Cyanide Metabolism and β -Cyanoalanine Formation by Washed, Non-proliferating Cultures of *Chromobacterium violaceum*: Studies with Radiolabelled Cyanide

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The fate of radiolabelled cyanide added to washed non-proliferating suspensions of the cyanogenic organism *Chromobacterium violaceum* was studied. Within 6 h of addition, over 90% of the added cyanide had been metabolized to other compounds, except when L-glutamate alone was included in the suspension medium; in this case only 50% of the added cyanide had been metabolized after 6 h incubation. Less than 2% of radioactivity was incorporated into cell material; the rest remained in the suspension medium. The only identified compound into which radioactivity accumulated was β -cyanoalanine. Formation of β -cyanoalanine was stimulated by inclusion of L-glutamate and either *O*-acetyl-L-serine or L-serine in the incubation medium. Under optimal conditions, approximately 50% of added radiolabel accumulated in β -cyanoalanine. Compounds capable of supplying carbon for growth of *C. violaceum* were able to substitute, to some extent, for L-glutamate in stimulating β -cyanoalanine synthesis. Both *O*-acetyl-L-serine and L-serine inhibited β -cyanoalanine formation when included at concentrations in excess of 3 mM. L-Cysteine, L-threonine and L-alanine were unable to substitute for *O*-acetyl-L-serine or L-serine, and L-cysteine inhibited β -cyanoalanine synthesis. L-Methionine inhibited β -cyanoalanine formation by cells resuspended in cyanide, L-glutamate and *O*-acetyl-L-serine or L-serine. The effect of growth conditions on the ability of resuspended cells to synthesize β -cyanoalanine was studied.

INTRODUCTION

Amongst the prokaryotes cyanogenesis is limited to *Chromobacterium violaceum*, a few pseudomonads and the cyanobacterium *Anacystis nidulans* (Knowles, 1976; Castric, 1981; Pistorius *et al.*, 1979). Addition of glycine and L-methionine to the growth medium of *C. violaceum*, a *Pseudomonas* species and *Pseudomonas aeruginosa* stimulates cyanide production (Michaels & Corpe, 1965; Wissing, 1968; Castric, 1977; Rodgers & Knowles, 1978). Radiolabelling studies have established that, for *C. violaceum* and *P. aeruginosa*, the precursor of cyanide is glycine (Michaels *et al.*, 1965; Castric, 1977). The mechanism by which methionine stimulates bacterial cyanogenesis is not clear, although it is known that it is not a precursor of cyanide and that it can be replaced as a stimulator of cyanogenesis by a number of structurally related analogues and by metabolites such as cystathionine and *S*-adenosyl-L-methionine (Michaels *et al.*, 1965; Collins *et al.*, 1980).

Maximal cyanide production by growing cultures of *C. violaceum* occurs during the transition from exponential growth to the stationary phase of growth on glutamate plus minimal salts medium, after which the cyanide content of the growth medium decreases (Rodgers & Knowles, 1978). The decrease in cyanide concentration appears to be correlated with the induction of

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three cyanide-utilizing enzymes during the early stationary phase of growth (Rodgers & Knowles, 1978). These enzymes are β -cyanoalanine synthase (EC 4.4.1.9), γ -cyano- α -aminobutyric acid synthase (EC 4.4.1) and rhodanese (EC 2.8.1.1, thiosulphate: cyanide sulphurtransferase). However, in subsequent radiolabelling studies with *C. violaceum*, β -cyanoalanine was the only cyanide metabolite to be found, suggesting that β -cyanoalanine synthase is the only cyanide-utilizing enzyme operative *in vivo* (Rodgers, 1981). Earlier studies with washed, resuspended cells of *C. violaceum* demonstrated that they convert cyanide to β -cyanoalanine and/or γ -cyano- α -aminobutyric acid, depending on the incubation conditions (Brysk *et al.*, 1969; Brysk & Ressler, 1970). The present study is an attempt to elucidate the fate of radiolabelled cyanide added to washed, non-proliferating cultures of *C. violaceum*, and to characterize further the β -cyanoalanine synthase of this organism.

METHODS

Organism and growth conditions. *Chromobacterium violaceum* NCIB 9131 was grown on a mineral salts medium consisting of M-9 salts (Miller, 1972) without NH_4Cl , including 1 ml trace metals 1^{-1} (Bauchop & Elsdon, 1960) and with the Fe^{2+} concentration raised to 30 μM to enhance cyanogenesis (Rodgers & Knowles, 1978). Sodium L-glutamate (10 mM) was used routinely as a source of carbon and nitrogen. Unless otherwise stated, 2 mM-glycine and 0.5 mM-L-methionine were also included in the growth medium.

Bacteria were grown in 100 ml medium in 250 ml conical flasks, or in 500 ml medium in 2 l conical flasks; incubation was at 30 °C in a gyratory shaker (250 r.p.m.). A 1% (v/v) inoculum of a 20–24 h stationary phase culture, grown on medium containing 10 mM-L-glutamate was used.

Preparation and incubation of cell suspensions. Early stationary phase cultures (16 h) of *C. violaceum* were harvested by centrifugation at 18000 *g* for 10 min in a Sorvall RC-2 centrifuge at 4 °C, washed twice in M-9 salts and resuspended in fresh growth medium minus amino acids to give a concentration of 1.0 mg dry wt ml^{-1} . To all suspensions were added 50 μg chloramphenicol ml^{-1} (sufficient to prevent growth), 1 mM-KCN and 5 μCi (185 kBq) Na^{14}CN (sp. act. 60 mCi mmol^{-1}). Where stated, amino acids were added at concentrations indicated in the text. Cell suspensions (7.5 ml) were incubated in stoppered 50 ml conical flasks at 30 °C in a shaking water bath.

Analysis of products of cyanide metabolism. After incubation with Na^{14}CN , samples of cell suspensions were taken for determination of total radioactivity. Radioactivity was measured as outlined below. The remainder of the cell suspension was filtered using a 0.45 μm (pore size) membrane filter to remove bacteria. Filters were washed with 10 ml M-9 salts, dried and radioactivity in the whole cells determined. The cyanide content of the filtrate was assayed by one of two methods, either colorimetrically (Epstein, 1947) or by blowing off cyanide with a stream of air and trapping the cyanide in 1.0 M-NaOH. The trapped cyanide was neutralized with 1.0 M-HCl and its radioactivity determined. Both methods gave essentially the same results.

The CO_2 content of the filtrate was measured by the following method. CO_2 was precipitated with saturated $\text{Ba}(\text{OH})_2$ solution and the precipitate collected on a 0.45 μm (pore size) membrane filter and dried. The filter was placed in a stoppered flask with side-arm and centre well. Phenethylamine was present in the centre well and 2 ml 6 M- HNO_3 was introduced into the side-arm. The flask was tipped to bring the acid into contact with the filter and shaken for 45 min. Any CO_2 liberated by the acid was absorbed by the phenethylamine and its radioactivity was determined. Tests using a standard solution of $\text{NaH}^{14}\text{CO}_3$ showed that the recovery of CO_2 by this method is 75–80%.

The thiocyanate content of the filtrate was assayed by the method of Sörbo (1955). Other components of the filtrate were analysed by TLC as outlined below.

Thin-layer chromatography. Amino acids in the filtrate were separated by silica gel TLC (Eastman Chromagram precoated plates) in a solvent system of propan-1-ol/31.5% (w/w) ammonia (70:30, v/v). Amino acids were visualized by spraying with ninhydrin reagent and heating at 100 °C for 5 min. The ninhydrin reagent consisted of 1% (w/v) ninhydrin in acetone (100 vol.) plus 1 g cadmium acetate in 100 ml glacial acetic acid and 50 ml water (15 vol.). Amino acids gave purple spots with this reagent, except for β -cyanoalanine which gave a blue-green colour. Amino acids were identified by means of standards.

Filtrates were assayed for the presence of formic acid and formamide by means of cellulose TLC (Eastman Chromagram precoated plates) in a solvent system of butan-1-ol/glacial acetic acid/pyridine/water (100:20:10:30, by vol.). Formamide was visualized by spraying with a reagent consisting of 10% (w/v) NaOH, 10% (w/v) sodium nitroprusside and 10% (w/v) potassium ferricyanide, the mixture being diluted threefold with water. Formamide gives a pink colour with this reagent. Formic acid was visualized by spraying with 1% (w/v) AgNO_3 in 1 M-ammonia; a brown colour is obtained after heating for 10 min at 110 °C.

All spots visualized on the chromatograms were scraped off and their radioactivity determined. As a check for the presence of radioactive compounds not visualized by the above procedures, sequential 1 cm squares were scraped off the chromatograms and assayed for radioactivity.

Determination of radioactivity. Radioactivity of samples was measured by scintillation counting using an Intertechnique SL 400 scintillation counter. The scintillation mixture consisted of 500 ml toluene, 500 ml Triton X-100 and 7 g butyl-PBD; 3 ml of this mixture was used per sample. Efficiency of counting was determined by the internal standards method.

Materials. Na^{14}CN and $\text{NaH}^{14}\text{CO}_3$ were obtained from Amersham. Solid Na^{14}CN was dissolved in 50 mM- Na_2HPO_4 to give a solution of specific activity $1 \mu\text{Ci ml}^{-1}$ and stored at -20°C . Chloramphenicol, β -cyanoalanine and *O*-acetyl-L-serine were obtained from Sigma. All other chemicals were of analytical grade; glass-distilled water was used throughout.

RESULTS

The metabolic fate of cyanide

All experiments were performed at least three times and gave reproducible results; the results given are those from typical experiments and the stated experimental values are the averages of duplicate determinations. All incubations of resuspended cells of *C. violaceum* contained $50 \mu\text{g}$ chloramphenicol ml^{-1} , a concentration sufficient to prevent further growth. The observation of Nazly *et al.* (1981) that resuspended early-stationary phase cells of *C. violaceum* are unable to produce cyanide was confirmed by incubating such cells in the presence of 5 mM-L-glutamate plus combinations of 2 mM-L-serine, 1 mM-*O*-acetyl-L-serine, 2 mM-glycine and 0.5 mM-L-methionine (results not shown).

Table 1 gives the distribution of radioactivity amongst various components after a 6 h incubation of resuspended cells with radiolabelled cyanide, either alone or plus combinations of various amino acids. In all cases except one, only 10% or less of the radioactivity remained as cyanide. The fate of the metabolized cyanide depended on the incubation conditions, although under no conditions was more than 2% of the cyanide converted into cell components or CO_2 . In every case, radioactivity was incorporated into β -cyanoalanine to a greater or lesser extent. The inclusion of glutamate, *O*-acetyl-L-serine or L-serine alone increased the incorporation of radioactivity into β -cyanoalanine some 3- to 10-fold, whereas the inclusion of glutamate plus either *O*-acetyl-L-serine or serine increased the incorporation some 15- to 18-fold. The remainder of the radioactivity was present in the incubation medium as compound(s) other than β -cyanoalanine. For cultures incubated with either cyanide alone or cyanide plus serine, over 80% of the radioactivity was present as these other compounds; when serine was replaced by *O*-acetyl-L-serine, more than 60% of radioactivity was present in this form. Only for cultures incubated with glutamate, either alone or plus *O*-acetyl-L-serine or serine, was less than 50% radioactivity present as unidentified compounds.

Table 1. *Distribution of radioactivity from Na^{14}CN added to resuspended, non-proliferating cells of *C. violaceum**

Cells were resuspended in mineral salts medium plus chloramphenicol, amino acids and Na^{14}CN as described in the text. After 6 h incubation at 30°C , cultures were analysed for radioactivity and the distribution of radioactivity was calculated as a fraction of the total radioactivity present in the culture at 0 h.

Additional medium components	Distribution of radioactivity (% of total added)				
	Cyanide	Cell components	CO_2	β -Cyanoalanine	Unidentified compounds in medium
None	10.5	0.6	1.7	2.8	84.4
L-Glutamate (5 mM)	50.5	1.0	1.5	17.7	29.3
<i>O</i> -Acetyl-L-serine (1 mM)	5.5	1.0	1.2	27.7	66.6
L-Serine (2 mM)	5.1	0.8	1.5	7.8	85.8
L-Glutamate (5 mM) + <i>O</i> -acetyl-L-serine (1 mM)	0.5	0.9	1.6	51.2	45.8
L-Glutamate (5 mM) + L-serine (2 mM)	8.0	1.4	0.8	42.6	47.8

Attempts were made to identify the other radiolabelled compounds by means of TLC. Silica gel TLC and staining for amino acids gave only two spots, corresponding to glutamate (when included in the suspension medium) and β -cyanoalanine; γ -cyano- α -aminobutyric acid could not be detected. Cellulose-TLC and staining for formamide and formic acid proved negative although ninhydrin again gave two spots, corresponding to glutamate (when appropriate) and β -cyanoalanine. No radioactivity, other than that corresponding to β -cyanoalanine, could be detected on TLC plates. As a check, samples of incubation medium filtrates were applied to silica gel plates; these spots were scraped off immediately after drying under hot air as in the previous experiments, and their radioactivity measured. For each incubation condition the amount of radioactivity lost during application of culture filtrates to silica gel plates was approximately equal to the radioactivity present as cyanide and the unidentified compounds (results not shown). Culture filtrates were also assayed for thiocyanate, this test gave negative results.

β -Cyanoalanine formation

Because β -cyanoalanine was the major identifiable product of cyanide metabolism, further experiments concentrated on the production of this compound. Figure 1 depicts a study of

Fig. 1. Cyanide metabolism (●) and β -cyanoalanine formation (○) by resuspended, non-proliferating cells of *C. violaceum*. Cells were resuspended in M-9 salts plus 50 μ g chloramphenicol ml^{-1} and 5 μCi Na^{14}CN (1 mM). The following additions were made, (a) nothing, (b) 5 mM-L-glutamate, (c) 1 mM-*O*-acetyl-L-serine, (d) 5 mM-L-glutamate plus 1 mM-*O*-acetyl-L-serine, (e) 2 mM-L-serine, (f) 5 mM-L-glutamate plus 2 mM-L-serine.

cyanide metabolism and β -cyanoalanine formation by cells resuspended in 1 mM-cyanide, either alone or plus combinations of 5 mM-glutamate, 1 mM-*O*-acetyl-L-serine and 2 mM-serine. It is important to stress that in identical incubation conditions, except for the absence of cells, there was no loss of cyanide. The rate of cyanide metabolism was essentially the same under all conditions, except for incubation in the presence of glutamate alone (Fig. 1*b*) where inhibition was observed. In the presence of glutamate alone, cyanide metabolism was not completed until 7 h incubation, whereas in all other conditions metabolism was complete after 4 h incubation. In agreement with the results listed in Table 1, Fig. 1 shows that β -cyanoalanine production was stimulated by inclusion of glutamate and/or *O*-acetyl-L-serine or serine in the medium. Maximal rates of β -cyanoalanine production occurred in the presence of glutamate plus either *O*-acetyl-L-serine or serine (Fig. 1*d, f*). In all cases, β -cyanoalanine synthesis terminated after 2 h and concentrations remained more or less constant for up to 10 h incubation.

The effect of growth conditions on the ability of resuspended cells to form β -cyanoalanine, when incubated with 1 mM-cyanide, 5 mM-glutamate and 6 mM-serine, was examined. Cells grown on glutamate supplemented with glycine and methionine produced β -cyanoalanine at a rate of 187 nmol h⁻¹ (mg dry wt cells)⁻¹, compared to cells grown on glutamate supplemented with glycine or methionine, which produced 101 and 33 nmol β -cyanoalanine h⁻¹ (mg dry wt cells)⁻¹, respectively; cells grown on glutamate alone produced 28 nmol β -cyanoalanine h⁻¹ (mg dry wt cells)⁻¹. Resuspended cells incubated with 1 mM-*O*-acetyl-L-serine instead of serine gave similar ratios of activities.

With resuspended cells incubated with 1 mM-cyanide, 5 mM-glutamate and either 1 mM-*O*-acetyl-L-serine or 2 mM-serine, β -cyanoalanine formation was proportional to the cell density up to a concentration of 1 mg dry wt cells ml⁻¹ (results not shown). β -Cyanoalanine synthesis by resuspended cells incubated with 1 mM cyanide and either 1 mM-*O*-acetyl-L-serine or 2 mM-serine was stimulated by the addition of glutamate up to a concentration of 6 to 8 mM (results not shown). Other compounds known to be growth substrates for *C. violaceum* (Sneath, 1956) were tried as replacements for glutamate in the production of β -cyanoalanine by resuspended cells incubated with 1 mM-cyanide and 1 mM-*O*-acetyl-L-serine. These compounds (concentration 5 mM) were D-glucose, citric acid, sodium succinate, mannitol and sodium acetate. With all of these compounds, β -cyanoalanine production was reduced by approximately half when compared with glutamate. No products of cyanide metabolism other than β -cyanoalanine could be detected.

The effect of *O*-acetyl-L-serine and serine concentration on the rate of β -cyanoalanine formation by resuspended cells is shown in Fig. 2. With both amino acids, β -cyanoalanine production was stimulated by concentrations of up to 3 mM, above which inhibition was

Fig. 2. Effect of *O*-acetyl-L-serine (●) or L-serine (○) concentration on formation of β -cyanoalanine by resuspended, non-proliferating cells of *C. violaceum*. Cells were resuspended in M-9 salts, 50 μ g chloramphenicol ml⁻¹, 5 μ Ci Na¹⁴CN (1 mM), 5 mM-L-glutamate and either *O*-acetyl-L-serine or L-serine. β -Cyanoalanine formation was measured after 1 h incubation at 30 °C.

observed. By measuring activities at substrate concentrations of less than 2 mM it was possible to calculate apparent K_m values of 0.44 mM (mean of 15 determinations, s.d. 0.17) for *O*-acetyl-L-serine and 1.59 mM (mean of 11 determinations, s.d. 0.50) for serine. Both Lineweaver-Burk and Eadie-Hofstee plots were used in calculating these constants. The ability of amino acids other than *O*-acetyl-L-serine or serine to act as substrates for β -cyanoalanine production was tested. Cells were incubated with 1 mM-cyanide and 5 mM-glutamate plus 1 mM-amino acid. Neither L-threonine nor L-alanine caused any stimulation of β -cyanoalanine formation when compared to that produced in their absence, whereas L-cysteine caused a sevenfold decrease (results not shown). Another sulphur amino acid, L-methionine, inhibited β -cyanoalanine synthesis by cells incubated with 1 mM-cyanide, 5 mM-glutamate and 1 mM-*O*-acetyl-L-serine or 2 mM-serine. Approximately 25% of the original activity remained in the presence of 4 mM-methionine, with inhibition being slightly higher when serine was used as substrate.

DISCUSSION

The only product of cyanide metabolism by *C. violaceum* that could be detected in significant amounts was β -cyanoalanine; another product(s) seemed to be present in the culture filtrate but could not be identified. The loss of radioactivity on application of culture filtrates to thin-layer plates suggested that this compound is volatile. Presumably, at least two pathways of cyanide metabolism operate in *C. violaceum*: an unknown pathway(s) and the β -cyanoalanine-producing one. The unknown pathway is predominant in the absence of compounds other than cyanide, but β -cyanoalanine is also produced in the presence of compounds such as glutamate that can be metabolized to the substrate for β -cyanoalanine synthase.

The results obtained support earlier indications that, of the three cyanide-utilizing enzymes detected by *in vitro* studies, β -cyanoalanine synthase is the only one operative *in vivo* (Rodgers, 1981). Brysk *et al.* (1969) first demonstrated that resuspended cells of *C. violaceum* will produce labelled β -cyanoalanine when incubated with serine or succinate and $K^{14}CN$. They were also able to detect labelled asparagine in smaller quantities, but this compound could not be detected in the present study. Brysk & Ressler (1970) found that washed, resuspended cells of *C. violaceum* produced both β -cyanoalanine and γ -cyano- α -aminobutyric acid when incubated with cyanide and one of glutamate, serine or threonine, whereas in the present study only β -cyanoalanine was produced. This difference may be due to the use of different strains of *C. violaceum*, and the use of cells harvested at different stages in the growth cycle.

The β -cyanoalanine synthase of blue lupin (*Lupinus angustifolia*) has been purified and shown to be highly specific for L-cysteine (Hendrickson & Conn, 1969). The only other substrate for this enzyme was *O*-acetyl-L-serine, but the rate of β -cyanoalanine formation was only 5% of that observed with cysteine. In the same study *O*-acetyl-L-serine sulphydrylase (EC 4.2.99.8) was purified and shown to catalyse β -cyanoalanine formation from *O*-acetyl-L-serine and cyanide at 0.1% of the rate catalysed by β -cyanoalanine synthase with cysteine and cyanide as substrates. Other than *C. violaceum*, the only bacteria known to produce β -cyanoalanine from cyanide are *Escherichia coli*, *Bacillus megaterium* and an *Enterobacter* species; all three organisms are acyanogenic (Dunnill & Fowden, 1965; Castric & Strobel, 1969; Sakai *et al.*, 1981). The β -cyanoalanine-synthesizing activity of *B. megaterium* was investigated in some detail by Castric & Conn (1971). Cell extracts formed β -cyanoalanine from cyanide and L-cysteine, *O*-acetyl-L-serine or, to a lesser extent, L-serine. Induction studies and partial purification suggested that the β -cyanoalanine-synthesizing activity was a secondary activity of the enzyme *O*-acetyl-L-serine sulphydrylase. Washed, resuspended cells of the *Enterobacter* sp. produced β -cyanoalanine when incubated with cyanide plus *O*-acetyl-L-serine, L-serine or, to a lesser extent, L-cysteine (Sakai *et al.*, 1981).

The present study indicates that, although all the systems have some features in common, the β -cyanoalanine-synthesizing activity of *C. violaceum* is different from those of blue lupin, *B. megaterium* and the *Enterobacter* sp. in that both *O*-acetyl-L-serine and L-serine are good substrates for the *C. violaceum* system, whereas L-cysteine actually inhibits β -cyanoalanine formation. It is not possible to say whether *O*-acetyl-L-serine or L-serine is the actual substrate

for β -cyanoalanine formation because they may be interconverted in the presence of serine acetyltransferase (EC 2.3.1.30), an enzyme of the cysteine biosynthetic pathway in *E. coli* and *Salmonella typhimurium* (Smith, 1971). The lower apparent K_m for *O*-acetyl-L-serine, compared with L-serine may suggest that this is the actual substrate for β -cyanoalanine synthase, but it must be borne in mind that the measured K_m values may be a reflection of the uptake systems for the two amino acids.

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