β -Cyanoalanine Formation by *Chromobacterium violaceum*

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Nonproliferating cells of *Chromobacterium violaceum* incubated with glycine, methionine, and succinate as substrates accumulated β -cyanoalanine in the culture fluid. Tracer experiments showed that carbons-2, -3, and -4 of β -cyanoalanine are derived from the 2-carbon of glycine. When methionine-methyl-¹⁴C, succinate-1,4-¹⁴C, or succinate-2,3-¹⁴C was used as substrate, β -cyanoalanine did not become labeled. If K¹⁴CN and serine were used as substrates, the cyano group of β -cyanoalanine was labeled. Radioactive β -cyanoalanine, labeled in the 3-carbon, was formed when glycine and H¹⁴CHO were used as substrates. ¹⁴C-formic acid did not replace formaldehyde. Asparagine also accumulated in the incubated mixture and was found to be labeled in the amide carbon. Incubation of cells with β -cyanoalanine-4-¹⁴C produced labeled aspartic acid in cell hydrolysates.

The metabolism of cyanide ion has been studied in a number of laboratories in recent years. Sorghum seedlings were reported to fix cyanide in the amide carbon of asparagine (1), and *Vicia sativa* was found to incorporate radioactivity from H¹⁴CN into β -cyanoalanine and its γ -glutamyl dipeptide (13). β -Cyanoalanine formation has been observed in extracts of *Escherichia coli* incubated with serine and KCN (4).

Michaels, Hankes, and Corpe (9) reported that washed cells of *Chromobacterium violaceum*, when incubated aerobically with a methyl donor compound, an energy source, and glycine-2-¹⁴C, produced H¹⁴CN exclusively from the methyl carbon of glycine. Subsequent experiments by the present authors have indicated that biologically produced cyanide ion can be incorporated into cell material. With the aid of tracer experiments, it was possible to map out a tentative series of reactions for the assimilation of cyanide into cell material by way of the intermediate β -cyanoalanine.

MATERIALS AND METHODS

Cultures of *C. violaceum* strain 9 were grown on glutamate medium which contained L-glutamic acid (4.413 g/liter) as the sole carbon and nitrogen source in a basal salts solution adjusted to pH 7.0 with NaOH (8). Two 500-ml Erlenmeyer flasks, each containing 100 ml of glutamate medium, were inoculated and

mounted on a rotary shaker in an incubator room at 30 C. After 18 hr of growth, the organisms were used to inoculate 9-liter serum bottles containing 5 liters of glutamate medium. The large cultures were grown for 22 hr in a stream of sterile air from a compressed air line. The bacteria were harvested by centrifugation at 6,000 rev/min in a Sorvall SS-3 centrifuge, washed twice with a salts solution buffered at pH 7.0 (glutamate medium minus glutamate), and resuspended in the salts solution to a concentration equivalent to 10 mg (dry weight) of cells per ml.

Tracer experiments were carried out with the following radioisotopes: K¹⁴CN, glycine-2-¹⁴C, L-methionine-methyl-¹⁴C, and sodium formate-¹⁴C, obtained from Volk Radiochemical Co. (Burbank, Calif.), and succinic acid-1,4-¹⁴C, succinic acid-2,3-¹⁴C, and formaldehyde-¹⁴C, obtained from New England Nuclear Corp. (Boston, Mass.). One-liter suction flasks containing washed cells [0.5 mg (dry weight)/ml] in 30 ml of salts medium were inoculated with various radioactive compounds together with glycine (5 μ moles/ml), methionine (1 μ mole/ml), and succinate (28.4 μ moles/ml). The flasks were stoppered and placed on a reciprocal shaker set at 100 strokes/min for 5 hr. At the termination of each experiment, compressed air was passed through the reaction flasks into 10% NaOH gas-washing bottles to remove and trap gaseous cyanide. The cells were removed by centrifugation at 6,000 rev/min, washed with distilled water, lyophilized in tared containers, and weighed.

The clear culture supernatant fluids were acidified with 6 N H₂SO₄, reconnected to the gas-washing bottles, and flushed with compressed air to remove residual cyanide.

Samples of supernatant fluids from incubation mixtures were passed through 0.9 by 20-cm columns of

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Dowex 50W-X8 (H^+) resin (100 to 200 mesh). The resins were washed with distilled water, and the amino acids were eluted with 3 N NH_4OH . The NH_4OH eluates were dried in vacuo, dissolved in a known volume of water, and spotted on strips of Whatman 3MM paper moistened with one of the following buffers: (i) 0.05 M sodium borate, pH 9.2 (15); (ii) 2% ammonium carbonate, pH 9.1 (14); (iii) pyridine-acetic acid-water (9.7:2.3:180 and 1:10:190), pH 5.7 and pH 3.5 (15); and (iv) 1.5 M formic acid-2 M acetic acid (1:1), pH 2.0 (15).

Electrophoresis was carried out in an E-C apparatus (E-C Co., Philadelphia, Pa.), with a potential difference of 36 v/cm, for 2 hr. Papers were dried, dipped in 0.6% ninhydrin in acetone, and heated at 105 C for 5 to 7 min.

Descending paper chromatography was also carried out on the amino acid residues in the following solvent systems (15): ethyl alcohol-ammonia-water (18:5:1), and *n*-butyl alcohol-acetic acid-water (12:3:5).

β -Cyano-L-alanine was determined quantitatively by taking advantage of the green color it gave with ninhydrin on paper chromatograms. Green spots were cut out of paper electrophoretograms run in pH 9.1 buffer and eluted with 5 ml of 70% ethyl alcohol for 30 min in colorimeter tubes. The optical density of the solution was determined in a Bausch & Lomb Spectronic-20 colorimeter set at 630 nm, and the values obtained were compared with a standard curve prepared with purified β -cyano-L-alanine (Calbiochem, Los Angeles, Calif.). Beer's law was obeyed in a concentration range of 0 to 50 units of β -cyanoalanine per 5 ml of ethyl alcohol.

Samples of the amino acid residues were also examined in an amino acid analyzer by the procedure of Spackman, Stein, and Moore (16). The outlet tube of the analyzer column was connected by means of a 2-ml flow cell, with a Nuclear-Chicago flow counter adapter, to a Nuclear-Chicago model 722 liquid scintillation system prior to reaction with ninhydrin in the amino acid analyzer. A composite curve was thus obtained which correlated ninhydrin-positive peaks with those that were radioactive. Electrophoretograms and chromatograms prepared from supernatant fluids of labeled incubation mixtures were examined by autoradiography with the use of type KK industrial X-ray film. Radioactive spots or bands identified as β -cyanoalanine and asparagine were then cut out of the electrophoretograms or chromatograms; the compounds were eluted from the paper with the aid of the appropriate carrier and were repeatedly crystallized from 95% ethyl alcohol. Weighed samples of the purified compounds thus isolated were individually hydrolyzed to aspartic acid with 6 N HCl at 100 C. The aspartic acid from each was separated by paper chromatography and isolated by the crystallization procedure of Klosterman and Painter (5).

Cells from tracer experiments were washed, lyophilized, and weighed in tared containers. Weighed samples of cell material were hydrolyzed with 6 N HCl for 15 hr. Hydrolysates were dried in vacuo to remove HCl and were analyzed by the methods previously described for supernatant fluids.

Total radioactivity in supernatant fluids and alkali traps were measured with a Nuclear-Chicago Unilux liquid scintillation counter. The combustion procedure of Van Slyke, Steele, and Plazin (18) was used to measure radioactivity in the cells and in radioactive compounds isolated from the supernatant fluids.

The activities in the carboxyl carbons of β -cyanoalanine, asparagine, and aspartic acid were determined by decarboxylation with ninhydrin (17). Aspartic acid obtained by hydrolysis of β -cyanoalanine was further degraded to determine the distribution of radioactivity in all four carbons. Aspartic acid was first decarboxylated to alanine (7), and the latter was converted to acetate (3). Acetate was further degraded to CO_2 by the procedure of Phares (12), and the specific activities of the evolved CO_2 were determined (18).

RESULTS

Supernatant fluids from buffered incubation mixtures containing glycine, methionine, and succinate were examined by chromatography and electrophoresis. When the chromatograms and electrophoresis strips were treated with ninhydrin, an intense green spot was observed, in addition to the violet areas representing the substrate amino acids. The green material was identified as β -cyanoalanine by comparison with a standard. In time-course studies (Brysk, Ph.D. Thesis, Columbia Univ., New York, N.Y., 1967), under optimal conditions for production, β -cyanoalanine accumulated in the culture fluid to a maximal concentration in 5 hr of incubation. With longer periods of incubation, the β -cyanoalanine began to disappear from the supernatant fluid.

Washed cells of *C. violaceum* were incubated with various substrates and $K^{14}CN$; then cells and supernatant fluids, freed from cyanide ion, were assayed for radioactivity (Table 1). Cells incubated with serine, succinate, and $K^{14}CN$ incorporated the greatest amount of radioactivity. Supernatant fluids from this incubation mixture not only contained more radioactivity than the others but also contained maximal microgram levels of β -cyanoalanine. The presence of a methyl donor compound, such as methionine or *N,N*-dimethylglycine, enhanced synthesis of β -cyanoalanine when glycine was used as substrate.

The labeled substances in the supernatant fluid were similar whether $K^{14}CN$ was used with serine or with glycine plus methionine. Chromatography followed by autoradiography showed that β -cyanoalanine and asparagine were labeled. Examination of desalted culture fluids in an amino acid analyzer coupled with a radioactivity monitor revealed two radioactive peaks whose elution pattern corresponded to those expected for β -cyanoalanine and asparagine. The cyano carbon of β -cyanoalanine contained 88% of the radioactivity of the molecule, and the amide carbon of the

TABLE 1. Incorporation of $K^{14}CN$ by *Chromobacterium violaceum* exposed to different substrate systems in basal salts in the presence of sodium succinate^a

Substrate system ^b	Total activity recovered				β -Cyanoalanine ($\mu\text{g/ml}$)
	Supernatant fluid		Cells		
	Amt (μc)	Per cent incorporated	Amt (μc)	Per cent incorporated	
DL-Serine . . .	46.32	23.32	1.48	0.75	5.0
Glycine	11.63	5.90	0.16	0.08	trace
Glycine and <i>N,N</i> -dimethylglycine	19.85	10.06	0.14	0.07	1.3
Glycine and DL-methionine	24.59	12.46	0.09	0.05	1.3

^a Concentration of sodium succinate was 28.4 $\mu\text{moles/ml}$.

^b Concentrations of glycine and serine were 5 $\mu\text{moles/ml}$, and of methionine and *N,N*-dimethylglycine, 1 $\mu\text{mole/ml}$. The organisms were grown in glutamate-basal salts medium according to the procedure of Michaels and Corpe (8). In addition to the compounds listed, 197.32 μc of $K^{14}CN$ was added to each flask.

asparagine contained 95% of the radioactivity (Table 3).

The contribution of the various components of the reaction mixture in the synthesis of β -cyanoalanine was examined by use of radioactive compounds (Table 2). Radioactive ^{14}C -sodium formate and ^{14}C -formaldehyde were tested as possible precursors in the reaction of glycine with a one-carbon compound in the synthesis of serine. Radioactive β -cyanoalanine was found only when

glycine-2- ^{14}C (Fig. 1) or ^{14}C -formaldehyde was used.

The incorporation of radioactivity in the amino acid fraction of acid-hydrolyzed cells was greatest with radioactive glycine or succinate (Table 2). By autoradiography of two-dimensional chromatograms of hydrolyzed cells grown on glycine-2- ^{14}C , the following labeled amino acids were tentatively identified: phosphoserine, aspartic acid, threonine, serine, glutamine, proline, glutamic acid, glycine, alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, arginine, lysine, and histidine. The identity of these amino acids was confirmed by the examination of peaks from the amino acid analyzer (Brysk, Ph.D. Thesis, Columbia Univ., New York, N.Y., 1967). In contrast to these results, cells incubated with β -cyanoalanine-4- ^{14}C were labeled only in aspartic acid.

The labeling pattern of β -cyanoalanine isolated from the culture fluid containing glycine-2- ^{14}C showed a distribution of radioactivity of 2.5 and 29% in the 1- and 4-carbons (Table 3) and of 8 and 25% in the 2- and 3-carbons (Brysk, Ph.D. Thesis, Columbia Univ., New York, N.Y., 1967). This accounts for approximately 65% of the radioactivity of the molecule. If the labeling pattern followed that presented in Fig. 2, one would expect no radioactivity in C-1 and 33% each in C-2, -3, and -4. The low recovery of activity in carbon-2 probably reflects an incomplete degradation in the Schmidt reaction.

DISCUSSION

Nonproliferating cells of *C. violaceum* were shown to produce cyanide from the 2-carbon of glycine in a buffered, incubated mixture also containing methionine and succinate (9). In the present work, the nitrile β -cyanoalanine was detected in this buffered, incubated mixture. This

TABLE 2. Incorporation of ^{14}C -substrates by *C. violaceum* incubated with glycine, methionine, and sodium succinate

Labeled substrate ^a	Radioactivity added ($\mu\text{C}/\text{flask}$)	Radioactivity recovered				Radioactivity of β -cyanoalanine
		Supernatant fluid		Cells		
		Amt (μC)	Per cent	Amt (μC)	Per cent	
Glycine-2- ¹⁴ C.....	57.59	30.11	52.29	10.22	17.74	+
L-Methionine-methyl- ¹⁴ C.....	67.04	59.66	88.99	1.83	2.74	—
Succinic acid-1,4- ¹⁴ C.....	81.00	13.25	16.38	5.74	7.09	—
Succinic acid-2,3- ¹⁴ C.....	102.60	32.90	32.08	33.41	32.57	—
Sodium formate- ¹⁴ C.....	199.80	187.45	93.81	0.307	0.0015	—
Formaldehyde- ¹⁴ C.....	185.43	165.13	89.04	2.09	0.011	+

^a Concentrations of substrates were: glycine, 5 $\mu\text{moles/ml}$; methionine, 1 $\mu\text{mole/ml}$; sodium succinate, 28.4 $\mu\text{moles/ml}$; sodium formate, 3.61 $\mu\text{moles/ml}$; and formaldehyde, 0.169 $\mu\text{mole/ml}$.

FIG. 1. Amino acid analyzer scan from a reaction mixture containing glycine-2- ^{14}C , methionine, and succinate recorded at 570 nm (scale at left). Broken line is concurrent scintillation counter measurement (scale at left). Compounds shown are phosphoserine (P. Ser), β -cyanoalanine (BCNA), methionine sulfoxides (MO), serine (Ser), asparagine (ANH_2), glutamic acid (Glu), glycine (Gly), and methionine (Meth).

TABLE 3. Analysis of carbons-1 and -4 of β -cyanolalanine and asparagine formed by *C. violaceum* in the presence of sodium succinate^a

Test compound	Culture medium ^a	Analysis of test compounds		Analysis of aspartic acid produced by acid hydrolysis of each test compound	
		Specific activity ($\mu\text{C}/\text{mmole}$)	Percentage of activity in carbon-1	Specific activity ($\mu\text{C}/\text{mmole}$)	Percentage of activity in carbons-1 and -4
β -Cyanoalanine	$\text{K}^{14}\text{CN} + \text{serine}$	0.1374	7.08	0.0176	95.54
	Glycine-2- $^{14}\text{C} + \text{methionine}$	0.183	2.44	0.0173	31.21
	Formaldehyde- $^{14}\text{C} + \text{glycine} + \text{methionine}$	0.334	4.19	0.0361	4.61
Asparagine	Serine + K^{14}CN	0.1862	2.26	0.0233	97.2

^a Concentrations of substrates were: glycine, 5 $\mu\text{moles}/\text{ml}$; methionine, 1 $\mu\text{mole}/\text{ml}$; sodium succinate, 28.4 $\mu\text{moles}/\text{ml}$; sodium formate, 3.61 $\mu\text{moles}/\text{ml}$; and formaldehyde, 0.169 $\mu\text{mole}/\text{ml}$.

nitrile was previously found to accumulate naturally in seeds of *Vicia sativa* and to be synthesized from serine and HCN; serine supplied carbons 1 to 3 of β -cyanoalanine (10). Similar results were observed with extracts of *E. coli* in which the reaction was dependent on adenosine triphosphate (4).

C. violaceum, when incubated with serine and K^{14}CN , accumulated β -cyanoalanine with the radioactivity localized in the cyano carbon. When glycine-2- ^{14}C was used in the previously described buffered incubation mixture, β -cyanoalanine was labeled in the 2-, 3-, and 4-carbons. Although it was not tested, one might anticipate that the carboxyl carbon of β -cyanoalanine would be labeled if glycine-1- ^{14}C was used as a substrate. Hence, glycine not only provided the cyano group but contributed directly to the synthesis of the

cyanide acceptor compound. Since serine served directly or indirectly as a cyanide acceptor, it appeared likely that its synthesis from glycine occurred in *Chromobacterium*. Serine did not accumulate in the reaction mixture in amounts readily detectable on paper chromatograms, but its presence was recognized in amino acid analyzer scans.

Methyl donor compounds were reported to enhance the formation of cyanide by *C. violaceum* (8). In the present work, methionine and *N,N*-dimethylglycine were found to enhance the synthesis of β -cyanoalanine as well. It seemed reasonable to assume that the methyl group of methionine could serve as a source of a one-carbon tetrahydrofolate derivative for the synthesis of serine from glycine. However, the inclusion of methionine-methyl- ^{14}C in the reaction mixture gave no

FIG. 2. Proposed pathway for the incorporation of cyanide by *Chromobacterium violaceum*.

label in β -cyanoalanine. The function of methionine in the synthesis of cyanide or β -cyanoalanine is obscure.

The synthesis of serine from glycine via the tetrahydrofolate system is known to occur in bacteria. One-carbon compounds such as formate and formaldehyde have been shown to participate in this reaction (6). Glycine can be oxidized by *C. violaceum* with the formation of glyoxylate (Brysk, Ph.D. Thesis, Columbia Univ., New York, N.Y., 1967). Glyoxylate has been reported to be oxidized to one-carbon compounds such as formate (2) and formaldehyde (11). In separate experiments ^{14}C -formate and ^{14}C -formaldehyde were employed in the buffered incubation mixture, but radioactive β -cyanoalanine was formed only when ^{14}C -formaldehyde was present. Apparently, formaldehyde was derived from glycine by way of glyoxylic acid and then condensed with glycine to yield serine.

Succinate, or another oxidizable four-carbon compound, was shown to enhance HCN formation by *C. violaceum* (8). In the present study, neither succinate-1,4- ^{14}C nor succinate-2,3- ^{14}C contributed carbons to β -cyanoalanine. Succinate was presumably oxidized, providing energy, perhaps as adenosine triphosphate, needed to drive one or more reactions leading directly or indirectly to HCN or β -cyanoalanine formation.

The evidence on the synthesis of β -cyanoalanine by *C. violaceum* leads to the postulated pathway depicted in Fig. 2. In this pathway, glycine supplies all the carbons of β -cyanoalanine.

Time-course experiments showed that β -cyanoalanine accumulated in the culture fluid for a limited period of time, and then the amount present decreased (Brysk, Ph.D. Thesis, Columbia

Univ., New York, N.Y., 1967). This suggested that the nitrile was being either incorporated into cell substance or degraded. In a tracer experiment with K^{14}CN and serine, radioactive asparagine accumulated in the supernatant fluid together with β -cyanoalanine. The asparagine was degraded and found to be labeled exclusively in the amide carbon. Asparagine was presumably derived from β -cyanoalanine by an incomplete hydrolytic reaction in which an amide was formed instead of the corresponding acid:

The β -cyanoalanine has been reported to be a precursor for asparagine in species of *Vicia* and *Lathyrus* (13) and to replace the requirement for asparagine in a mutant of *Neurospora* (14).

Aspartic acid was the only labeled amino acid found in cells previously incubated with β -cyanoalanine-4- ^{14}C . Whether it was synthesized by the cells from β -cyanoalanine or asparagine is uncertain, as hydrolysis of either of these compounds resulted in the formation of aspartic acid.

The limited distribution of label from cyanide of β -cyanoalanine suggests that the synthesis of the nitrile provides the only metabolic route for the incorporation of cyanide into the cells.

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