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Cyanide Formation from Oxidation of Glycine by a *Pseudomonas* Species

FRODE WISSING

Department of Biochemistry, The Royal Dental College, DK 8000 Århus C, Denmark

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With whole cells of a hydrogen cyanide-producing bacterium strain C, of the genus *Pseudomonas*, it was found that the oxygen necessary for the oxidation of glycine to cyanide could be replaced by various artificial electron acceptors. The order of reactivity was: oxygen > phenazine methosulphate > methylene blue > 2,6-dichlorophenolindophenol > ferricyanide. Cyanide production was inhibited by pyrrolnitrin, a well-known inhibitor of many flavine enzymes. The molar ratio of added glycine to cyanide produced was found to be 1.09. With whole bacteria the apparent K_m (glycine) for the cyanide production was found to be 5.0×10^{-4} M.

In the bacterial oxidation of glycine to hydrogen cyanide and carbon dioxide, it has been shown by Michaels et al. (9) that the liberated hydrogen cyanide originates from the aminomethyl group of glycine and that the cyanide produced was equal to the amount of CO_2 produced on a molar base. Further, it has been established by Brysk et al. (1) that the methylene C—N bond is retained during the reaction. Another important question is whether oxygen is necessary for the reaction to proceed. Thus, Clawson and Young (2) reported that free hydrogen cyanide is apparently produced only under aerobic conditions. Patty (11) also states that only small amounts of cyanide are produced under anaerobic conditions.

The experiments reported in this paper were carried out to establish whether O_2 is essential for the reaction and whether artificial electron acceptors may substitute for oxygen. Furthermore, inhibitor experiments were performed to elucidate the kind of enzymes which are involved in the reaction. The molar ratio between added glycine and cyanide produced was determined to establish the stoichiometry of the biochemical reactions leading to cyanide formation. A proposed reaction scheme is presented and discussed.

At present no information exists as to the successful preparation of a cyanide-producing system in vitro. Attempts in this laboratory to prepare such a system have also been unsuccessful, and as a consequence the experiments reported in this paper were performed with whole bacteria.

MATERIALS AND METHODS

Experimental material. The bacterium used in the present study was a cyanide-producing strain (designated strain C) of a *Pseudomonas* species isolated from a water reservoir in a greenhouse and has previously been described (15). The bacteria were maintained at 20 C either on nutrient agar containing 8 g of nutrient (Difco), 5 g of NaCl, and 15 g of agar per 1,000 ml of tap water, or on a minimal medium made from 10^{-1} M KH_2PO_4, 2×10^{-2} M NH_4Cl, 10^{-3} M $MgSO_4$, 2×10^{-2} M succinic acid, and 15 g of agar per liter. Finally the solution was adjusted to pH 7.5 with 1 M NaOH. After autoclaving, each of the media was enriched with 10 ml of a separately autoclaved solution containing 2 M glycine, 0.02 M $FeCl_3$, and 0.1 M $Na_4P_2O_7$. By this procedure no precipitation occurred. It was found that decreasing activity in cyanide production could be avoided when bacteria were transferred from one medium to another every 3rd day.

The bacteria used for the experiments were obtained from an 18-h culture by using the minimal medium without agar. It was of importance for the cyanide-producing activity that the inoculum obtained from two 8-cm petri dishes consisted of bacteria not more than 18 to 20 h old. The culturing was performed in a 5-liter reaction jar with a sterile air flow of 500 ml per min onto the concave surface of the vigorously stirred media. This technique prevented foaming and gave an average yield of 1.5 g of bacteria per liter.

The bacteria were stored at 2 to 5 C as a 10% suspension, on a wet weight basis, in a 0.067 M KH_2PO_4/Na_2HPO_4 buffer (pH 7.50). Phosphate buffer was found to be the most suitable form for storage, giving a loss of cyanide-producing activity of only 5 to 7% in 8 days. Normally a preparation was discarded after 4 days. The amount of bacteria was

determined from the optical density at 450 nm (OD_{450}). The relationships between OD_{450} and the protein content, dry weight, and number of bacteria by viable counting were found to be: OD_{450} = 1.000 ~ 0.064 mg of protein/ml ~ 0.24 mg dry weight/ml ~ 2 × 10^{15} cells/ml.

Experimental set up. The determination of cyanide production from 2-ml portions of a bacterial suspension was made by means of the apparatus shown schematically in Fig. 1. The reaction chamber consisted of a round-bottomed glass test tube (inner diameter 15 mm, overall height 80 mm) with a 14-mm ground conical socket joint fused with a glass mantle for thermostating by means of a water-circulating system. A rubber stopper fitting the reaction chamber had three apertures: one for an inlet tube, one for an outlet tube, and one for a 100-μliter constriction pipette which served as a substrate reservoir. Air was passed through traps containing NaOH and water. The air was distributed through a three-way microvalve so that its flow could be directed either through the inlet tube or, for emptying purposes, through the constriction pipette. Before air or nitrogen passed the reaction chamber, the flow rate (420 ml/min) was measured by a flow rate meter. The outlet tube was connected to another three-way microvalve, leading the aeration gas (air or nitrogen) to either one of two test tubes which contained 1,000 μliters of 0.1 M NaOH each for the collection of cyanide. Magnetic stirring served for aeration and facilitated the removal of cyanide produced. The round-bottomed reaction chamber permitted such a rate of stirring that a 2-ml bacterial suspension could be forced up cylindrically, giving a nearly laminar flow and thus improving the exchange of cyanide from liquid to air. By using the method described, a recovery rate for known amounts (2 to 40 nmol) of cyanide in 2 ml of 0.1 M pyrophosphate buffer (pH 8.3) was 96 to 97% during 4 min.

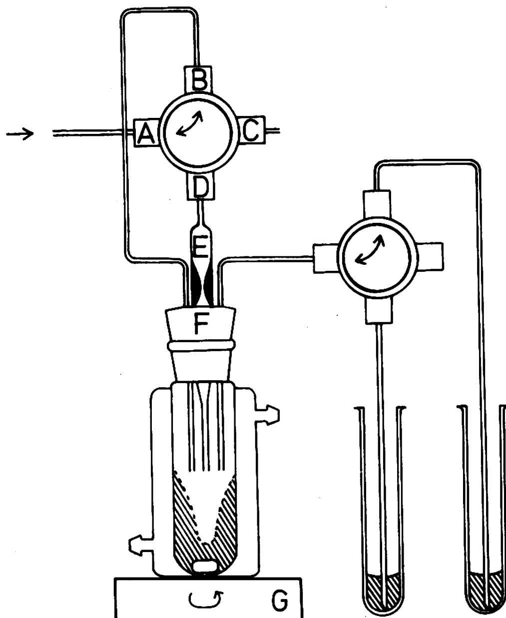

FIG. 1. *Apparatus for measuring cyanide production in a bacterial suspension. Air or nitrogen is passed through the reaction chamber via a microvalve (position A–B) and is further distributed by another microvalve to either one of two test tubes containing NaOH for the collection of cyanide. The position C–D of the microvalve allows filling the pipette E, which can be emptied in position A–D. The reaction chamber is closed with a rubber stopper (F). The suspension is stirred by a magnetic stirring device (G).*

General procedure. Bacteria from the 10% storage suspension were washed in the same phosphate buffer used for storage and centrifuged just prior to each experiment to remove cyanide accumulated by the bacteria on standing. The bacteria were then diluted by resuspending them in 0.1 M pyrophosphate buffer (pH 8.3) to give, unless otherwise stated, 0.5 mg of protein per ml. The cyanide production from 2 ml of this bacterial dilution was followed for 20 to 24 min to obtain a steady-state cyanide production before the addition of substrate. All experiments were performed at 25 C under aerobic conditions, except in the experiments with electron acceptors which were performed anaerobically.

Electron acceptors. At the start of the experiment, an excess of glycine (10 μmol) was added to the bacterial suspension. The electron acceptors were added anaerobically through the constriction pipette after an additional 24 min of nitrogen flushing.

Inhibitors. Each inhibitor was added at the start of the experiment and was followed by glycine (10 μmol) 24 min later.

Initial velocities. The cyanide production was linear with time for the first 4 min after the addition of substrate in the presence of oxygen, and from a plot of this the initial velocities were determined as nanomoles of cyanide produced per minute per milligram of protein. In the experiments with electron acceptors and inhibitors, the cyanide production was, however, not linear with time for the first 4 min. Therefore, no initial velocities could be determined in these two cases.

Analysis. Cyanide production was always followed for periods of 4 min by collection in 1 ml of 0.1 M NaOH. It was determined by the method of Epstein (4). Protein concentration was determined with a sensitive biuret method adapted for impure systems such as whole bacteria as described by Koch and Putnam (6). Bovine serum albumin was used as a standard.

Chemicals. Pyrrolnitrin was kindly supplied by Eli Lilly & Co., Indianapolis, Ind. Methylene blue, 2,6-dichlorophenolindophenol, and potassium ferricyanide were purchased from Fluka, Switzerland. Phenazine methosulphate and acriflavine were obtained from the British Drug House, England. Benzoic acid and *o*-phenanthroline were purchased from E. Merck, Germany. The nitrogen was a commercial O_2-free preparation. All other chemicals were of analytical grade.

RESULTS

Linearity between bacterial protein and cyanide produced. In the present investigation, different concentrations of bacterial protein were used. The existence of a linear relationship between bacteria, expressed as milligrams of protein per milliliter, and the amount of cyanide produced per minute is shown in Fig. 2. The activities were determined as initial velocities with an excess of glycine added to the incubation mixture.

Apparent K_m. Whole bacteria were used to determine initial velocities for cyanide production by adding varying amounts of glycine. When a direct addition of glycine from the 100-µliter constriction pipette was employed, reproducible results could be obtained. Figure 3 shows the resulting Lineweaver-Burk diagram from which the apparent K_m was calculated to be 5.0×10^{-4} M.

Importance of oxygen. Anaerobic conditions were used in some experiments to investigate the effect of oxygen on cyanide production. A change to aerobic conditions immediately initiates the production of cyanide (Fig. 4). Glycine was added at the beginning of the experiment, and from Fig. 4 and 5 it can be seen that a low level of cyanide production was always observed when an anaerobic equilibrium condition was established. No change in this low level of cyanide production could be observed, whether extracellular glycine was present or not. The use of short thick-walled polyethylene tubings or deoxygenation of the O_2-free nitrogen by passing it through 0.1 M NaOH with 50 g of sodium dithionite and 0.5 g of anthraquinone sulfonate

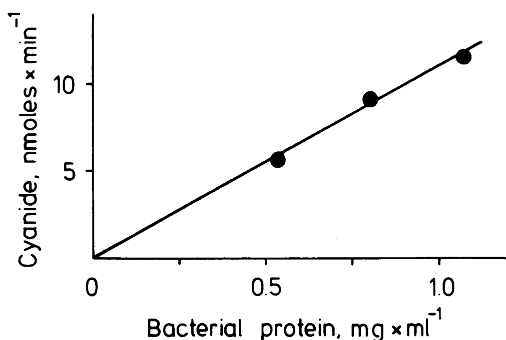

FIG. 2. *Correlation between bacteria, expressed as milligrams of protein per milliliter, and the amount of cyanide produced per minute.*

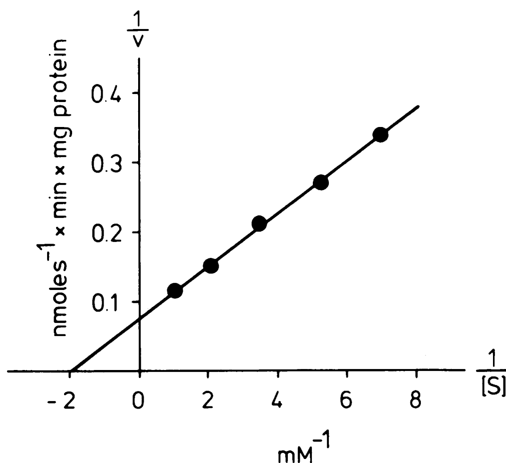

FIG. 3. *Lineweaver-Burk diagram obtained with whole bacteria and determined from the initial velocities of cyanide production with varying amounts of glycine.*

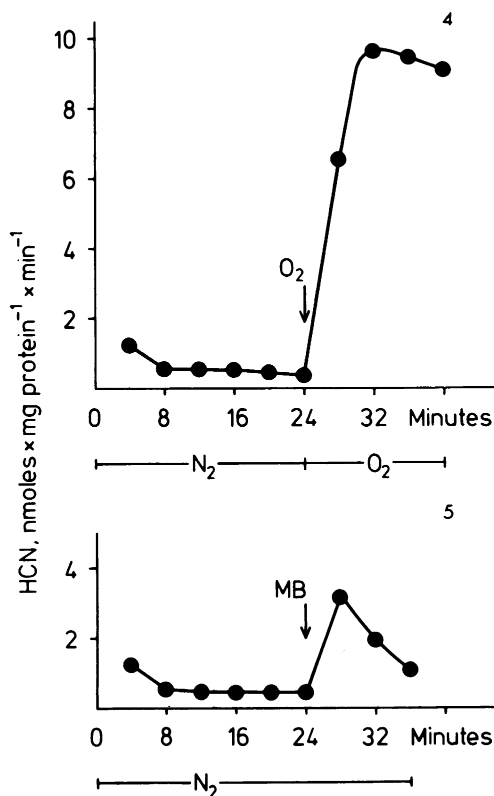

FIG. 4. *Response of cyanide production to oxygen relative to time. The arrow indicates the time when the nitrogen flow is changed to air.*

FIG. 5. *Response of cyanide production to methylene blue (0.4 mM), added under anaerobic conditions, relative to time. The color of methylene blue disappeared gradually as the cyanide production decreased.*

per liter did not influence the anaerobic endogenous cyanide production.

Electron acceptors. In the presence of a number of artificial electron acceptors, cyanide production occurred during anaerobic conditions (Table 1). The order of reactivity was: phenazine methosulfate (PMS) > methylene blue > 2,6-dichlorophenolindophenol (DCIP) > ferricyanide. There was apparently no obvious relationship to the redox potentials. The experiments with ferricyanide were corrected for cyanide liberated by ferricyanide itself. The result of an experiment with methylene blue as an acceptor is shown in Fig. 5.

Inhibitors. A variety of inhibitors of flavine enzymes was tested with respect to their effect on cyanide production. The results are summarized in Table 2. Pyrrolnitrin and acriflavine showed a pronounced inhibitory effect at a final concentration of 1 mM. At the same concentration, phenanthroline likewise showed a strong inhibitory effect, whereas cupric sulfate and benzoic acid exerted a relatively moderate inhibitory action on cyanide production. Amytal and rotenone did not show any inhibition at all.

TABLE 1. *Effect of oxygen and artificial electron acceptors on bacterial cyanide formation*[a]

Acceptor	Concn (mM)	HCN nmol/ min/mg of protein	Redox potential[b] ($E^{\circ\prime}$ [V])
Oxygen	0.23	6.6	+0.8
PMS	0.4	4.1	+0.08
Methylene blue	0.4	2.7	0
DCIP	0.4	0.98	+0.16
Ferricyanide	4.0	0.02	+0.43

[a] Determined from the cyanide produced within the first 4 min after the addition of electron acceptors. Results are based on double experiments.

[b] From reference 3.

TABLE 2. *Effect of various inhibitors on the bacterial cyanide production*[a]

Inhibitor	Concn (mM)	Inhibition (%)
Pyrrolnitrin	1.0	79.6
	0.1	37.5
Acriflavine	1.0	98.4
	0.1	13.7
o-Phenanthroline	1.0	72.5
Cupric sulfate	1.0	26.9
Benzoic acid	1.0	13.7
Rotenone	0.1	1.9
Amytal	1.0	−1.0

[a] Determined from the cyanide produced within the first 4 min after the addition of glycine. The bacteria were preincubated with the inhibitor for 20 min before the addition of glycine. Results are based on double experiments.

Molar ratio. The molar ratio between added glycine and cyanide recovered was determined by collecting cyanide at intervals of 4 min after the addition of a known amount of glycine. Depending on the amount of added glycine, it was found that after about 2 h, the cyanide production had decreased to the original level observed without glycine. The total amount of cyanide produced was determined and corrected for the endogenously produced cyanide. The results with varying amounts of glycine are shown in Fig. 6. The molar ratio found to be 1.09 is based on a regression line.

DISCUSSION

The studies on cyanogenesis in microorganisms are at present met with two drawbacks. Firstly, no metabolic intermediates have as yet been identified from the reaction: glycine to cyanide. However, Ward and Thorn (14) reported the presence of a cyanogenic material in extracts from the fungus *Marasmius oreades*. Secondly, there are no reports on a cell-free cyanide-producing system from either fungi or bacteria. Therefore, fundamental information can at present only be obtained from experiments with whole organisms.

A hypothetical pathway for bacterial cyanide formation from glycine, suggested in Fig. 7, is

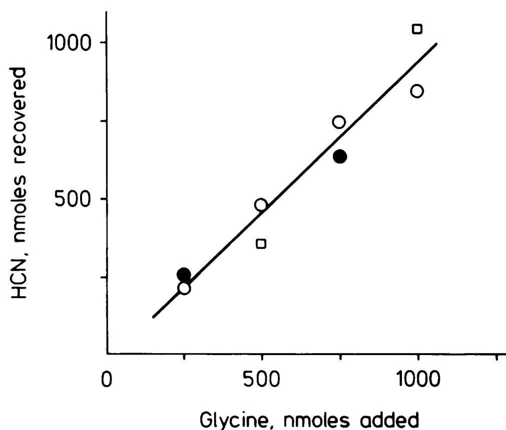

FIG. 6. *Regression line showing the molar ratio between added glycine and cyanide recovered, when whole bacteria were fed varying amounts of glycine. Cyanide production was followed in time from the addition of glycine until it had ceased and was then integrated and corrected for endogenous cyanide. Similar dots refer to experiments made with the same bacterial preparation.*

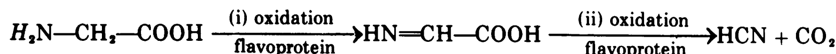

FIG. 7. *Hypothetical pathway for cyanide formation from glycine by bacteria.*

based on information in the literature (1, 2, 9, 11, 15) and the results reported here.

(i) The observation that glycine is metabolized by bacteria to give cyanide has often been reported (2, 9, 11, 15), and the cyanide-producing enzyme system in the bacteria investigated here was found to have an apparent K_m of 5.0×10^{-4} M, but the rate-limiting step is not known since intact bacteria were studied. For comparison, other glycine oxidative enzymes such as glycine oxidase (12) and D-amino acid oxidase (EC 1.4.3.3.) (10) have K_m values of 4×10^{-2} M and 6×10^{-2} M, respectively. The D-amino acid oxidase is apparently not involved in cyanide production, as concluded from the K_m value observed.

(ii) The cyanide produced has its origin in the aminomethyl group of glycine, and cyanide and carbon dioxide are produced in equimolar amounts (9). Thus, glycine is apparently split between the carbon atoms 1 and 2.

(iii) The methylene C—N bond is retained during the reaction (1), excluding possible transaminations or deaminations.

(iv) A molar ratio of 1.09 between added glycine and cyanide recovered shows that one molecule of glycine gives rise to only one molecule of hydrogen cyanide. Thus, the observations are in accordance with the stoichiometry of the hypothetical pathway.

The conversion of glycine to cyanide is an oxidative process and is dependent on oxygen (2, 11). This has been confirmed with the present strain C of a *Pseudomonas* (Fig. 4), which gives a linear increase in cyanide production with time after the addition of oxygen. Cyanide production could also be initiated by substituting PMS, methylene blue, or DCIP for oxygen (Table 1). Dixon (3), in a comparative study of different flavine enzymes, has shown that these electron acceptors and some others act as acceptors for flavoproteins. It was therefore of interest to investigate the effect of such inhibitors, which are known to inhibit flavine enzymes, on the cyanide-producing reaction. Pyrrolnitrin (8, 13) and *o*-phenanthroline and acriflavine showed a strong inhibitory action at a final concentration of 1 mM. On the other hand, amytal and rotenone, acting at the substrate side of flavine enzymes, did not inhibit cyanide production at all. Furthermore, only some inhibitory effect was observed with cupric sulfate known as an inhibitor of glycine oxidase (12) or with benzoic acid known as an inhibitor of the D-amino acid oxidase (7). Conclusively, the D-amino acid oxidase is apparently not involved in cyanide formation. The results of the experiments with electron acceptors and inhibitors suggest that at least one flavoprotein participates in the oxidation of glycine, as suggested in Fig. 7.

The bacteria did not contain cytochromes of the *a* type but cytochromes of the *b*, *c*, and *o* type could be identified by spectrophotometric analysis with intact bacteria (unpublished results). At present it is uncertain whether the respiratory chain is involved in cyanide production or not.

In the oxidation of glycine to cyanide, the C—N single bond is converted to the C≡N triple bond via a C=N double bond, probably in the form of an imino acid. The very short life time of free imino acids (5) may explain the difficulty in proving the existence of an intermediate in the reactions leading to the production of cyanide from glycine by bacteria.

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