Cyanate Specifically Inhibits Arginine Biosynthesis in *Escherichia coli* K12: a Case of By-product Inhibition?

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Growth of *Escherichia coli* K12 cultivated in minimal medium was strongly inhibited by 2 mM-cyanate. This inhibition could be specifically reversed by arginine. Citrulline (but not ornithine, *N*- α -acetylornithine or *N*-acetylglutamate) could also restore a normal growth rate. Since growth inhibition by cyanate was followed by an accumulation of ornithine within the cell it was concluded that cyanate specifically inhibits the formation of citrulline from ornithine. The effect of cyanate on the growth of defined strains was consistent with a specific inhibition of carbamoylphosphate synthase. A kinetic study of carbamoylphosphate synthase and ornithine carbamoyltransferase *in vitro* supported this conclusion. Since carbamoylphosphate is probably the only source of endogenous cyanate it is postulated that carbamoylphosphate synthase activity can be regulated by cyanate resulting from the dissociation of carbamoylphosphate in metabolic circumstances leading to its overproduction.

INTRODUCTION

The toxicity of cyanate for cells and organisms has been known for a long time. Following the cytological studies of Dustin (1947) the toxicity of cyanate for biological systems was described by Schütz (1949). Poisoning effects of cyanate were described in plants, animals and humans; its toxicity for micro-organisms was reported by Taussig (1960). So far, the effect of cyanate on proteins and enzymes *in vitro* has been characterized by an apparent lack of specificity. It is consequently not known whether the effects observed *in vivo* could be directed towards more specific targets at the molecular level.

Cyanate is a well known carbamoylating agent which reacts through isocyanic acid with nucleophilic groups, especially with amino (Stark, 1965; Williams & Jencks, 1974) and thiol groups (Stark, 1964). Many proteins and enzymes have been shown to undergo structural and functional alterations after carbamoylation mediated by cyanate. Some enzymes are inactivated by a specific reaction with a group essential for catalysis, e.g. papain (Sluyterman, 1967), bovine glutamate dehydrogenase (Veronese *et al.*, 1972), tobacco ribulose-1,5-bisphosphate carboxylase (Chollet & Anderson, 1978), and carbamoylphosphate synthase from *Escherichia coli* (Anderson & Carlson, 1975). Cyanate is also a pseudohalide ion which can form complexes with transition metal ions (Forster & Goodgame, 1965). Some enzymes are inactivated by cyanate after chelation of a metal ion in the active site, e.g. carbonic anhydrase (Engberg & Lindskog, 1984), superoxide dismutase (Bertini *et al.*, 1981), and cobalt(II) carboxypeptidase A (Bertini *et al.*, 1985).

This article deals with the effect of cyanate on the physiology of *E. coli* K12 and more precisely with the specific inhibition of arginine biosynthesis exerted by cyanate.

Abbreviations: ACTase, aspartate carbamoyltransferase; CP, carbamoylphosphate; CPSase, carbamoylphosphate synthase; OCTase, ornithine carbamoyltransferase.

METHODS

Bacterial strains. All strains used in the present study were derivatives of *Escherichia coli* K12. They are listed in Table 1. BGM 023 (*cnt3*) is a mutant of strain R5, lacking inducible cyanase (see the preceding paper: Guilloton & Karst, 1987). BGM 171 is a spontaneous *argR* mutant derived from BGM 023 by selection on 'arginine-free' medium (Novick & Maas, 1961) supplemented with canavanine according to Maas (1961). BGM 180, which harbours the *carB8* deletion, was obtained as follows: a *thr* derivative (BGM 167) of BGM 023 was obtained after UV irradiation and penicillin enrichment; this strain was transduced to Thr⁺ by bacteriophage P1 cultivated on strain CSH 73 (*carB8*) (Lissens *et al.*, 1980) and strain BGM 180 (*cnt3 carB8*) was scored as a double Arg⁻ Ura⁻ auxotroph among the Thr⁺ transductants. Strain 58161 (*carB8 thr metB/pMC40 carA⁺ carB⁺*) was provided to us by Marjolaine Crabeel and André Piérard; this strain harbours the pMC40 recombinant plasmid with the adjacent *carAB* genes subcloned by insertion into the *Hind*III site of pBR322. BGM 186 was obtained by transformation of BGM 180 by pMC40 and selection for Amp^R; BGM 186, prototrophic for arginine and uracil, has a specific activity of carbamoylphosphate synthase 13 times greater than strain BGM 023 when cultivated in minimal medium. BGM 209 (*cnt3 carB8*) is a spontaneous citrulline-utilizing mutant obtained by plating strain BGM 180 on a solid minimal medium containing L-citrulline (2000 µg ml⁻¹), according to Legrain *et al.* (1976).

Genetic techniques. Generalized transduction mediated by bacteriophage P1 was performed according to Miller (1972); bacteriophage P1vir was kindly supplied by M. Schwartz (Institut Pasteur, Paris, France). Plasmid pMC40 was isolated from strain 58161/pMC40 by the small-scale boiling method and transformation experiments were done by the calcium chloride procedure described by Maniatis *et al.* (1982).

Media and growth conditions. L broth and synthetic A medium described by Miller (1972) were used throughout this study. Bacterial cells were cultivated with shaking in 25 × 250 mm test tubes at 37 °C; optical densities (OD) were monitored at 575 nm directly in the test tubes using a Coleman Junior AII spectrophotometer. OD₅₇₅ was proportional to cell density up to 0.6. For physiological studies, overnight cultures in minimal medium were subcultured in the same medium, grown to mid-exponential phase, then diluted to an OD₅₇₅ of 0.1, the pH being adjusted to 7.00. Cells were cultivated to an OD₅₇₅ of 0.2 before addition of cyanate or other solutions and growth was followed for a further 150 min.

Cyanate assay. Cyanate was assayed by a spectrophotometric method using a reaction with 2-aminobenzoic acid (Guilloton & Karst, 1985).

Enzyme assays. *Carbamoylphosphate synthase (CPSase; EC 6.3.5.5).* CPSase activity was measured by coupling carbamoylphosphate (CP) formation with citrulline synthesis in the presence of ornithine and an excess of partially purified ornithine carbamoyltransferase (Piérard & Wiame, 1964). The assay medium was prepared in 0.1 M-TES(K) buffer (pH 7.5). Reaction was stopped by addition of cold TCA. After centrifugation of precipitated proteins, citrulline was estimated in the supernatant using a modification of the method of Archibald (1944). Routine assay of CPSase was done with cell-free extracts from cells (mid-exponential phase culture) broken in the cold by ultrasonic treatment in the presence of acid-washed glass beads (0.12 mm diam.). The extracts were centrifuged at 15 000 g and filtered at 4 °C just before enzyme assays through Sephadex G25 columns equilibrated with 0.1 M-TES(K) buffer (pH 7.5). Cyanate inhibition of CPSase was studied with a partially purified preparation (specific activity 6 U mg⁻¹) obtained from strain BGM 023 according to Anderson *et al.* (1970): the enzyme fraction obtained after step 6 was treated with solid ammonium sulphate to achieve 65% saturation and the precipitate was dissolved in 0.2 M-potassium phosphate buffer containing 0.5 mM-EDTA (pH 7.9). This enzyme preparation was filtered just before use through a Sephadex G25 column equilibrated with 100 mM-TES(K)/100 mM-KCl buffer (pH 7.5).

Ornithine carbamoyltransferase (OCTase; EC 2.1.3.3). Activity of OCTase in growing cells was routinely assayed on samples of cultures treated with toluene for 20 min at 37 °C. Activity of OCTase was measured at 37 °C in a medium (final volume 0.5 ml) containing 100 mM-Tris/HCl buffer (pH 8), 10 mM dilithium CP and 10 mM-L-ornithine. Citrulline was estimated by a modification of the method of Archibald (1944). For the kinetic studies, the assay medium contained, in a volume of 0.95 ml, various amounts of L-ornithine and dilithium CP in a buffer composed of 50 mM-Tris/acetate and 50 mM-KCl (pH 8). KNCO and zinc acetate solutions were prepared in the same buffer. Test tubes were equilibrated at 37 °C and the reaction was initiated by adding 50 µl of an enzyme preparation diluted in Tris/acetate buffer. The reaction was stopped with 1 ml of the acid mixture described by Prescott & Jones (1969) and citrulline was estimated according to the same authors, following the optimization procedure of Pastra-Landis *et al.* (1981). In order to minimize the dissociation of CP, frozen stock solutions were diluted in cold buffer 1 min before addition to assay mixtures and CP was added 2 min before addition of enzyme preparation. Steady-state conditions were observed during the kinetic measurements and enzymically synthesized citrulline did not exceed 10% of the limiting substrate. The solutions were mixed in the following order: buffer, ornithine, zinc acetate, CP and enzyme solution, because incubation of enzyme preparations with zinc in the absence of substrates led to a time-dependent decrease of activity. Citrulline formation in the presence of cyanate was corrected for the spontaneous carbamoylation of ornithine by using blanks without enzyme.

A partially purified preparation of OCTase was used as auxiliary enzyme in the assay of CPSase. OCTase from

Table 1. *E. coli* strains

Strain	Genotype	Source
R5	Hfr <i>lacY mal mtl thi</i>	J. H. Miller
CSH 73 (<i>carB8</i>)	HfrH $\Delta(\textit{ara-leu})$ <i>carB8 cnt</i> $\Delta\textit{lac}$ $\Delta(\textit{ppc-argECBH})$ <i>argR11 thi</i>	N. Glansdorff
58161/pMC40	HfrP4X <i>thr carB8 leu met</i> <i>recA/pMC40 carAB</i> ⁺	A. Piérard
BGM 023	as R5 but <i>cnt3</i>	This work
BGM 167	as R5 but <i>thr cnt3</i>	
BGM 171	as R5 but <i>cnt3 argR</i>	
BGM 180	as R5 but <i>carB8 cnt3</i>	
BGM 186	as R5 but <i>carB8 cnt3/pMC40 carAB</i> ⁺	
BGM 209	as R5 but <i>carB8 cnt3</i> ; citrulline-utilizing strain	

strain CSH 73 (*argR carB8*) (Lissens *et al.*, 1980) was purified according to Legrain & Stalon (1976); the preparation consisted of the 40–90% saturation ammonium sulphate precipitate dissolved in 50 mM-potassium phosphate buffer containing 0.5 mM-MgEDTA (pH 7.5) and dialysed overnight against the same buffer. Such preparations contained at least 30000 units ml⁻¹ and were free of CPSase activity.

Aspartate carbamoyltransferase (ACTase; EC 2.1.3.2). ACTase activity was estimated according to Gerhart & Pardee (1962). Carbamoylaspartate values were corrected in each assay for the spontaneous carbamoylation of aspartate by cyanate introduced in the assay medium or originating from dissociation of CP.

Enzyme units. One unit of enzyme activity is the amount of enzyme which catalyses the formation of 1 μ mol product h⁻¹. Specific activities are expressed as units (mg protein)⁻¹. Protein in cell extracts was estimated by the biuret method; protein in cell cultures was estimated from OD₅₇₅ measurements (an OD₅₇₅ of 0.5 corresponded to 0.17 mg protein ml⁻¹).

RESULTS

Nature of the metabolic step blocked by cyanate

We observed that growth of *E. coli* was inhibited by cyanate more strongly in minimal medium than in L broth (see the preceding paper: Guilloton & Karst, 1987). This different behaviour could be due to the presence in L broth of molecules which specifically prevent cyanate inhibition. In a preliminary study we analysed the effects of various metabolites (amino acids, purines and pyrimidines) on this growth inhibition on solid minimal medium supplemented with cyanate. We found that growth was restored specifically by arginine.

Fig. 1 shows that arginine allowed strain R5 to grow at a fairly normal rate after addition of 2 mM-KNCO; this concentration of cyanate strongly inhibits cell growth and cyanase synthesis in non-supplemented minimal medium (Guilloton & Karst, 1987). Arginine could be replaced by citrulline but not by ornithine, *N*- α -acetylornithine or *N*-acetylglutamate. By means of automated amino acid analysis we observed that the growth inhibition effected by 2 mM-cyanate induced a very large increase in the cellular ornithine pool. Cyanate inhibition was thus suspected to be due to a specific inhibition of the formation of citrulline from ornithine. However, OCTase activity was found to be insensitive to cyanate *in vitro* (see below). The effect of cyanate on the physiology of *E. coli* had therefore to be understood on the basis of other metabolic interferences such as a decreased availability of CP.

The production of CP for the arginine and pyrimidine biosynthetic pathways in *E. coli* is achieved by a single CPSase (Fig. 2). This enzyme is encoded by the two adjacent genes, *carA* and *carB* (Mergeay *et al.*, 1974), which form an operon. Although mutations in the *carAB* locus generally lead to a double auxotrophy for arginine and uracil, some CPSase mutants behave as arginine bradytrophs (Mergeay *et al.*, 1974) or auxotrophs (Bolivar *et al.*, 1976). Such mutants have also been described in *E. coli* B/r (Abd el al *et al.*, 1969) and in *Salmonella typhimurium* (Abd el al *et al.*, 1978). It was also reported that arginine bradytrophism could be a consequence of mutations in genes whose expression is required for CPSase synthesis (Bussey & Ingraham, 1982; Neuhard *et al.*, 1982). On the other hand CPSase from *E. coli* can be inhibited *in vitro* by

Fig. 1. Effect of 2 mM-cyanate on growth of strain R5, and influence of arginine and intermediates of arginine biosynthesis. Overnight cultures in minimal medium were subcultured as described in Methods. KNCO (final concn 2 mM) and metabolites (final concn $20 \mu\text{g ml}^{-1}$) were added to the cultures when the OD_{575} reached 0.2. Arg, arginine hydrochloride; Cit, citrulline; Orn, ornithine hydrochloride; NAO, *N*- α -acetylornithine; NAG, *N*-acetylglutamic acid. C1 and C2 are controls which received no cyanate and 2 mM-cyanate, respectively. The cultures that received Arg and Cit showed a lag of 30 min during which cyanate was completely hydrolysed; added cyanate was not hydrolysed in the other cultures.

Fig. 2. Biosynthetic pathways of arginine and pyrimidines in *E. coli*. The relevant genes and enzymes are shown. The enzymes are identified by the genetic symbols of the corresponding structural genes as follows: *carAB*, carbamoylphosphate synthase (EC 6.3.5.5); *argI,F*, ornithine carbamoyltransferase (EC 2.1.3.3); *pyrB*, aspartate carbamoyltransferase (EC 2.1.3.2); *upp*, UMP pyrophosphorylase (EC 2.4.2.9).

cyanate (Anderson *et al.*, 1973). Cyanate reacts specifically with a SH group required for glutamine binding and deamidation (Anderson & Carlson, 1975). It was thus of interest to investigate the interaction of cyanate with arginine and pyrimidine biosynthetic pathways.

Effect of cyanate, arginine and uracil on the physiology of strain BGM 023

Mutant strain BGM 023 (*cnt3*) was chosen to study the effect of cyanate on cell physiology. This strain (derived from strain R5) lacks inducible cyanase and thus does not hydrolyse added cyanate. Fig. 3 shows the effects of cyanate (1 and 2 mM) on growth of strain BGM 023 and its

Fig. 3. Effect of cyanate, arginine and uracil on growth of strain BGM 023. An overnight culture in minimal medium was diluted in the same medium and cultured as described in Methods. KNCO (1 mM, *a*, or 2 mM, *b*), with or without arginine ($100 \mu\text{g ml}^{-1}$) and/or uracil ($50 \mu\text{g ml}^{-1}$) was added to the cultures when the OD_{575} reached 0.2 (arrows). OD_{575} was monitored for a further 150 min. The corresponding doubling times are shown in Table 3.

interaction with arginine ($100 \mu\text{g ml}^{-1}$) and uracil ($50 \mu\text{g ml}^{-1}$). The doubling time (*g*) of strain BGM 023 in minimal medium was 54–55 min. Addition of 1 mM-KNCO to exponentially growing cultures moderately inhibited growth (Fig. 3*a*). After transient inhibition a stable *g* value of 130 min could be observed. Arginine partially abolished cyanate inhibition (*g* = 78 min). Uracil induced a progressive enhancement of inhibition; 30 min after addition of uracil and cyanate a stable *g* value of 300 min could be observed. Addition of both uracil and arginine virtually abolished cyanate inhibition (*g* = 66 min). In the presence of 2 mM-KNCO, growth was strongly inhibited (*g* = 429 min) (Fig. 3*b*). A partial restoration of growth could be obtained with arginine (*g* = 114 min). Addition of both arginine and uracil allowed cells to grow at a higher rate (*g* = 85 min). Uracil alone had little effect (*g* = 437 min).

These results confirm that arginine reactivates cyanate-inhibited growth. Addition of uracil resulted in a rather complex set of interactions, depending on the concentration of cyanate. Moderate inhibition of growth by 1 mM-KNCO was enhanced by uracil, but addition of both uracil and arginine resulted in a stronger reactivation of growth than did addition of arginine alone. These effects could be interpreted as the consequences of an inhibition by cyanate of both arginine and pyrimidine biosynthesis at the level of CPSase. Uracil added to cultures of *E. coli* is readily converted by UMP pyrophosphorylase (O'Donovan & Neuhaud, 1970; Piérard *et al.*, 1972) into UMP, which effects feedback inhibition of CPSase (Piérard *et al.*, 1965). Since UMP binds to the heavy subunit of CPSase (Trotta *et al.*, 1971) and cyanate reacts with the light subunit (Anderson & Carlson, 1975), a cumulative effect of UMP and cyanate on CPSase could result in a stronger inhibition of CP formation and in a shortage of CP for arginine biosynthesis.

Derepression of ACTase, OCTase and CPSase in the presence of 1 mM cyanate

Further evidence supporting the above hypothesis was obtained by estimation of these enzyme activities after addition of 1 mM-KNCO to exponentially growing cultures of BGM 023. Addition of 1 mM-KNCO resulted in derepression of OCTase and ACTase: 2 h after the

Fig. 4. Derepression of OCTase and ACTase induced by 1 mM-KNCO in strain BGM 023. An overnight culture was subcultured in minimal medium as described in Methods, and 1 mM-KNCO was added when the OD₅₇₅ reached 0.2 (arrow). Samples were withdrawn at various times, treated with toluene for 20 min at 37 °C, and OCTase and ACTase specific activities were measured as described in Methods. The specific activities are expressed relative to the values at the beginning of the experiment. The specific activities of OCTase and ACTase were initially 80 and 6.5 units mg⁻¹, respectively; they had 600 and 110 units mg⁻¹, respectively, at the end of the experiment. ▲, OCTase; △, ACTase; □, OD₅₇₅.

Table 2. Specific activities [$\mu\text{mol h}^{-1} (\text{mg protein})^{-1}$] of OCTase, ACTase and CPSase in strain BGM 023 and derivatives

Strain (relevant genotype)	OCTase	CPSase	ACTase
BGM 023 (<i>cnt3</i>)	80	0.65	6.5
BGM 171 (<i>cnt3 argR</i>)	1520	1.50	ND
BGM 186 (<i>cnt3 carB8/pMC40 carAB</i> ⁺)	150	8.35	ND
BGM 209 (<i>cnt3 carB8</i>)(Cit ^{wt})	720	ND	135

ND, Not determined.

addition the specific activities had increased by factors of 7.5 and 17, respectively (Fig. 4). The assay of CPSase showed that this enzyme was also subject to derepression (1.3 units mg⁻¹ vs 0.65 units mg⁻¹ 1 h after cyanate addition). These results show that both pyrimidine and arginine biosynthetic pathways are inhibited by cyanate.

Decreased arginine requirement produced by argR mutation or CPSase amplification

Since the arginine requirement of strain BGM 023 in the presence of cyanate was probably caused by a decreased availability of CP for the reaction catalysed by OCTase, enhancement of CPSase or OCTase might reduce the cyanate inhibition of growth. In order to obtain isogenic strains with enhanced CPSase or OCTase activities, strain BGM 023 was genetically modified as described in Methods. Strain BGM 171 is an *argR* mutant of BGM 023 and strain BGM 186 is a *carB8* derivative of BGM 023 harbouring plasmid pMC40 (*carAB*⁺). Specific activities of OCTase and CPSase in these strains are presented in Table 2. It should be noted that *argR* mutation derepresses OCTase and, to a lesser extent, CPSase. This derepression can be accounted for by the participation of the arginine repressor in the repression of CPSase in wild-type strains (Piérard *et al.*, 1976). On the other hand, amplification of CPSase in strain

Table 3. *Effect of cyanate, arginine and uracil on generation times of strain BGM 023 and derivatives*

Overnight cultures in minimal medium were subcultured and diluted as described in Methods. KNCO (1 mM, *a*, or 2 mM, *b*), with or without arginine (100 µg ml⁻¹) and/or uracil (50 µg ml⁻¹) were added when the OD₅₇₅ reached 0.2. The table shows the generation times of the different strains calculated from OD₅₇₅. The values in parentheses show the generation times expressed relative to the generation time of a control culture without additions. See also Fig. 3.

Strain (relevant genotype)	Generation time (min)				
	None	KNCO	Addition(s):		
			KNCO + Arg	KNCO + Ura	KNCO + Arg + Ura
			(a) 1 mM-KNCO		
BGM 023 (<i>cnt3</i>)	54 (1)	130 (2.4)	78 (1.4)	300 (5.5)	66 (1.2)
BGM 171 (<i>cnt3 argR</i>)	60 (1)	107 (1.8)	78 (1.3)	108 (1.8)	73 (1.2)
BGM 186 (<i>cnt3 carB8</i> / pMC40 <i>carAB</i> ⁺)	67 (1)	98 (1.5)	87 (1.3)	96 (1.4)	84 (1.3)
			(b) 2 mM-KNCO		
BGM 023 (<i>cnt3</i>)	55 (1)	429 (7.8)	114 (2.1)	437 (7.9)	85 (1.6)
BGM 171 (<i>cnt3 argR</i>)	63 (1)	248 (3.9)	130 (2.1)	160 (2.5)	106 (1.7)
BGM 186 (<i>cnt3 carB8</i> / pMC40 <i>carAB</i> ⁺)	62 (1)	145 (2.3)	123 (2.0)	135 (2.2)	107 (1.7)
BGM 209 (<i>cnt3 carB8</i>) (Cit ^{ut})	64 (1)	101 (1.6)	ND	104 (1.6)	ND

ND, Not determined.

BGM 186 harbouring plasmid pMC40 (*carAB*⁺) slightly derepresses OCTase. Growth of these modified strains was tested in the presence of 1 mM- and 2 mM-KNCO and the influence of arginine and uracil was determined (Table 3). Firstly, both strains showed reduced sensitivity to cyanate. Secondly, the uracil sensitivity induced by 1 mM-KNCO in strain BGM 023 was no longer obtained in its isogenic derivatives BGM 171 and BGM 186. Some differences between these two strains can be pointed out. Uracil induced a marked growth reactivation of strain BGM 171 (*argR*) in the presence of 2 mM-KNCO; this behaviour may be interpreted as the consequence of a cyanate inhibition of pyrimidine biosynthesis. On the other hand strain BGM 186 was less sensitive to cyanate than strain BGM 171; thus it can be assumed that amplification of CPSase is more efficient than derepression of arginine biosynthesis in the reduction of cyanate sensitivity. Hence these results are in agreement with a specific inhibition of CPSase by cyanate *in vivo*.

Absence of specific cyanate inhibition of pyrimidine biosynthesis in a mutant using citrulline as a source of CP

carB8 strains are double auxotrophs for arginine and uracil. They possess no detectable CPSase activity (Mergeay *et al.*, 1974) since they bear the extensive *carB8* deletion which represents approximately 75% of the *carB* gene (Crabeel *et al.*, 1980). Legrain *et al.* (1976) described the isolation of *carB8* strains able to grow on citrulline-supplemented medium. In such mutant strains CP is produced by phosphorolysis of citrulline, the reverse reaction of OCTase. Citrulline-utilizing strains show derepressed levels of OCTase and belong to various classes of mutants: *argG* bradytrophs or strains in which synthesis of OCTase is specifically derepressed (Legrain *et al.*, 1976). Strain BGM 180 (*carB8 cnt3*) was plated on minimal medium supplemented with L-citrulline (2 mg ml⁻¹); spontaneous citrulline-utilizing mutants appeared at a frequency of 10⁻⁷. Specific activity of OCTase and ACTase in one of these mutants, BGM 209, was measured; Table 2 shows that the two enzyme activities were derepressed by factors of 9 and 21, respectively. The effect of 2 mM-KNCO on the growth rate of BGM 209 was then tested. The results (Table 3*b*) showed that this strain has reduced sensitivity to cyanate and that the generation time is identical in the presence of 2 mM-KNCO or 2 mM-KNCO + uracil

Fig. 5. Inhibition of CPSase by cyanate. A partially purified enzyme preparation from strain BGM 023 was used. The incubation medium was as described in Methods except that glutamine and ATP concentrations were 0.5 mM and 6 mM, respectively. The reaction was initiated by addition of partially purified CPSase and stopped by TCA. Citrulline formed by coupling CPSase with added OCTase preparation was estimated by a modification of the method of Archibald (1944). ●, Control without cyanate; ○, 0.5 mM-KNCO; □, 1 mM-KNCO; △, 2 mM-KNCO.

(50 $\mu\text{g ml}^{-1}$). This result suggests very strongly that the growth inhibition induced by cyanate in other strains is the consequence of an inhibition of CPSase.

In vitro inhibition of CPSase activity by cyanate

CPSase inhibition has been observed with 9 mM-KNCO (67% inhibition) and 18 mM-KNCO (86% inhibition) in the presence of 5 mM-glutamine (Anderson & Carlson, 1975). Since these concentrations of cyanate are far above those used in this study it was of interest to know whether CPSase activity could be inhibited by cyanate in the 2 mM range. The effect of cyanate on CPSase activity was tested in the presence of 0.5 mM-glutamine, a concentration near the K_m value (Piérard & Wiame, 1964) (Fig. 5). Cyanate inhibition proceeded gradually: 2 mM-KNCO inhibited CPSase by 50% within 3 min. The relatively long-lasting pre-steady-state of cyanate inhibition is caused by the slowness of carbamoyl-CPSase formation and dissociation compared with the rapid equilibrium characterizing the glutamine-CPSase complex (Anderson & Carlson, 1975). It is noteworthy that CPSase activity was assayed in the presence of 10 mM-ornithine. Thus, the presence of this allosteric activator does not prevent CPSase inhibition.

Kinetics of OCTase in the presence of cyanate

The results presented in this study are consistent with CPSase inhibition by cyanate. However, they do not exclude the possibility of an inhibition of citrulline formation by OCTase as well. The saturation kinetics of OCTase activity in a cell-free extract of BGM 023 was followed as a function of ornithine in the absence or presence of 2 mM-KNCO and in the presence of 0.1 mM-CP (non-saturating concentration). Cyanate did not modify the saturation of the enzyme by ornithine. This study was repeated in the presence of a saturating concentration of CP (2 mM); again no cyanate inhibition was observed.

It has been reported that zinc(II) inhibits OCTase activity and that saturation kinetics as a function of ornithine exhibits a positive cooperativity (Kuo *et al.*, 1982). The above kinetic experiments were repeated in the presence of 0.4 mM-zinc acetate (see Methods). Zinc did inhibit OCTase activity but, in our hands, the saturation kinetics remained strictly Michaelian; graphical analysis indicated a competition between zinc and ornithine. Cyanate (2 mM) did not afford an additional inhibition.

Identical kinetic results were obtained with a partially purified preparation of OCTase [specific activity 12500 units (mg protein)⁻¹] obtained from strain CSH 73 (*carB8 argR*). Thus it seems unlikely that OCTase is inhibited by cyanate *in vivo*.

DISCUSSION

The results presented here are consistent with a specific inhibition of CPSase by cyanate added to cultures of *E. coli*; this inhibition leads to an inhibition of arginine biosynthesis. Since cyanate-mediated inhibition leads to an arginine requirement, it is likely that a shortage of CP is more critical for arginine than for pyrimidine biosynthesis.

Effects of cyanate on the kinetic properties of another enzyme of CP metabolism have been reported. Nelbach (1972) found that prolonged incubation of purified ACTase with high concentrations of CP led to a carbamoylated enzyme, still active but no longer regulated by CTP or ATP. She also reported that carbamoylation was effected by cyanate originating from dissociation of CP. We investigated this enzyme modification since a desensitization of ACTase might induce a metabolic deviation of CP towards pyrimidine biosynthesis and thus lead to a reduced availability of CP for arginine biosynthesis. A partially purified preparation of ACTase from BGM 023 was incubated for 30 min at 37 °C with various concentrations of cyanate. Incubation with cyanate caused a slight decrease in $K_{0.5}$ for aspartate, and also decreased the allosteric activation by ATP and inhibition by CTP. Nonetheless these effects were obtained with relatively high concentrations of cyanate (e.g. 10 mM). Therefore it seems unlikely that such a modification of the kinetic properties of ACTase can actually induce by itself the observed requirement for arginine or citrulline.

The growth of *Lactobacillus arabinosus* and *Streptococcus lactis* is inhibited by carbamoyl compounds such as CP (Ravel *et al.*, 1957) and S-carbamoylcysteine (Ravel *et al.*, 1958); this growth inhibition can be reversed by arginine and citrulline but not by ornithine. CP and carbamoylcysteine are unstable compounds which dissociate readily in dilute solutions; the half-life of CP is 38 min at 37 °C (Allen & Jones, 1964) and this compound dissociates into inorganic phosphate and cyanate. Carbamoylcysteine is unstable at pH >6 and dissociates into cysteine and cyanate with a half-life of approximately 92 min at 30 °C (Stark, 1964). Besides a specific action of CP or carbamoyl-cysteine themselves on the micro-organisms, an action of cyanate originating from dissociation of these compounds was therefore highly probable. The long duration of these experiments (27 h) allowed a complete dissociation of these carbamoyl compounds, as discussed by Stark (1964) in the case of carbamoylcysteine. It is thus likely that in *L. arabinosus* and *S. lactis*, as in *E. coli*, CPSase is the specific target of cyanate.

Taking the above results into account, we propose that endogenous cyanate, arising from spontaneous dissociation of carbamoyl compounds, could participate in CPSase regulation. Among the carbamoyl compounds encountered in the metabolism of *E. coli*, citrulline and carbamoylaspartate are stable molecules, and urea (synthesized during growth of *E. coli*: Morris & Koffron, 1967) dissociates very slowly and to a limited extent (Marier & Rose, 1964). Thus CP is probably the only carbamoyl compound which can give rise to endogenous cyanate. The existence of a pool of CP implies cyanate formation *in vivo*. On the other hand our observations showed that CP biosynthesis is a specific target of cyanate. We propose that CPSase is subject *in vivo* to a 'by-product' regulation mechanism in which cyanate originating from the dissociation of CP exerts a reversible inhibition of the enzyme. This inhibition would be essentially effective in the regulation of arginine biosynthesis. This regulation mechanism would provide the cell with an energy-saving device depending on cellular CP level, which would modulate the allosteric regulation of CPSase by UMP (inhibitor) (Piérard *et al.*, 1965), ornithine (antagonistic activator) (Piérard, 1966), and purine nucleotides (allosteric activators) (Anderson & Meister, 1966). Direct evidence for the proposed mechanism will require a reliable and sensitive assay of endogenous cyanate in bacteria.

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