Cyanide Production and Degradation During Growth of *Chromobacterium violaceum*

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(Received 12 April 1978)

Cyanogenesis by growing cultures of *Chromobacterium violaceum* was stimulated by the inclusion of glycine and methionine in the growth medium. Increases in the ferrous ion and phosphate concentrations of the growth medium stimulated cyanide production. *Chromobacterium violaceum* possesses a number of cyanide-utilizing enzymes: β-cyanoalanine synthase, γ-cyano-α-aminobutyric acid synthase and rhodanese. Studies on the activities of these enzymes in cell-free extracts of cultures growing under both high and low cyanide-evolving conditions are presented. Addition of chloramphenicol to high and low cyanide-evolving cultures towards the end of exponential growth had a profound effect on the medium cyanide concentrations. These observations are shown to have been caused by chloramphenicol blocking the induction of the cyanide-utilizing enzymes.

**INTRODUCTION**

A wide range of living organisms are cyanogenic, including many eukaryotic microorganisms (Knowles, 1976). However, the only bacteria known to produce cyanide are the soil organisms *Chromobacterium violaceum* and a few pseudomonads. Cyanide production by *C. violaceum* and *Pseudomonas aeruginosa* is stimulated by addition of glycine and methionine to the medium (Michaels & Corpe, 1965; Wissing, 1968; Castric, 1977). In the snow mould fungus, glycine stimulates cyanogenesis (Ward & Thorn, 1966). In *C. violaceum* the carbon and nitrogen of cyanide are derived from the C-2 methylene group and the amino group of glycine, respectively; the carboxyl group is lost as CO₂ (Michaels et al., 1965). A similar situation prevails in the snow mould fungus (Ward & Thorn, 1966), but in *P. aeruginosa* both the C-1 and C-2 groups of glycine are incorporated into cyanide (Castric, 1977). The role of methionine in bacterial cyanogenesis is not known, but it possibly acts as an intermediary methyl group donor.

Cyanide-producing micro-organisms are able to assimilate or detoxify cyanide by a variety of pathways (Knowles, 1976). *Chromobacterium violaceum* converts cyanide to β-cyanoalanine and then aspartate, presumably as an assimilatory pathway (Brysk et al., 1969a). It also forms γ-cyano-α-aminobutyric acid (Brysk & Ressler, 1970) although no further metabolism appears to occur, suggesting that it is a detoxication product. As shown below, *C. violaceum* also forms rhodanese as a possible detoxifying agent.

It is the aim of the work presented in this paper to investigate further the conditions for cyanide production by *C. violaceum* and the relationship between the formation and utilization or degradation.

**METHODS**

*Organism and growth conditions.* *Chromobacterium violaceum* (NCIB 9131, D252, supplied by Dr Dorothy Jones of the University of Leicester) was grown on a minimal medium consisting of M-9 salts (Miller, 1972) with ammonium salts omitted, 1 ml trace metals l⁻¹ (Bauchop & Elsden, 1960) with the Fe⁴⁺ concentration...
Cyanide production ofor growth of *C. violaceum* was optimal with 2 to 3 mM-glycine and 0.2 to 0.5 mM-methionine in the medium (not shown). These concentrations of glycine and methionine are lower than those previously reported for maximal cyanogenesis by *C. violaceum* (Michaels & Corpe, 1965) and *P. aeruginosa* (Castric, 1977), suggesting that cyanide is such a product (Castric, 1975).

Cyanide production for growth of *C. violaceum* on 10 mM-glutamate was optimal with 2 to 3 mM-glycine and 0.2 to 0.5 mM-methionine in the medium (not shown). These concentrations of glycine and methionine are lower than those previously reported for maximal cyanogenesis by *C. violaceum* (Michaels & Corpe, 1965) and *P. aeruginosa* (Castric, 1977). Cyanide production, and growth rate and final yield were relatively unaffected by growth in media with pH values in the range 6.0 to 8.0. Although the growth rate was, as expected,
Cyanogenesis by C. violaceum

Fig. 1. Growth and cyanide evolution by C. violaceum. For clarity, only growth in medium containing 10 mM-glutamate is shown (△). Cyanide evolution was followed during growth on 10 mM-glutamate (□), and on 10 mM-glutamate supplemented with 2 mM-glycine (■), 0.5 mM-methionine (○) and 2 mM-glycine plus 0.5 mM-methionine (●).

Fig. 2. Effect of ferrous ion (as FeSO₄) concentration (○) and phosphate concentration (●) on the maximum level of cyanide evolved by cultures of C. violaceum growing on 10 mM-glutamate supplemented with 2 mM-glycine and 0.5 mM-methionine. In the experiment where FeSO₄ concentration was varied the phosphate content was maintained at 68 mM, and where the phosphate content was varied the FeSO₄ concentration was 30 µM.

affected by variation of the growth temperature in the range 25 to 35 °C, growth yield and cyanide production were essentially unaffected by the growth temperature.

Like P. aeruginosa (Castric, 1975), cyanogenesis by C. violaceum was affected by the Fe²⁺ concentration of the medium (Fig. 2). Although higher concentrations of Fe²⁺ gave greater yields of cyanide, we have routinely used 30 µM-FeSO₄ in growth media as higher levels caused precipitation. Increasing the medium phosphate concentration also caused an increase in cyanogenesis (Fig. 2). Above 100 mM-phosphate the growth yield declined somewhat, and we have routinely used 68 mM-phosphate in the medium. Chromobacterium violaceum appears to be different from P. aeruginosa in its response to phosphate as Meganathan & Castric (1977) have reported that cyanogenesis is inhibited in the latter organism by phosphate concentrations greater than 10 mM.

Figure 3 shows the induction of β-cyanoalanine synthase (a, b), γ-cyano-α-aminobutyric acid synthase (c, d) and rhodanese (e, f) during the late-exponential and early-stationary phases of growth of C. violaceum on glutamate (a, c, e) or glutamate plus glycine and methionine (b, d, f). In each case, the enzyme activity increased after about 8 h growth and reached a maximal value about 2 h after the stationary phase had been attained (12 h growth). Inclusion of glycine plus methionine in the medium caused a significant reduction in the induction of β-cyanoalanine synthase and γ-cyano-α-aminobutyric acid synthase, but not rhodanese, during the exponential–stationary phase (trophophase–idiophase)
transition period. Inclusion of glycine alone was also found to cause a reduction in the degree of induction of \(\beta\)-cyanoalanine synthase and \(\gamma\)-cyano-\(\alpha\)-aminobutyric acid synthase (not shown).

Following induction of these enzymes, the medium cyanide concentration decreased rapidly for cells induced for cyanogenesis (Figs 4 and 5b), presumably due to cyanide assimilation or detoxication especially via \(\beta\)-cyanoalanine synthase (Brysk et al., 1969a; Brysk et al., 1969b).

Addition of chloramphenicol to the medium in the exponential phase of growth prior to the onset of cyanide production (5.5 h growth) caused an immediate cessation of growth and a dramatic reduction of cyanogenesis for cells growing with glycine and methionine in the medium (Fig. 4). Addition of chloramphenicol after 5.5 h growth on glutamate alone terminated growth but had no effect on the low level of cyanide produced (not shown).

For cells growing on glutamate alone, chloramphenicol added after 9.5 h growth caused a doubling of the medium cyanide content (Fig. 5a). When chloramphenicol was added
Cyanogenesis by *C. violaceum*

Fig. 4. Effect of addition of chloramphenicol during the exponential phase on growth and cyanide evolution of *C. violaceum* growing on 10 mM-glutamate supplemented with 2 mM-glycine and 0.5 mM-methionine. Chloramphenicol (50 μg ml⁻¹) was added after 5.5 h growth (arrowed). Growth in the absence (○) and presence (●) of chloramphenicol and cyanide evolution in the absence (△) and presence (▲) of chloramphenicol were followed.

Fig. 5. Effect of addition of chloramphenicol during the late-exponential phase on growth and cyanide evolution of *C. violaceum* growing on 10 mM-glutamate (a) and on 10 mM-glutamate supplemented with 2 mM-glycine and 0.5 mM-methionine (b). Chloramphenicol (50 μg ml⁻¹) was added to both cultures after 9.5 h growth (arrowed). Growth in the absence (○) and presence (●) of chloramphenicol and cyanide evolution in the absence (△) and presence (▲) of chloramphenicol were followed in both cultures.

After cyanogenesis had commenced during growth on glutamate plus glycine and methionine (9.5 h growth), the rate of decrease of medium cyanide content due to assimilation or detoxication decreased dramatically (Fig. 5b). In both cases, addition of chloramphenicol prevented induction of the enzymes for cyanide assimilation or detoxication (Table 1). If chloramphenicol was added at a time later than 9.5 h growth, the medium cyanide content was not affected showing that chloramphenicol did not inhibit the cyanide-utilizing enzymes.
Table 1. Effect of addition of chloramphenicol to late-exponential phase cultures of C. violaceum on the induction of the cyanide-utilizing enzymes

Chloramphenicol was added to a final concentration of 50 μg ml⁻¹ after 9.5 h growth. The percentage increase in specific activity refers to the difference between activities measured at 8 h and at 12 h growth (see Fig. 3).

<table>
<thead>
<tr>
<th>Medium</th>
<th>Chloramphenicol</th>
<th>β-Cyanoalanine synthase</th>
<th>γ-Cyano-α-aminobutyric acid synthase</th>
<th>Rhodanese</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM-Glutamate</td>
<td>−</td>
<td>124</td>
<td>116</td>
<td>120</td>
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<tr>
<td></td>
<td>+</td>
<td>28</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>10 mM-Glutamate + 2 mM-glycine + 0.5 mM-methionine</td>
<td>−</td>
<td>52</td>
<td>58</td>
<td>340</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>71</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Wissing (1975) has recently obtained a bacterial cell-free extract capable of producing cyanide from glycine, but so far nothing is known about the enzymic step(s) involved in the conversion, although speculative pathways have been proposed (Knowles, 1976). On the other hand, several enzymes involved in cyanide assimilation and detoxication by cyanogenic micro-organisms have been identified, yet little is known about their properties and whether, for example, their activities are regulated by the cellular or medium cyanide, glycine or methionine levels (Knowles, 1976). Thus it is not possible to quantify the levels of the enzyme(s) involved in cyanide production during growth of C. violaceum and, whereas the activities of the assimilatory or detoxifying enzymes can be assayed **in vitro**, their activities **in vivo** cannot be estimated. Nonetheless, from measurements of the medium cyanide concentration, an assessment can be made concerning the balance between cyanide production and degradation in growing cultures of C. violaceum.

Experiments involving addition of glycine and methionine to cultures of C. violaceum several hours before measuring the ability to form cyanide from glycine have shown that glycine and methionine act as inducers of cyanogenesis (Michaels & Corpe, 1965; Michaels et al., 1965), in addition to glycine being a precursor of cyanide. We have found that when chloramphenicol is added before the onset of cyanogenesis, to cultures growing in the presence of glycine and methionine, cyanide production is inhibited. This observation confirms that glycine and methionine at concentrations greater than the intracellular pool levels act as inducers of cyanide production, and shows that induction of the cyanide-producing enzyme(s) occurs in the late-exponential phase of growth. Furthermore, if chloramphenicol is added later, when maximal cyanogenesis is occurring, the level of cyanide production is only slightly affected but the following decrease in its concentration in the culture is inhibited. This indicates that the enzymes for cyanide assimilation or detoxication are induced only after cyanide production has occurred. In agreement with this supposition, assays of β-cyanoalanine synthase, γ-cyano-α-aminobutyric acid synthase and rhodanese show that they increase in concentration at the start of the stationary phase, after cyanogenesis has occurred. It is possible that either cyanide or glycine acts as an inducer for the synthesis of these enzymes. However, addition of glycine alone to the medium, even though it does not cause induction of cyanogenesis, actually causes a small degree of inhibition of synthesis of the cyanide assimilating or detoxifying enzymes. It is therefore more likely that cyanide itself is an inducer of these enzymes. When growing on glutamate alone, where relatively little cyanide is produced, the assimilatory and detoxifying enzymes are induced in the stationary phase; possibly the low levels of cyanide formed under these conditions are still sufficient to act as inducers for the enzymes. Alternatively, the intra-
cellular glycine or methionine pools could be sufficient to cause induction of the enzymes, especially if the pool sizes increase at the end of the exponential growth phase as the demand for glycine and/or methionine for other purposes decreases (see Drew & Demain, 1977).

The low concentrations of cyanide found in the medium of cultures grown on glutamate in the absence of glycine and methionine is presumably due to the relatively lower rate of its production by the uninduced levels of the cyanide-forming enzyme system in comparison with its rate of removal by the degrading enzymes. Prevention of induction of the latter enzymes might be expected to result in an increase in cyanide concentration in the medium, as is observed (Fig. 5a).

The medium cyanide concentration will also depend on factors affecting the activities of the cyanide-degrading enzymes. Preliminary studies on the enzymology of these enzymes have shown that $\beta$-cyanoalanine synthase activity is about 60% inhibited by 2-0 mm-methionine, yet higher concentrations of methionine cause little further inhibition. Glycine, although it repressed induction of this enzyme in the stationary phase (Table 1), has no effect on its activity. The activities of rhodanese and $\gamma$-cyano-$\alpha$-aminobutyric acid synthase were unaffected by glycine or methionine (P. B. Rodgers, unpublished observations).

This work was supported by the Science Research Council via grant no. GR/A/11543 to C. J. K. and a research studentship to P. B. R. Mrs P. A. Collins provided excellent technical assistance. We thank Dr L. F. Wright for helpful discussions.

REFERENCES


