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Ring cleavage and degradative pathway of cyanuric acid in bacteria

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1. The degradative pathway of cyanuric acid [1,3,5-triazine-2,4,6(1*H*,3*H*,5*H*)-trione] was examined in *Pseudomonas* sp. strain D. 2. The bacterium grew with cyanuric acid, biuret, urea or NH_4^+ as sole source of nitrogen, and each substrate was entirely metabolized concomitantly with growth. 3. Enzymes from strain D were separated by chromatography on DEAE-cellulose and three reactions were examined. 4. Cyanuric acid (1 mol) was converted stoichiometrically into 1.0 mol of CO_2 and 1.1 mol of biuret, which was conclusively identified. 5. Biuret (1 mol) was converted stoichiometrically into 1.1 mol of NH_4^+, about 1 mol of CO_2 and 1.0 mol of urea, which was conclusively identified. 6. Urea (1 mol) was converted into 1.9 mol of NH_4^+ and 1.0 mol of CO_2. 7. The reactions proceeded under aerobic or anoxic conditions and were presumed to be hydrolytic. 8. Data indicate that the same pathway occurred in another pseudomonad and a strain of *Klebsiella pneumoniae*.

INTRODUCTION

Three defined degradative pathways, one each for three groups of *s*-triazines, converge at, or are funnelled to, cyanuric acid (Jutzi *et al.*, 1982; Cook & Hütter, 1984; Cook *et al.*, 1984; see also Grossenbacher *et al.*, 1984). Cyanuric acid is known to be utilized as the sole and growth-limiting source of nitrogen by bacteria (Cook & Hütter, 1981*a*), and, more slowly, by fungi (e.g. Zeyer *et al.*, 1981). Ring nitrogen is released quantitatively as NH_4^+ in a bacterium (Cook & Hütter, 1981*a*), and the fate of the ring carbon is CO_2 in bacteria, fungi and anaerobic sludge (Cook & Hütter, 1981*a*; Zeyer *et al.*, 1981; Saldick, 1974).

We now present qualitative and quantitative evidence for the degradative pathway of cyanuric acid in bacteria. Parts of this work have been presented in preliminary form (Beilstein & Hütter, 1980; Cook *et al.*, 1983*b*).

EXPERIMENTAL

Materials

Cyanuric acid, biuret and urea (purity > 99%) were obtained from Fluka (Buchs, Switzerland). Each contained negligible NH_4^+. Cyanuric acid and urea were chromatographically pure; biuret was observed to contain ammelide (0.15%). The mass spectra of cyanuric acid and biuret confirmed the identity of the material and 1 mol of urea yielded 2.0 mol of NH_4^+ when treated with urease. [U-^{14}C]Cyanuric acid (107 GBq/mol) was provided by Ciba–Geigy (Basel, Switzerland), and [*carbonyl*-^{14}C]allophanic acid (1.1 TBq/mol) was a gift from Dr. Y. Lemoine (Laboratoire de Microbiologie, Université Libre de Bruxelles, Bruxelles, Belgium). T.l.c. plates (Kieselgel 60 F-254) were from Merck (Darmstadt, Germany) and autoradiography was done using Kodirex X-ray film (Kodak, Rochester, NY, U.S.A.). Jack-bean urease (EC 3.5.1.5; 0.9 mkat/kg of freeze-dried material) was purchased from Boehringer (Mannheim, Germany). Other materials have been described elsewhere (Jutzi *et al.*, 1982) or were the best quality available commercially.

Apparatus, analyses and identification of metabolites

Spectrophotometry and experiments under anaerobic conditions (Jutzi *et al.*, 1982), h.p.l.c. (Beilstein *et al.*, 1981; Grossenbacher *et al.*, 1985) and mass spectrometry (Cook & Hütter, 1981*a*) were done with apparatus described previously. Warburg manometry was done with thermostatically controlled (V 85; Braun, Melsungen, Germany) single- or double-side-arm flasks (about 15 ml).

Cyanuric acid or biuret in neutral solution was determined by reversed-phase h.p.l.c. as previously described (Beilstein *et al.*, 1981; Cook *et al.*, 1983*a*; Grossenbacher *et al.*, 1985). Biuret was identified by mass spectrometry. Urea was routinely quantified by the diacetyl mono-oxime method of Wilding & Blanton (1982), with an essentially linear standard curve in the range 0–200 nmol/assay. The identity of urea as a metabolic product was confirmed by the specific reaction with urease (Gutmann & Bergmeyer, 1974) and by co-chromatography (h.p.l.c.) with authentic material (Grossenbacher *et al.*, 1985). Urea was also detected by t.l.c. after separation on silica gel with a mobile phase of ethyl acetate/acetic acid/methanol/water (5:1:1:1, by vol.). NH_4^+ was measured by the Berthelot reaction (Weatherburn, 1967). Protein was assayed by the method of Kennedy & Fewson (1968) as described by Cook & Hütter (1981*a*). CO_2 was determined manometrically at 30 °C with double-arm Warburg flasks after acidification of the reaction mixture (Umbreit *et al.*, 1972); the flasks and manometers were calibrated by release of CO_2 from authentic urea by urease, and $NaHCO_3$ solution was calibrated in the double-arm flasks and used to calibrate single-arm flasks in which oxygen consumption was measured (Umbreit *et al.*, 1972).

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Organisms and growth conditions

Three strains of bacteria were used (Cook & Hütter, 1981*a*): two strains of *Pseudomonas* (A and D, deposited as N.R.R.L. B-12227 and B-12228 respectively) and *Klebsiella pneumoniae* strain 99.

Cyanuric acid, biuret, urea or NH_4^+ was the sole source of combined nitrogen (about 2.5 mM-N) for growth in the mineral-salts medium described by Cook & Hütter (1981*a*). Strains A and D (or 99) were supplied with 10 mM-lactate (glucose) as carbon source. Cells were grown for kinetic experiments or to produce cell material and stored frozen as described by Jutzi *et al.* (1982).

Cell-free extracts and enzyme assays

Stored cells were thawed, suspended and disrupted in a French pressure cell as previously described (Jutzi *et al.*, 1982). Disrupted cells were centrifuged, and the supernatant fluid was used for enzyme assays directly or after column chromatography; treatment of the extract with Sephadex G-25 had a negligible effect on enzyme activity. None of the enzyme assays was optimized.

The rate of enzymic degradation of cyanuric acid, biuret or urea was measured as substrate disappearance in discontinuous assays (Jutzi *et al.*, 1982). Many variants of this technique were used. Warburg manometry was done with a total fluid volume of 3.0 ml. Anaerobic reactions were done as described by Cook *et al.* (1984). Preliminary location of enzyme activities in eluates from DEAE-cellulose columns was done in several ways: degradation of cyanuric acid was assayed directly after working-up reactions stopped with $HClO_4$ (Jutzi *et al.*, 1982); degradation of biuret or urea was monitored by release of NH_4^+ in reactions stopped with trichloroacetic acid (Cook *et al.*, 1984). When the lability of biuret degradation was recognized, the preliminary location was accelerated by examining fractions during chromatography and stopping 30 min reactions by removal of protein in Centriflo filters (M_r cut-off, 25000; Amicon, Lexington, MA, U.S.A.) according to the manufacturer's instructions. Precise location of enzyme activity after chromatography on DEAE-cellulose was by substrate disappearance as described by Jutzi *et al.* (1982). Allophanate amidohydrolase [a component of the urea carboxylase (EC 6.3.4.6) complex] was assayed as described by Whitney & Cooper (1972).

Separation of enzyme activities by column chromatography

DEAE-cellulose columns (35 cm × 2.5 cm) were used to separate the enzymes degrading cyanuric acid from other reactions in the pathway; the supernatant fluid (about 500 mg of protein in 20 ml) from centrifugation of crude extract at 110000 × *g* (1.5 h at 4 °C) was applied to the column (Jutzi *et al.*, 1982). Only in strain D, however, was it possible to elute reproducibly enzymes degrading biuret and urea, and this experiment required special conditions because the enzyme degrading biuret was labile. Whereas degradation of cyanuric acid or urea in crude extract was stable at 4 °C for many days, loss of degradation of biuret (in 6 days) could be decreased to 25% (control, 75%) only with 1.5 M-glycerol (or 10 mM-dithiothreitol or large amounts of bovine serum albumin); no better buffers were observed. Tris inactivated the enzyme, and the enzyme was essentially inactive in the 'biuretase' assay described by Nishihara *et al.* (1965). The presence of DEAE-cellulose slowly inactivated the enzyme, and this inactivation accelerated markedly on addition of dithiothreitol. Thus, in order to elute the enzyme degrading biuret, we used a short DEAE-cellulose column (9 cm × 2.5 cm), a large amount of cell extract (about 800 mg of protein) and a steep gradient (10–500 mM-potassium phosphate, pH 7.3; 250 ml total volume) without washing the column with starter buffer after loading the column.

Isolation of biuret as a product from cyanuric acid

The enzyme that degraded cyanuric acid was separated from other pathway enzymes on a large DEAE-cellulose column (as described above), and the most active portion (20 ml) was dialysed against 10 mM-potassium phosphate buffer, pH 7.3, containing 0.25 mM-$MgSO_4$ in order to decrease the ionic strength. The dialysis sac was then suspended in 100 ml of dialysis buffer containing 5 mM-cyanuric acid at 30 °C. When the reaction was 98% complete, the dialysis sac and contents were discarded. The solution of reaction product was evaporated to dryness and the product, biuret, recrystallized from ethanol and examined.

The lability of the enzyme degrading biuret and the slight contamination with urease prevented recovery of large amounts of urea from biuret.

RESULTS

Growth experiments

Cyanuric acid served as sole and limiting source of nitrogen for each of the three bacteria under study: two strains of *Pseudomonas* and one strain of *Klebsiella pneumoniae*. *Pseudomonas* strain D utilized cyanuric acid concomitantly with growth (Fig. 1), similar to the behaviour of *Pseudomonas* strain A (Jutzi *et al.*, 1982). In contrast, *Klebsiella pneumoniae* strain 99 grew biphasically: cyanuric acid was exhausted before the end of growth and a transient intermediate was observed, which was then utilized (Fig. 1). In all cases cyanuric acid was completely removed from the growth medium, and the molar growth yield, compared with the appropriate control utilizing NH_4^+, showed that all the *s*-triazine nitrogen had been incorporated into cell material (Table 1). In control experiments, cyanuric acid was stable in sterile growth medium and the organisms did not grow in the absence of a source of combined nitrogen.

The transient intermediate, observed in cultures of strain 99, was tentatively identified as biuret by co-chromatography and by u.v. spectrophotometry [$\lambda_{max.}$ = 216 nm at pH 13, in agreement with published data (Weast, 1977]; the spectrum of biuret and of the unknown showed the same blue shift with rising pH. A further preliminary experiment, done with a cell-free extract of strain 99, [U-^{14}C]cyanuric acid and including 500 mM-urea to dilute any radioactive urea, led to the detection by t.l.c. of a spot of radioactivity with the R_F of urea (Beilstein, 1982). We thus hypothesized that the degradative pathway involved biuret and urea as intermediates, and these substrates were tested as nitrogen sources for growth (Table 1). Whereas all strains could utilize urea quantitatively as a nitrogen source, only strain D could utilize biuret.

Complementary experiments with suspensions of non-growing cells showed that only strain D could

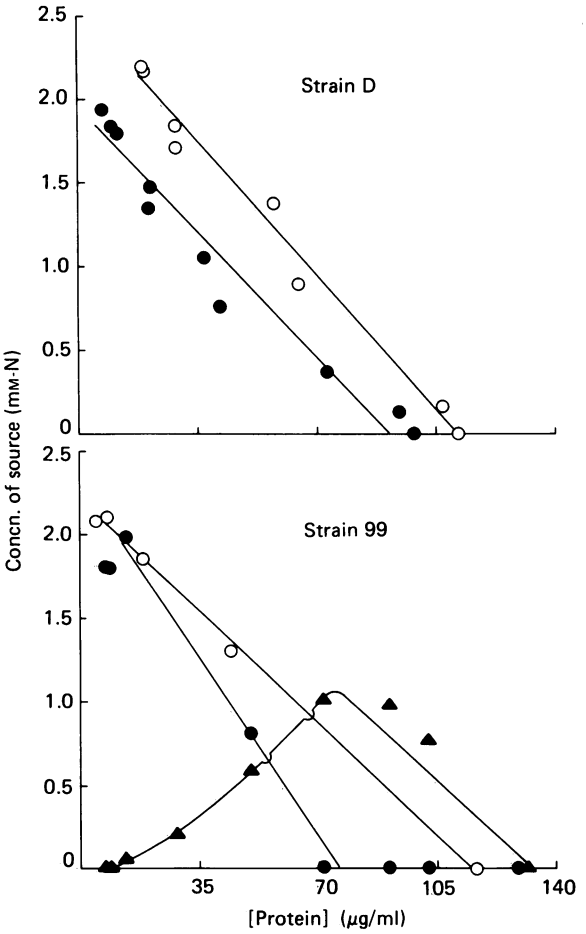

Fig. 1. Disappearance of cyanuric acid as a function of growth of *Pseudomonas* strain D and *Klebsiella pneumoniae* strain 99

A 60 ml culture of strain D or 99 was grown with cyanuric acid as the sole added source of nitrogen and harvested aseptically (23000 *g* at 4 °C for 10 min) during the growth phase. The pellet was suspended in cold sterile 10 mM-potassium phosphate buffer, pH 7.2, which contained 0.25 mM-$MgSO_4$ and was used immediately as inoculum for 300 ml cultures. Samples were taken at intervals to determine the concentrations of protein and nitrogen source. Control experiments: ○, NH_4^+; ●, cyanuric acid; ▲, biuret.

convert cyanuric acid, biuret and urea quantitatively into NH_4^+. Strains A and 99 could convert urea quantitatively into NH_4^+ and catalyse the complete disappearance of cyanuric acid to yield biuret and traces of NH_4^+, but these strains usually caused only negligible ($\leq 5\%$) disappearance of biuret.

Enzyme activities

Crude extracts of strain D degraded cyanuric acid, biuret and urea quantitatively to NH_4^+, each with a specific activity of the same order of magnitude as that observed in whole cells (Table 2; cf. specific activities in Table 1). The same enzymic activities could be observed in strain 99 and in part in strain A (Table 2), but the degradation of biuret was not reproducibly measurable, which led to the almost stoichiometric accumulation of biuret from cyanuric acid in some reactions. We suspected that we had suboptimal conditions for the extraction and/or assay of biuret degradation in strains 99 and A and worked thereafter largely with strain D.

It was possible to obtain complete conversion of cyanuric acid, biuret and urea into NH_4^+ with extracts of strains D and 99 (Table 2). Crude extracts of strain D were studied and found to release CO_2 stoichiometrically from each substrate (Table 2). This manometric determination of CO_2 complemented the earlier tentative identification by radiorespirometry in whole cells (Cook & Hütter, 1981*a*).

The supernatant fluid from centrifugation of the crude extract at 110000 *g* could be fractionated on DEAE-cellulose columns, and in each case an enzyme degrading cyanuric acid was eluted. A chromatogram from strain

Table 1. Molar growth yields of three bacteria utilizing different nitrogen sources, with growth rates and calculated specific degradation rates

Strain	Substrate*	Growth yield (g of protein/mol of substrate)	Nitrogen utilized (mol/mol of substrate)	Specific growth rate (μ) (h^{-1})	Specific degradation rate (mkat/kg of protein)
D	NH_4^+	43	1	0.30	1.9
	Cyanuric acid	142	3	0.33	0.6
	Biuret†	122	3	0.26	0.6
	Urea	87	2	0.30	1.0
A	NH_4^+	51	1	0.50	2.7
	Cyanuric acid	156	3	0.28	0.5
	Biuret†	6	0	0	0
	Urea	102	2	0.53	1.4
99	NH_4^+	59	1	0.87	4.1‡
	Cyanuric acid	181	3	0.46	1.3
	Biuret†	6	0	0	0
	Urea	118	2	0.39	0.9

* No residual substrate was observed after growth.
† This material contained small amounts of cyanuric acid and ammelide.
‡ This value was calculated by using 100 g of protein/mol of cyanuric acid, the yield observed during the degradation of cyanuric acid, and before utilization of the excreted biuret.

Table 2. Inorganic products and specific rates of degradation in crude extracts of cyanuric acid and putative intermediates

The specific rates were obtained from initial rates in reaction mixtures with low protein concentrations. When stoichiometry was studied, reaction mixtures contained high protein concentrations: in all appropriate cases, substrate completely disappeared. Abbreviation used: na, not assayed.

Strain	Substrate (~ 1 mM)	Specific activity (mkat/kg of protein)	Inorganic product (mol/mol of substrate)	
			NH_4^+	CO_2
D	Cyanuric acid	10	3.0	3.2*
	Biuret	1.0	3.0	1.8
	Urea	0.2	1.9	1.0
A	Cyanuric acid	2.2	0.3	na
	Biuret	0	< 0.1	na
	Urea	1.0	2.1	0.9
99	Cyanuric acid	4.1	3.0	na
	Biuret	0.2	2.9	na
	Urea	11	2.2	1.0

* In a reaction with a fraction separated on a DEAE-cellulose column, 1.0 mol of CO_2/mol of substrate was observed.

D is shown in Fig. 2 [data for strain A (Jutzi *et al.*, 1982) and strain 99 (Beilstein, 1982) are given in the cited references; quantitative experiments with the cyanuric acid-cleaving enzyme from strain D were done with a better preparation (see the Experimental section)].

The pathway

The disappearance of cyanuric acid catalysed by the separated enzyme proceeded to completion without release of NH_4^+. When [U-^{14}C]cyanuric acid was the substrate and the reaction mixture was acidified after exhaustion of the cyanuric acid, 37% of the radioactivity was driven off and could be trapped in a solution of KOH (Beistein, 1982); we interpreted this to mean that one-third of the ring carbon was released as CO_2, and we then confirmed this observation manometrically (Table 2). The organic product of ring cleavage (of unlabelled substrate) was isolated and tentative identification as biuret (h.p.l.c. and u.v. spectrum) was confirmed by mass spectrometry (M^+, 103; M^+ – CONH, 60; $CONH^+$, 43). The reaction could thus be seen to yield 1.1 mol of biuret plus 1.0 mol of CO_2/mol of cyanuric acid.

The enzyme degrading biuret could be chromatographed routinely only with extract from strain D (Fig. 2), but even here, the activity was lost within a few hours. The enzyme yielded only urea as an organic product, which was identified by reaction with urease, with diacetyl mono-oxime and by co-chromatography with authentic material (h.p.l.c.). The reaction was stoichiometric, yielding 1.0 mol of urea and 1.1 mol of NH_4^+/mol of biuret. The second carbon atom from biuret was released as CO_2, as can be seen in the difference between formation of CO_2 from biuret and urea by strain D (Table 2).

All three strains under study were urease-positive in taxonomic tests (Cook & Hütter, 1981*a*). Crude extracts from each strain released about 2 mol of NH_4^+ and, where tested, 1 mol of CO_2/mol of urea (Table 2). The urease of strain D could be chromatographed on DEAE-cellulose columns (Fig. 2), in contrast with the enzyme from strain 99.

The reactions in the degradative sequence proceeded in the absence of molecular oxygen. Suspensions of strain

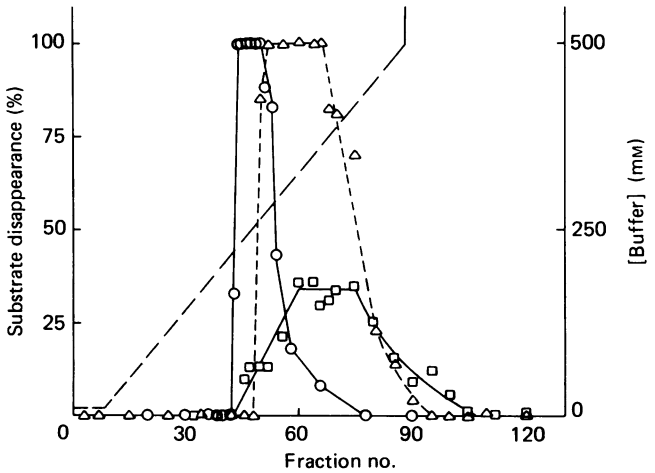

Fig. 2. Separation on DEAE-cellulose of three enzymes of cyanuric acid degradation from strain D

A crude extract of strain D, which had been grown with cyanuric acid as nitrogen source, was centrifuged to remove membrane particles and applied to the column as described in the Experimental section. Proteins were eluted by a gradient (——) of potassium phosphate buffer and 3 ml fractions were collected. Activity was measured as substrate disappearance: △, cyanuric acid; ○, biuret; □, urea.

D degraded cyanuric acid, biuret and urea quantitatively under anaerobic conditions (see also Jutzi *et al.*, 1982). In complementary aerobic manometric experiments, cell extracts did not utilize O_2 (above the control levels without substrate) while degrading cyanuric acid, biuret or urea.

The possibility that other intermediates might be involved has been examined. The enzyme cleaving cyanuric acid in strain D (a protein fraction from a DEAE-cellulose column) was used to study kinetically the reaction converting cyanuric acid into biuret. Samples taken at intervals were separated rapidly from protein, by

filtration on Centriflo filters, and measured immediately. The sum of [cyanuric acid] + [biuret] was constant (±5%) and no intermediate needed to be postulated. The possibility that allophanate would occur was thought unlikely, because the intermediate is known only in some urease-negative yeasts and algae (Dixon & Webb, 1979). All our strains contained urease (Table 2), and strain 99 contained negligible activities of allophanate amidohydrolase (10 μkat/kg of protein; cf. Table 2).

DISCUSSION

The overall products of cyanuric acid degradation were 3 mol of CO_2 plus 3 mol of NH_4^+/mol of substrate (Table 2). These data confirm the work of Cook & Hütter (1981*a*) in whole cells and confirm the tentative identification of CO_2. The organic intermediates have now been identified conclusively as biuret and urea, and the reactions have been shown to be hydrolytic. These data show the pathway in Fig. 3 to be present and confirm our working hypothesis (Cook & Hütter, 1981*b*).

The necessary enzymes are present in strain D, the reactions are all quantitative, there is essentially sufficient activity in cell extracts to explain the growth rate and there is physiological evidence that the pathway operates in growing cells. There may be other pathways of cyanuric acid degradation, but there seems no need to hypothesize them for strain D.

Presumably the same pathway is present in strains A and 99, but the data are less easily interpreted than in strain D. The failure of strains A and 99 to grow with biuret as a source of nitrogen, although the enzyme of biuret degradation was present [at least in strain 99 (Table 2)], could be explained as lack of a transport system: but crypticity does not explain how strain 99 utilized the biuret excreted during growth on cyanuric acid (Fig. 1). These problems (irreproducible degradation of, and presumed crypticity to, biuret) in strains A and 99 explain the incomplete carbon and nitrogen balances for the degradation of cyanuric acid reported by Cook & Hütter (1981*a*), when we could not determine biuret.

Each reaction in the pathway (Fig. 3) involves the hydrolysis of two carbonyl-carbon–nitrogen bonds. We believe all these reactions to be ureases assignable to EC 3.5.1.- and EC 3.5.2.-. Whereas urease (EC 3.5.1.5) has been extensively studied (Reithel, 1971; Dixon & Webb, 1979), the enzymology of biuret cleavage is scarcely described. Nishihara *et al.* (1965) describe a partially purified 'biuretase', and, from measurements of release of NH_4^+ from biuret, indicate degradation to NH_4^+ and presumably urea and CO_2. With our specific analytical chemistry for biuret and urea we are the first to be able to demonstrate substrate disappearance directly, and provide definitive proof of the identity of the products. The enzyme described by Nishihara *et al.* (1965) has a pH optimum at about pH 9.5 and thus differs markedly from our enzyme, which is essentially inactive under those conditions.

The ring-cleavage reaction is described for the first time. Callely (1978), in a comprehensive review of the degradation of heterocycles, makes no mention of the metabolism of *s*-triazines. The ring cleavage we describe seems to belong to the general group of amidohydrolases for nitrogen-containing heterocycles (EC 3.5.2.-; Callely, 1978).

We believe that the pathway shown in Fig. 3 also operates in at least one fungus. Zeyer *et al.* (1981) observed *Sporothrix schenckii* strain JZ 6.2 to utilize cyanuric acid, biuret and urea as sole nitrogen sources, and Beilstein (1982) observed the degradation of cyanuric acid, biuret and urea in extracts of strain JZ 6.2. It seems likely that the pathway (Fig. 3) is widespread in microbes that utilize cyanuric acid.

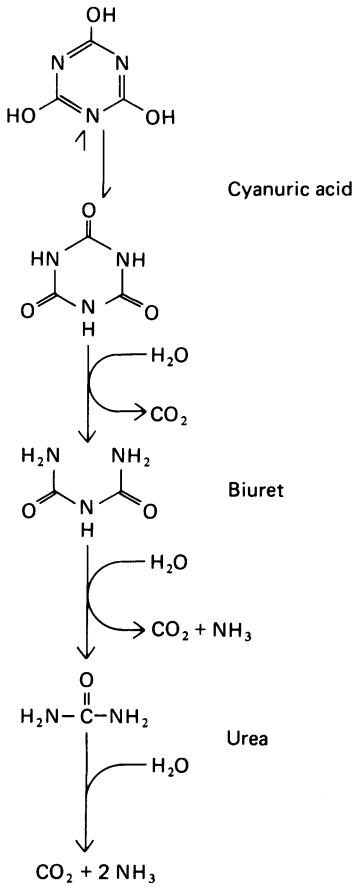

Fig. 3. Degradative pathway of cyanuric acid in *Pseudomonas* strain D

Cyanuric acid is present in solution (pH 7.2) mainly as the dione and trione tautomers (Smolin & Rapoport, 1959). The enol and trione tautomers are shown.

Cook & Hütter (1981*a*) postulate that the ring carbon of cyanuric acid is unavailable to heterotrophs because it is at the oxidation level of CO_2: the idea is strengthened by evidence for simple hydrolysis apparently without cofactors and by quantitative yields of CO_2 under aerobic and anaerobic conditions (the present paper; Zeyer *et al.*, 1981; Wolf & Martin, 1975; Saldick, 1974). However, cyanuric acid is claimed to be the major carbon and energy source for anaerobic growth of an unidentified facultative anaerobe (Jessee *et al.*, 1983), but we calculate a 7% carbon balance (the growth yield), much of which could have come from the cysteine that is shown to be utilized during growth, so we feel that claim to be premature.

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