The absorption spectrum thus obtained was compared with the absorption spectrum of authentic imidazole 4-aldehyde.

Results

Optimization of HCN yield

The amount of HCN obtained from histidine in the presence of amino acid oxidase and peroxidase varies with the nature of the buffer and with its concentration as well as with pH and with the amounts of the two enzymes, as illustrated in Fig. 1. In the present study, an effort was made primarily to find conditions which maximized the HCN yield from a given small amount of histidine, which was oxidized to completion. All of the data shown in Fig. 1 where obtained in this way. Superoxide dismutase was included in the reaction mixtures because it caused a small increase in the yield of HCN. Buffer and buffer concentration had different effects on the amino acid oxidase reaction alone, and on the reaction leading to HCN. In general, the yield of HCN increased with increasing buffer concentration. The highest yields we have observed (72%) were obtained with high concentrations of Hepes buffer.

Stoichiometry of O_2 uptake, and yield of NH_3, CO_2 and HCN

The oxidation of histidine in the presence of amino acid oxidase alone

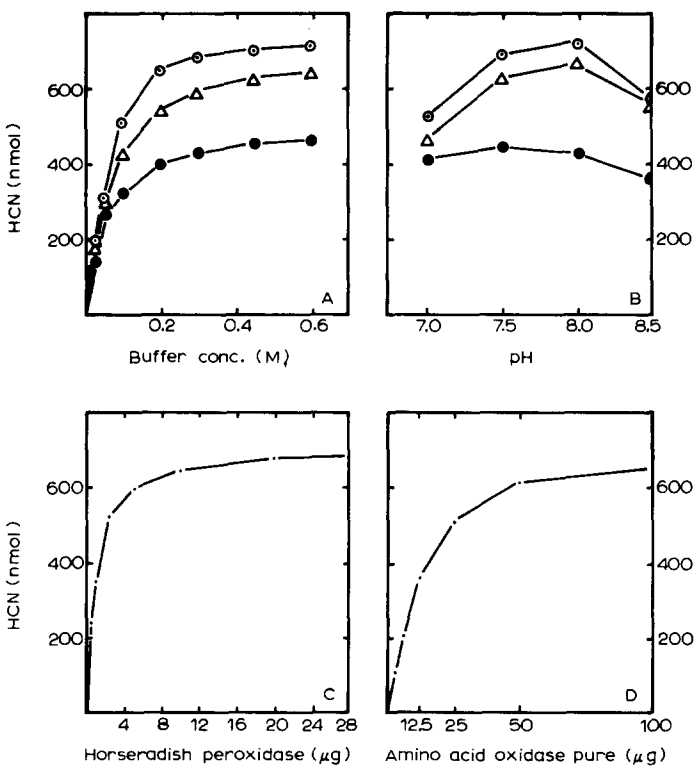

Fig. 1. Effect of conditions on yield of HCN. Except for the indicated variable, reaction mixtures contained 350 mM Hepes buffer, pH 7.5, 1 μmol L-histidine, 100 μg amino acid oxidase (pure), 25 μg superoxide dismutase and 40 μg peroxidase, in a total reaction volume of 1.2 ml. Time, 5.5 h. (A) Effect of buffer and buffer concentrations; ○———○, Hepes; △———△, Hepps; ●———●, potassium phosphate. (B) Effect of pH with 350 mM buffer; ○———○, Hepes; △———△, Hepps; ●———●, potassium phosphate. (C) Effect of peroxidase. (D) amino acid oxidase.

proceeds in the normal fashion expected for the oxidation of any L-amino acid substrate. One molecule of O_2 is taken up, and one molecule each of NH_3 and CO_2 are formed for each molecule of amino acid oxidized in accordance with Eqns. 1—3. This is illustrated in Table I, which shows the results of two sets of measurements, in which 2, 3 and 5 μmol of histidine were oxidized to completion. The upper half of the table shows the close agreement with the expected values in the absence of added peroxidase, and the lower half shows the results with peroxidase added. Added peroxidase caused a substantial increase in the amount of O_2 taken up, but no change in the amount of CO_2 formed. The NH_3 formation on the other hand decreased by an amount equal to the HCN formation, so that histidine oxidized = CO_2 formed = HCN plus NH_3 formed. The buffer concentration in these experiments was somewhat lower than that required to give a maximal HCN yield, because very high buffer concentrations interfered with the NH_3 determination. Expt. A was carried out with unfractionated snake venom, and Expt. B with crystalline amino acid oxidase. The results with both are the same. That is, there is nothing in the crude snake venom that interferes with the reaction. Other experiments showed that added NH_3 did not influence the reaction, and that the O_2 uptake and the HCN and CO_2 yields were also the same with both crude and purified enzyme (not shown). Experiments like those of Table I were also carried out in phosphate buffer, with similar results (not shown), except that the yield of HCN was lower (Fig. 1A) and NH_3 correspondingly higher.

In the measurements shown in Table I, the addition of peroxidase in Hepes buffer led to about a doubling of the O_2 uptake. This amounts to 1.5—1.6 μmol extra O_2 consumed/μmol of HCN produced. In phosphate buffer, the extra O_2 consumed after peroxidase addition amounted to 1.2—1.3 μmol extra

TABLE I

O_2 UPTAKE, AND YIELD OF NH_3, CO_2 AND HCN

The reaction mixtures contained 350 mM Hepes buffer of pH 7.5, the indicated amount of L-histidine, 25 μg superoxide dismutase, and 40 μg peroxidase (HRP), where indicated, in a total reaction volume of 1.2 ml. In Expt. A, 0.9 U amino acid oxidase were added; in Expt. B, 0.7 U amino acid oxidase were used. Incubation time was 5 h for Expt. A and 5.5 h for Expt. B. For other details see Materials and Methods.

Histidine (μmol)		O_2 taken up (μmol)	NH_3 formed (μmol)	CO_2 formed (μmol)	HCN formed (μmol)	NH_3 + HCN (μmol)
—HRP						
2	A	1.92	2.03	—	0	
	B	1.96	—	1.90		
3	A	2.82	2.97	—	0	
	B	2.90	—	2.97		
5	A	4.80	4.82	—	0	
	B	4.85	—	4.72		
+HRP						
2	A	4.35	0.72	—	1.32	2.04
	B	4.35	—	1.99	1.34	—
3	A	6.31	1.07	—	1.88	2.95
	B	6.54	—	2.97	1.96	—
5	A	9.89	2.09	—	2.88	4.95
	B	10.2	—	4.83	3.09	—

O_2/μmol HCN formed. Thus, there was no clear stoichoimetric relationship of extra O_2 consumption to HCN production.

Effect of O_2 tension

All of the experiments here described were carried out in 100% O_2. This high O_2 tension was not necessary, however, for maximal HCN yields. In fact, at low histidine concentrations, the yield was as good in air as in O_2, and a strong dependence on O_2 tension could be demonstrated only by going to O_2 pressures lower than 20%.

Effect of superoxide dismutase

Superoxide dismutase catalyzes the reaction shown in Eqn. 6:

$$2\ O_2^- + 2\ H^+ \rightarrow O_2 + H_2O_2 \qquad (6)$$

When superoxide dismutase was added to the histidine-amino acid oxidase system in the absence of peroxidase it had no effect on the reaction, but when added in the presence of peroxidase, it stimulated HCN formation and O_2 consumption slightly, and caused a larger increase in the amount of H_2O_2 which accumulated (Table II). This increase in H_2O_2 implies that O_2^- may be formed, though there are other possible interpretations. The stimulatory effect of superoxide dismutase on HCN production might be due to the fact that peroxidase reacts with O_2^- to yield the less reactive compound III [25,26]. Removal of O_2^- might therefore increase the effectiveness of a given amount of peroxidase. The effect of superoxide dismutase at suboptimal peroxidase concentrations was not greater, however, than the effect of superoxide dismutase with a saturating peroxidase concentration. If superoxide dismutase acts by causing a dismutation of O_2^- as shown in Reaction 6 one might expect it to cause a decline in the net rate of O_2 consumption, at least at the beginning of the reaction. No such initial inhibitory effect could be detected.

Attempts to measure O_2^- formation

Cytochrome *c* and nitroblue tetrazolium are often used as trapping agents

TABLE II

EFFECT OF SUPEROXIDE DISMUTASE ON O_2 UPTAKE, H_2O_2 FORMATION AND HCN FORMATION

Reaction mixtures contained 1.2 ml of 133 mM phosphate buffer, pH 7.5, 5 μmol of L-histidine, 0.7 U amino acid oxidase, 40 μg peroxidase (HRP) where indicated and superoxide dismutase (SOD) as indicated. Incubation time, 4 h.

SOD (μg)	HRP	O_2 uptake (μmol)	H_2O_2 (μmol)	HCN (μmol)
0	—	4.86	0.04	0.007
0	+	6.52	0.60	1.37
5	+	6.75	0.94	1.53
10	+	6.84	1.10	1.50
20	+	6.98	1.12	1.63
50	+	6.98	1.23	1.64
100	+	7.02	1.30	1.60
200	+	7.20	1.28	1.64

for O_2^- [27]. Both were reduced when added to the HCN-producing system. Cytochrome *c* is reduced by the system in a different way, however, since it can serve as a substitute for peroxidase to cause HCN formation (see later), and reduced cytochrome *c* can also be oxidized by peroxidase-H_2O_2. These interactions became so complex that cytochrome *c* did not appear suitable for demonstrating O_2^- formation. Added nitroblue tetrazolium was also reduced by the system. For a rapid rate of nitroblue tetrazolium reduction, the presence of O_2 and peroxidase was essential, in addition to amino acid oxidase and histidine. Maximal reduction rates were observed, however, at an O_2 concentration of about 5%. At higher O_2 tensions, the rate of nitroblue tetrazolium reduction decreased and was eventually strongly inhibited as though a reoxidation of the dye was occurring. An O_2 concentration of 5% was definitely suboptimal for HCN production. Added superoxide dismutase had variable effects, depending on conditions. Thus, the interaction with nitroblue tetrazolium also seemed too complex to be very useful.

Cooxidation of NH_2OH to NO_2^-

NH_2OH can interact with many oxidants to give N_2 and nitrous oxide as well as nitrite and nitrate. The products may be mixed, and vary with pH and the nature of the oxidizing agent [28]. When NH_2OH was added to histidine and amino acid oxidase in the absence of peroxidase, there was no change in O_2 consumption, and only a trace of NO_2^- was formed. In the presence of peroxidase, added NH_2OH led to NO_2^- formation, perhaps because of the occurrence of the reaction shown in Eqn. 5. Because NH_2OH was inhibitory, only small amounts could be used. With 1 μmol of histidine and 1 μmol of NH_2OH or less, the amount of NO_2^- formed was equal to the amount of HCN formed over the initial course of the reaction, until about 25% of the NH_2OH could be accounted for. Since no more NO_2^- was obtained, it appeared that the remainder of the NH_2OH was oxidized to other products. In fact, the manometric pressure changes suggested that there was a burst of gas evolution at the beginning of the reaction in the presence of NH_2OH. Thus, O_2 uptake could not be measured manometrically. Measurements of the O_2 uptake with the O_2 electrode indicated that NH_2OH inhibited the extra O_2 uptake and the HCN formation due to peroxidase to about the same extent.

The formation of NO_2^- from NH_2OH is not a specific indicator of O_2^- formation, since organic peroxides (but not H_2O_2) can also oxidize NH_2OH to NO_2^- [29]. To the extent that the NO_2^- formation is caused by O_2^-, it should be inhibited by superoxide dismutase. Table III shows that added superoxide dismutase inhibited NO_2^- formation to the extent of 63%, without inhibiting HCN formation. Heat-inactivated superoxide dismutase had no such inhibitory effect. The inhibition was largest at the beginning of the reaction and declined somewhat with time (not shown). About 70% inhibition was obtained in the first minute, with the highest concentration of superoxide dismutase.

Effect of catalase

As previously reported [6], catalase inhibits the peroxidase-dependent HCN-forming system. Table IV show this effect under the conditions presently used,

TABLE III

EFFECT OF SUPEROXIDE DISMUTASE ON YIELD OF NITRITE FROM HYDROXYLAMINE

The reaction mixtures contained 1 μmol L-histidine, 1 μmol hydroxylamine, 40 μg peroxidase, 0.6 U amino acid oxidase and superoxide dismutase (SOD) as indicated, in 1.2 ml 350 mM Hepps buffer, pH 7.5. Incubation time, 5 min.

SOD added (μg)	HCN (nmol)	NO_2^- (nmol)
None	72	78
3.1	69	41.5
6.2	75.5	37
12.5	74	31.5
25	73.5	28.5
50	72.5	29.0

at two different peroxidase concentrations, one saturating and one well below saturation. The catalase is more effective at the lower peroxidase concentration.

Addition of a few micrograms of catalase to the amino acid oxidase-histidine system, in the absence of peroxidase, has the expected effect of causing H_2O_2 dismutation and a consequent accumulation of imidazole pyruvic acid, since the H_2O_2 is no longer available for the oxidation of the keto acid to imidazole acetic acid and CO_2. As expected, the O_2 consumption is reduced by 50% and almost no CO_2 is formed after catalase addition, as shown in the first two lines of Table V. The lower two lines show the effect of catalase on the reaction in the presence of peroxidase. When this small amount of catalase is added together with peroxidase to the amino acid oxidase and histidine, a yellow pigment develops, which does not appear in the absence of peroxidase. A similar yellow pigment is formed when peroxidase is incubated with imidazole pyruvic acid alone, without amino acid oxidase. The pigment-forming reaction requires O_2 and is accompanied by CO_2 production. That is, peroxidase behaves as an oxidase toward imidazole pyruvic acid. Under the same conditions, imidazole acetic acid is not oxidized. Thus, the addition of catalase leads to a

TABLE IV

EFFECT OF CATALASE ON HCN YIELD

The reaction mixtures contained 1 μmol L-histidine, 25 μg superoxide dismutase, 0.35 U amino acid oxidase, and peroxidase (HRP) and catalase as indicated in 1.2 ml 350 mM Hepes buffer, pH 7.5. Incubation time, 5 h.

Added catalase (μg)	HCN (nmol)	
	40 μg HRP	1 μg HRP
None	604	396
1	570	380
10	545	113
100	302	16
500	62	4

side reaction which complicates the interpretation of O_2 uptake and CO_2 formation. This side reaction is largely but perhaps not entirely avoided when the imidazole pyruvic acid is converted to imidazole acetic acid by oxidation with H_2O_2, because this latter reaction is not instantaneous, and some imidazole pyruvic acid accumulates during the early part of the reaction, eventually disappearing with more prolonged incubation. In the bottom line of Table V, the values for O_2 consumption and CO_2 production must be corrected for the peroxidase-catalyzed oxidation of imidazole pyruvic acid, a reaction not affected by catalase. Oxidation of imidazole pyruvic acid under the conditions of Table V gave 1.69 μmol O_2 taken up and 1.07 μmol CO_2 formed. These values (especially the CO_2 value) could not be measured very accurately. If we apply these corrections to the numbers in the bottom line of Table V, we get 4.39 μmol O_2 taken up, and 2.06 μmol CO_2 given off. Comparison of the CO_2 value, 2.06, with the value for HCN, 1.54, suggests that the CO_2 evolution is somewhat larger than the HCN formation, but in view of the inaccuracies, these numbers might be equal. If HCN is formed from the α-carbon atom of the side chain and its attached N, we expect CO_2 to be formed from the carboxyl group of histidine. The data in Table V substantiate this; that is, CO_2 = HCN. Furthermore, there were $7.11 - 4.39 = 2.72$ μmol O_2 evolved by catalase action in the presence of peroxidase. This number does not differ significantly from 2.47, the difference measured in the absence of peroxidase. In both cases, 5 μmol H_2O_2 were available for catalytic decomposition: 1 mol H_2O_2/mol histidine oxidized. One must conclude that the H_2O_2 formed initially (Eqn. 1) is either not used in the peroxidase-catalyzed reaction, or if the reaction involves utilization of H_2O_2, it also provides for the regeneration of H_2O_2.

Substitutes for peroxidase

We have already reported that a variety of redox metals can substitute for peroxidase to cause HCN production from histidine in the presence of amino acid oxidase [5,6]. In addition to these, cytochrome *c*, haemin and ferricyanide are also effective, but less so than peroxidase (Table VI). All of the cytochrome *c* was completely reduced before the end of the incubation period, which suggested that the reoxidation of cytochrome *c* was rate limiting. Since ferricyanide is potentially a source of HCN, controls were carried out to show that the amounts of HCN obtained from ferri- or ferrocyanide alone were relatively trivial, compared to the amount of HCN generated from histidine.

TABLE V

EFFECT OF CATALASE ON STOICHIOMETRY

The reaction mixtures contained 5 μmol L-histidine, 20 μg peroxidase (HRP) as indicated, 0.9 U amino acid oxidase, 5 μg amino acid oxidase, 5 μg catalase as indicated, in 3 ml 147 mM phosphate buffer, pH 7.5. Incubation time, 4.5 h. Data are in μmol.

	O_2 uptake	CO_2	NH_3	HCN
No HRP, no catalase	4.93	4.68	4.90	0.002
No HRP, catalase	2.46	0.10	4.96	0.002
HRP, no catalase	7.11	4.71	3.28	1.63
HRP, catalase	6.08	3.13	3.22	1.54

TABLE VI

EFFECT OF VARIOUS COFACTORS

The reaction mixtures contained 1 μmol L-histidine, 25 μg superoxide dismutase, 0.35 U amino acid oxidase, and the indicated cofactors in 1.2 ml 350 mM Hepes buffer, pH 7.5. Reaction time, 4 h.

Additions (μmol)	HCN (nmol)
Peroxidase (0.001)	611
Cytochrome *c* (0.2)	98
Ferricyanide (6.0)	215
Haemin (0.2)	225
FMN (0.1)	2

Ferricyanide can act as a substitute for O_2 also, in the amino acid oxidase reaction. Under argon, much ferricyanide was reduced, but no HCN was formed. Thus, this HCN-producing reaction was completely dependent on O_2. In air, the yield of HCN with ferricyanide was one third of the yield in O_2. Illuminated flavin can also cause HCN production from histidine (not shown), but we are dealing in the present experiments only with dark reactions.

Possible intermediates and reaction products

Imidazole 4-acetonitrile and imidazole 4-pyruvic acid oxime were tested as possible intermediates, by addition to the HCN-forming system or to the enzymes, with and without added H_2O_2. No HCN formation from these compounds was observed.

In addition to the products of the side chain of histidine, reaction products containing the imidazole ring were sought. Chromatography on paper of reaction mixtures run to completion in the absence of catalase showed the expected imidazole acetic acid as a prominent red-brown spot. A pale yellow spot which cochromatographed with imidazole 4-carboxylic acid could also be identified. Imidazole 4-aldehyde gives a pale yellow color with the Pauly reagent, but the intensity is so low that it is difficult to detect on paper unless relatively very large amounts are present. This compound could easily be detected, however, by means of its characteristic absorption band in the ultraviolet.

No trace of imidazole 4-alcohol could be found, either after long-term or short-term incubations. If imidazole alcohol was added to the active enzyme system, it was reasonably stable and could easily be detected chromatographically at the end of the reaction. We therefore concluded that imidazole alcohol was not among the reaction products. After treatment of the sample with borohydride, however, the alcohol could be detected, thus confirming the identification of the imidazole aldehyde.

Incubation of imidazole pyruvic acid with peroxidase led to a small rate of O_2 consumption and a slow rate of formation of something very similar to imidazole aldehyde. Control measurements indicated that the rate of aldehyde production from imidazole pyruvic acid was insufficient to explain the relatively rapid initial rate of aldehyde formation during the HCN-producing reaction. Furthermore, addition of catalase in amount sufficient to cause only a small inhibition of HCN production led to an increase in aldehyde yield of

only about 15%. Since added catalase led to a large increase in imidazole pyruvic acid accumulation, it should have led to a relatively large increase in aldehyde production if the imidazole aldehyde had its main origin in imidazole pyruvic acid.

A large number of measurements were made in an attempt to correlate imidazole aldehyde production with HCN production. In 150 mM phosphate buffer of pH 7.5 or 8.0, there was good agreement between imidazole aldehyde and HCN yield, provided the amount of histidine oxidized was about 2 μmol or less. Increasing the phosphate concentration to 300 mM resulted, however, in a decline in the yield of aldehyde and a rise in the yield of HCN, whereas diminishing phosphate concentrations led to diminishing yields of HCN, with little change in aldehyde yield, so that the ratios of aldehyde:HCN varied widely. In strong Hepes buffer, under conditions giving HCN yields of greater than 50%, the yield of aldehyde was in the range of 20—30% of the amount of histidine oxidized.

In Hepes buffer of high concentration, another absorption band appeared during the reaction, with a maximum at 262 nm (in alkali after borohydride). It disappeared on acidification, like the aldehyde absorption band, but it was not bleached by borohydride. The intensity of this band was about twice as high as that of the aldehyde, under conditions optimal for HCN production; and its intensity at 20 min in different buffer concentrations was proportional to the yield of HCN. This band at 262 nm disappeared slowly in alkali (0.1 N NaOH), with a half-life of about 5 h at room temperature. No trace of this 262 nm band could be detected, however, when the incubation was in phosphate buffer. In Hepps, the 262 nm band was replaced by one of lower intensity, with a maximum at 258 nm, which suggested that the 262 nm (or 258 nm) band might be due to something in the buffer solution. Such unidentified side products may explain the low yield of aldehyde.

HCN production from other amino acids

All aromatic amino acids which can be oxidized by the amino acid oxidase will produce HCN when the system is supplemented with peroxidase [5,6]. Under conditions which led to a yield of 0.65 μmol HCN from 1.0 μmol of L-histidine, 0.50 μmol HCN was obtained from 1 μmol L-1′-methyl histidine, and 0.51 μmol HCN was obtained from L-3′-methyl histidine. Under the same conditions, 0.099, 0.138 and 0.011 μmol HCN were obtained, respectively, from 1 μmol of L-phenylalanine, of L-tryptophan, and of L-tyrosine. α-DL-Methyl histidine was not oxidized by the amino acid oxidase, nor did it give HCN. Supplementation of the histidine-oxidizing system with imidazole derivatives did not increase the yield of HCN.

With all the aromatic amino acids, peroxidase stimulated the O_2 uptake substantially, from the beginning of the reaction (not shown). Such a stimulation was not observed with leucine, methionine, or isoleucine, three of the better non-aromatic substrates for amino acid oxidase. Though the occurrence of peroxidase-stimulated O_2 uptake correlated with the occurrence of HCN production, there was no quantitative correlation between the amount of extra O_2 consumed and the HCN yield.

Table VII records the HCN and NO_2^- yield from the aromatic amino acids in

TABLE VII

COMPARISON OF NO_2^- AND HCN FORMATION WITH VARIOUS AMINO ACID SUBSTRATES

The reaction mixtures contained 1 μmol amino acid, 1 μmol hydroxylamine, 0.2 U amino acid oxidase and 40 μg peroxidase in 1.2 ml 350 mM Hepps buffer, pH 7.5.

Substrate	NH_2OH added				Without NH_2OH	
	nmol HCN after 2 min	nmol NO_2^- after 2 min	nmol HCN after 4.5 h	nmol NO_2^- after 4.5 h	nmol HCN after 2 min	nmol HCN after 4.5 h
L-Histidine	16.0	16.1	439.7	225.0	33.2	546
L-Tryptophan	54.5	55.7	120.0	134.4	128.7	180
L-Tyrosine	18.3	35.0	28.3	53.5	20.2	0
L-Phenylalanine	29.7	38.0	63.0	88.8	77.4	65
L-Leucine	0.22	11.6	0	9.2	0	0
L-Isoleucine	0.22	11.7	0	9.8	0	0
L-Methionine	0.22	11.1	0	28.6	0	0

the presence and absence of NH_2OH after 2 min and after 4.5 h. Tyrosine, phenylalanine and especially tryptophan are all better HCN producers than histidine, when initial rates are measured. This can be explained in part by the fact that histidine is oxidized less rapidly than the other amino acids.

With tyrosine and phenylalanine, the yield of NO_2^- was larger than the yield of HCN at 2 min, but for tryptophan and histidine the NO_2^- yield and the HCN yield at 2 min were in reasonable good agreement. Unexpectedly, some NO_2^- was obtained from NH_2OH with the non-aromatic amino acids, though no HCN was formed.

Measurements were made of the initial course of NO_2^- formation from NH_2OH with all the amino acids listed in Table VII both in the presence and absence of superoxide dismutase (not shown). The sensitivity to superoxide dismutase inhibition varied greatly. Except for the histidine and the tryptophan oxidation, there was no correlation between HCN production and superoxide dismutase-sensitive NO_2^- production.

Discussion

The best known cyanogenic systems in nature are those that synthesize the cyanogenic glycosides in plants. Conn and his associates have investigated the mechanism of synthesis of dhurrin from tyrosine in sorghum [30—32]. Their system has at least a superficial similarity to the amino acid oxidase-peroxidase system here described, in the sense that precursor and product are analogous. The mechanisms involved appear to be quite different, however. There are many differences, but we mention only one: in Conn's system, *p*-hydroxyphenyl acetonitrile could easily be shown to be an intermediate. It is oxidized to *p*-hydroxymandelonitrile, which hydrolyzes to *p*-hydroxybenzaldehyde and HCN, in the absence of the glycosylation reaction. In contrast, we were not able to convert imidazole 3-acetonitrile to HCN with the amino acid oxidase-peroxidase system, though imidazole 3-aldehyde was identified as one of the products of histidine oxidation.

Of the various oxidase reactions described in the literature, it appears that the oxidation of indole 3-acetic acid by O_2 in the presence of peroxidase [33] may provide the most helpful analogies for further studies of the reactions here described. In the present studies, the peroxidase can be considered to be acting as an oxidase in the sense that it causes an increase in O_2 consumption. It would be premature to speculate on the mechanism of peroxidase action in the absence of further information.

Since the oxidation of histidine in our system is totally dependent on the presence of amino acid oxidase, it seems probable that the reaction begins with the normal enzyme oxidation shown in Eqn. 1. To cause HCN formation, the peroxidase must attack the imino (or enamine) product [34] prior to the release of NH_3 (Eqn. 2). To the extent that Reaction 2 occurs before peroxidase attack, a portion or all of the imidazole pyruvic acid may be oxidized by H_2O_2 to imidazole acetic acid, as in the absence of peroxidase. Under the conditions used, imidazole acetic acid appeared to be stable, i.e. it was not a substrate for oxidative degradation by peroxidase. This was not the case, however, for imidazole pyruvic acid. Much of the present uncertainty about the stoichiometry of the O_2 uptake relates to the uncertainty about how the imidazole pyruvic acid is degraded.

If we regard imidazole 4-aldehyde as a product of the reaction leading to HCN, and if we further assume that the equivalence of HCN production with NO_2^- formation (when NH_2OH is added) can be regarded as evidence for the reduction of 2 O_2 to 2 O_2^-, as in Eqn. 5, then we can represent the net reaction as in Eqn. 7. If we add the dismutation of 2 O_2^- to O_2 and H_2O_2 (Eqn. 6) to Eqn. 7, we get a net O_2 uptake of one molecule/molecule of HCN generated, with no net change in H_2O_2.

$$R \cdot CH_2 \cdot CHN \cdot COOH + H_2O_2 + 2\,O_2 \rightarrow R \cdot CHO + HCN + CO_2 + 2\,O_2^- + H_2O + 2\,H^+ \quad (7)$$

Eqn. 7 is completely provisional, and should be regarded as a working hypothesis, presented in the absence of a better alternative. There is serious doubt about whether the imidazole aldehyde should be regarded as the major reaction product of the reaction leading to HCN, since imidazole pyruvic acid also gives imidazole aldehyde in the presence of peroxidase and O_2 under the conditions used to maximize HCN formation. So far as we could ascertain, the quantity of imidazole aldehyde formed by this route was definitely smaller than that formed from histidine. It is not excluded, however, that part of the reaction complex proceeds as a primed chain reaction [33]. Rate measurements with separate reaction components may not be applicable to rates in the entire complex system, since peroxidase-oxidase reactions are potentially very complex kinetically [35].

Eqn. 7 suggests that of the four oxidation equivalents required for the indicated reaction, two are provided by one molecule of H_2O_2 which is reduced to water, and two more are provided by two molecules of O_2 which are reduced to 2 O_2^-. The major evidence for the use of H_2O_2 is that the reaction is sensitive to catalase. Relatively massive amounts of catalase are required, however, for effective inhibition, and an alternative explanation is possible: viz. that catalase inhibits by binding an essential intermediate organic peroxide. In that case, the

increase in H_2O_2 accumulation observed with peroxidase may merely reflect the fact that peroxidase diverts a high proportion of imidazole pyruvic acid precursor to another reaction path, so that less imidazole pyruvic acid is available to reduce the H_2O_2 formed in the amino acid oxidase reaction.

The evidence for O_2^- formation from O_2 may likewise be questioned. A definite conclusion rests mainly on the assumption that the superoxide dismutase enzyme is highly specific for O_2^- and this assumption may not be true. An organic peroxyradical may be involved in the superoxide dismutase-sensitive oxidation of NH_2OH to NO_2^-.

In spite of these numerous reservations, we regard Eqn. 7 provisionally as a reasonable description of the stoichoimetry. There is always somewhat more O_2 taken up than the equation indicates, but we would ascribe this to side reactions. Since the amounts of imidazole acetic acid and imidazole carboxylic acid which are formed have not been determined quantitatively, we cannot judge how much of the imidazole group of the oxidized histidine has been accounted for. A ring opening has not been excluded, but the HCN must have its origin in the side chain of the histidine. The biology of oxygen radicals has recently been reviewed [36]. Peroxidase is often quite rightly listed as a reagent which removes H_2O_2 by reducing it to water. In the present case it should be noted that peroxidase causes the generation of O_2^- or something similar to it in reactivity.

More than twenty years ago, it was reported that a particulate fraction made from the hepato-pancreas of mussels (*Mytillus edulis* L.) formed imidazole 4-carboxylic acid, imidazole 4-aldehyde and imidazole 4-methanol from L-histidine as well as the products expected from the action of the L-amino acid oxidase, which was present [13]. The responsible enzyme system is probably related to the amino acid oxidase-peroxidase system here under investigation. Though we found no imidazole alcohol in our system, this product could very well be produced by a secondary reduction in the crude particulate enzyme preparation from mussels.

In our opinion, the HCN-forming reaction here described may have physiological significance in a wide variety of cells. It is not likely, however, that large amounts of HCN are formed in this way. The more probable assumption is that the HCN production is part of a control system. The unphysiological conditions here employed were designed to maximize the HCN-producing reaction of histidine and thus make it more amenable to study. But wherever amino acid oxidases occur, and they are widely distributed, there is a possibility that a little HCN may be formed from one of the aromatic amino acids, provided the flavoprotein has the required proximity to cytochrome *c* or peroxidase.

Our own experimental work is centered around the role of amino acid oxidase in photosynthetic microorganisms. Pistorius et al. [37] have recently shown that the cyanobacterium *Anacystis nidulans* contains an L-amino acid oxidase which contributes to its dark respiratory system, and which can form a little HCN from L-histidine in vivo. The possible role of amino acid oxidases in respiration, generally, deserves further study.

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