The degradative pathway of the *s*-triazine melamine

The steps to ring cleavage

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(Received 7 June 1982/Accepted 2 September 1982)

1. The degradative pathway of melamine (1,3,5-triazine-2,4,6-triamine) was examined in *Pseudomonas* sp. strain A. 2. The bacterium grew with melamine, ammeline, ammelide, cyanuric acid or NH_4^+ as sole source of nitrogen, and each substrate was entirely metabolized. Utilization of ammeline, ammelide, cyanuric acid or NH_4^+ was concomitant with growth. But with melamine as substrate, a transient intermediate was detected, which was identified as ammeline by three methods. 3. Enzymes from strain A were separated by chromatography on DEAE-cellulose, and four activities were examined. 4. Melamine was converted stoichiometrically into equimolar amounts of ammeline and NH_4^+ . 5. Ammeline was converted stoichiometrically into equimolar amounts of ammelide and NH_4^+ ; ammelide was identified by four methods. 6. Ammelide was converted stoichiometrically into equimolar amounts of cyanuric acid and NH_4^+ ; cyanuric acid was identified by four methods. 7. Cyanuric acid was converted by an enzyme preparation into an unidentified product with negligible release of NH_4^+ . 8. The specific activities of the degradative enzymes (≥ 0.3 mkat/kg of protein) were high enough to explain the growth rate of the organism. 9. The bacterium converted 0.4 mM-melamine anaerobically into 2.3 mM- NH_4^+ . 10. Two other pseudomonads and two strains of *Klebsiella pneumoniae* were also examined, with similar results. 11. The degradative pathway of melamine appears to be hydrolytic, and proceeds by three successive deaminations to cyanuric acid, which is further metabolized.

s-Triazines are used widely as commercial chemicals (e.g. Cook & Hütter, 1981b), some of which are produced in very large quantities, e.g., melamine (Reitter & Lihotzky, 1978). There are many publications claiming metabolic conversions of *s*-triazines (see, e.g., Esser *et al.*, 1975), but Calley (1978), in his extensive review of the microbial degradation of heterocycles, mentions no biodegradation of *s*-triazines. However, many *s*-triazines are biodegradable (Cook & Hütter, 1981a,b), including melamine (see Table 1 and Fig. 3), and we now present qualitative and quantitative confirmation of the melamine degradative pathway suggested previously (Cook & Hütter, 1981b). A portion of these data has been presented in preliminary form (Jutzi *et al.*, 1981).

Experimental

Materials

The *s*-triazines used, their chemical names and sources are shown in Table 1. The purity was $\geq 98\%$

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Abbreviations used: h.p.l.c., high-pressure liquid chromatography.

(Table 1; Beilstein *et al.*, 1981). DE-52 DEAE-cellulose was purchased from Whatman, Maidstone, Kent, U.K. Pharmacia (Uppsala, Sweden) provided Sephadex G-25 (as PD-10 columns). Bovine serum albumin (fraction V) was from Sigma Chemical Co., St. Louis, MO, U.S.A. Noble agar was bought from Difco, Detroit, MI, U.S.A. Trifluoroacetic acid and trifluoroacetic anhydride were obtained from Fluka (Buchs, Switzerland), and *N*-methyl-*N*-trimethylsilyltrifluoroacetamide was from Pierce, Rockford, IL, U.S.A. The amine-bonded phase (Nucleosil 10) was from Macherey-Nagel, Düren, Germany. All other chemicals were of reagent grade or better.

Apparatus, analyses and identification of metabolites

Spectrophotometric analyses were done with a Uvikon 800 (Kontron, Zürich, Switzerland) spectrophotometer. H.p.l.c. was done with the apparatus of Beilstein *et al.* (1981). G.l.c. was done with a 5840A chromatograph equipped with a flame ionization detector (Hewlett-Packard, Palo Alto, CA, U.S.A.). Derivative formation was carried out in 1 ml

Table 1. *s*-Triazines

Melamine was from Fluka, Buchs, Switzerland, and ammeline and ammelide were from Ciba-Geigy AG, Basel, Switzerland. Cyanuric acid was obtained from both Fluka and Ciba-Geigy. The original preparation of ammeline was found to contain about 5% ammelide and 5% melamine; the impurities were removed by double recrystallization from 20 mM- Na_2CO_3 . Mass spectra of authentic materials, presented as m/z (with intensity relative to base peak >10 in parentheses) are: ammeline, 42(16), 43(58), 85(13), 127 (M^+ , 100); cyanuric acid, 43(52), 44(61), 70(14), 86(18), 129 (M^+ , 100).

Common name	Chemical name
Melamine	1,3,5-Triazine-2,4,6-triamine
Ammeline	4,6-Diamino-1,3,5-triazin-2(1H)-one
Ammelide	6-Amino-1,3,5-triazine-2,4(1H,3H)-dione
Cyanuric acid	1,3,5-Triazine-2,4,6(1H,3H,5H)-trione

Reacti-Vials (Pierce) that were capped with Tuf-Bond septa (Pierce) and heated on a thermostatically controlled heating block (Silli-Therm; Pierce). Tentative identification of an organic compound was confirmed by mass spectrometry of purified material (Beilstein *et al.*, 1981). Experiments under anaerobic conditions were done at room temperature in a glovebox (12425; Coy, Ann Arbor, MI, U.S.A.) in which 1–2 p.p.m. O_2 was observed. Cells were grown in flasks with cotton plugs. Cell suspensions were incubated in closed screw-capped tubes.

s-Triazines in neutralized solutions were routinely measured and tentatively identified by h.p.l.c. by the method of Beilstein *et al.* (1981); isocratic elution was done with 100 mM potassium phosphate buffer, pH 6.7. H.p.l.c. with an amine-bonded phase (Jessee *et al.*, 1981) was also done; the capacity ratio for ammelide varied from 2.3 to 5.6. The tri(trimethylsilyl)-derivatives of the *s*-triazines were synthesized and assayed by g.l.c. (Stoks & Schwartz, 1979); aqueous samples were dried in a stream of dry N_2 and were treated with trifluoroacetic anhydride to remove traces of water. The scaled-up reactions were done in 100 μl (final volume) reaction mixtures and the derivatives were stable for at least 10 days. NH_4^+ was measured by the nitroprusside reaction (Weatherburn, 1967). The protein assay of Kennedy & Fewson (1968) was used as described by Cook & Hütter (1981a).

Organisms and growth conditions

Five strains of bacteria were used (Cook & Hütter, 1981a): three strains of *Pseudomonas* (A, D and F, deposited as N.R.R.L. B-12227, B-12228 and B-12229 respectively) and two strains of *Klebsiella pneumoniae* (90 and 99).

s-Triazines served as sole and growth-limiting sources of nitrogen for growth in the minimal-salts medium described by Cook & Hütter (1981a). The concentration of combined nitrogen was routinely about 2.5 mM. Strains A, D and F were supplied with 10 mM-lactate as carbon source. Strains 90 and 99 were supplied with 10 mM-glucose, or, anaerobically, 50 mM-glucose.

Growth kinetics were followed in aerated 300 ml cultures in 1 litre side-arm flasks at 30°C in the apparatus of Harvey *et al.* (1968) essentially as described by Cook & Hütter (1981a). Cultures (900 ml) could also be grown in the side-arm flasks, and harvested cells were stored at –20°C (Cook & Hütter, 1981a).

Cell suspensions, cell-free extracts and enzyme assays

Cells stored at –20°C were thawed, suspended in chilled 10 mM-potassium phosphate buffer, pH 7.3, which contained 0.25 mM- MgSO_4 , and washed. The washed pellet was suspended at 250 mg wet wt./ml in the chilled buffer, and the cells were disrupted by two passages through an ice-cold French pressure cell (Aminco, Silver Spring, MD, U.S.A.) at about 130 MPa. The suspension of disrupted cells was centrifuged at 27000 g for 30 min at 4°C, and the clear supernatant fluid was used for assays of enzyme activity, either directly or after column chromatography.

Enzyme activity was measured as substrate disappearance at 30°C. Reaction mixtures (10 ml) contained 100 μmol of potassium phosphate buffer, pH 7.3, 2.5 μmol of MgSO_4 , substrate (4 μmol of melamine, 5 μmol of ammeline, 6 μmol of ammelide or 8 μmol of cyanuric acid) and protein (0.5–2 mg of crude extract) with which the reaction was started. Samples (2.0 ml) were taken at intervals and the reaction was stopped with 88 μl of cold 12 M- HClO_4 . The precipitate was removed by centrifugation (23000 g for 20 min at 4°C). Supernatant fluid (1.5 ml) was neutralized with 0.15 ml of 5 M-KOH and the KClO_4 was removed by centrifugation (23000 g for 20 min at 4°C). Supernatant fluid (1.35 ml) was acidified with 0.15 ml of 1 M-HCl and stored frozen (in 8 ml screw-cap glass vials with Teflon-lined caps) before analysis for *s*-triazines and NH_4^+ . The reaction rate was calculated as the slope of the graph of substrate concentration versus time. When fractions eluted from column chromatography of the crude extract were examined, the final volume of the assay was 2.0 ml and the reaction was stopped after 3 h; the concentrations used were as in the kinetic experiments.

Some experiments with cell suspensions were done anaerobically. The reaction mixture (10 ml) was as described above for enzyme activity and contained about 8 mg of protein.

Separation of enzyme activities by column chromatography

The DEAE-cellulose column (35 cm × 2.5 cm) was equilibrated at 4°C with 10 mM-potassium phosphate buffer, pH 7.3, which contained 0.25 mM-MgSO₄. Protein (about 500 mg) from crude extract of strain A was applied to the column and washed with about 1 bed volume of the initial buffer at 83 ml/h. Proteins were eluted with a linear gradient (1.7 litres total volume) of potassium phosphate buffer, pH 7.3 (10–500 mM), which contained 0.25 mM-MgSO₄ and 14 ml portions were collected.

Isolation of the products of enzymic reactions with s-triazines

Ammeline, which was precipitated from cultures of strain A supplied with melamine, was collected by centrifugation (20000g for 20 min at 4°C) and the supernatant fluid was discarded. The pellet, which consisted of a white crystalline layer overlayed by cells, was treated with 0.1 M-HCl to dissolve ammeline, and the turbid solution was filtered (0.2 µm pore diameter filter membrane) to remove the cell material. The filtered solution was neutralized with 1 M-NaOH; the precipitated ammeline was collected on a sintered glass filter.

Cyanuric acid was formed from ammelide in an enzyme reaction (50 ml) using the appropriate pooled fractions from the DEAE-cellulose column. Portions of solid ammelide were added, and the reaction was followed by release of NH₄⁺. When no more NH₄⁺ was released, protein was precipitated with HClO₄, which was then removed as KClO₄. The sample was brought to pH 13 with 1 M-KOH and concentrated on a rotary evaporator to give about 5 ml of 70 mM-cyanuric acid, but, unlike the trial experiment, cyanuric acid and KClO₄ both precipitated on cooling, and the precipitate was collected by membrane filtration. The large crystals of KClO₄ were removed manually and cyanuric acid was purified by three recrystallizations from water.

Results

Pseudomonas sp. strain A grew with ammeline, ammelide, cyanuric acid or NH₄⁺ as sole and growth-limiting source of nitrogen, and growth was concomitant with substrate utilization (Fig. 1a); the nitrogen source was utilized quantitatively, and the growth yield was about 50 g of protein/mol of nitrogen, similar to data from end-point determinations (Cook & Hütter, 1981a). Melamine was also used quantitatively as a source of nitrogen (Fig. 1b), but melamine disappeared (at about 73 µg of protein/ml) before the end of growth (at about 120 µg of protein/ml), and a transient intermediate, which had been excreted during the utilization of

Fig. 1. Disappearance of nitrogen source as a function of growth of *Pseudomonas* strain A

The organism was grown from a washed inoculum in batch culture with a single, growth-limiting nitrogen source. Samples were taken at intervals and analysed for protein, nitrogen source and possible excretion products. The s-triazines were stable in sterile control experiments, and no products in control experiments interfered with the assays of nitrogen sources. The nitrogen sources were: O, NH₄⁺; △, cyanuric acid; □, ammelide; ▲, ammeline; ●, melamine.

melamine, was consumed during further growth. At the end of growth with melamine as the sole supplied source of nitrogen, the growth yield was 45 g of protein/mol of nitrogen, similar to the control experiment with NH₄⁺ as a source of nitrogen (Fig. 1a). When strain A was grown with 5 mM-melamine as a source of nitrogen, the transient intermediate precipitated as white crystals.

The transient intermediate was detected by h.p.l.c., and it co-chromatographed with authentic

Table 2. Specific activities of the postulated reactions in melamine degradation, and the effect of the nitrogen source used for growth on activities in crude extracts

The value given is the arithmetic mean of *n* (indicated in parentheses) experiments. Abbreviation: n.d., not detectable.

Nitrogen source for growth	Strain examined	Specific activity with named substrate (mkat/kg of protein)			
		Melamine	Ammeline	Ammelide	Cyanuric acid
Melamine	A	2.3 (3)	0.3 (2)	0.9 (2)	1.9 (3)
Ammeline	A	3.9 (2)	0.4 (2)	1.3 (2)	2.0 (2)
Ammelide	A	4.1	0.4	1.1	0.9
Cyanuric acid	A	4.5 (2)	0.3	1.0	2.9 (2)
NH ₄ ⁺	A	3.5 (2)	0.3	1.0	1.0 (2)
NO ₃ ⁻	A	3.5	0.3	1.1	1.2
Ammeline	D	n.d.	0.36	0.64	3.0
Cyanuric acid	99	n.d.	n.d.	0.29	4.5

Fig. 2. Separation of enzymes of *s*-triazine degradation from strain A on DEAE-cellulose

Crude extract (about 500 mg of protein in 10 mM-phosphate buffer) of strain A, which had been grown with melamine as sole source of nitrogen, was applied to the column, and proteins were eluted by applying a linear gradient of phosphate buffer, as detailed in the Experimental section. Activity was measured as substrate disappearance: ●, melamine; ▲, ammeline; □, ammelide; △, cyanuric acid.

ammeline. Its u.v. spectrum was identical with that of authentic ammeline ($\lambda_{\text{max.}} = 230 \text{ nm}$) and the identification of the intermediate as ammeline was confirmed by mass spectrometry (not shown; cf. Table 1).

We anticipated ammelide and cyanuric acid as subsequent intermediates in the degradative pathway (Cook & Hütter, 1981b), but neither was produced in detectable amounts during growth (Fig. 1) or in experiments with non-growing cells (Cook & Hütter, 1981a), and in experiments with crude extracts from strain A only negligible amounts were observed. However, these same crude extracts catalysed reactions in which melamine, ammeline, ammelide or cyanuric acid disappeared (Table 2), and the specific activities were essentially independent of the nitrogen source supplied for growth (Table 2).

The *s*-triazines were stable in the absence of crude extract, and crude extract contained no compounds that interfered with the determinations of *s*-triazines or of NH₄⁺. The extract showed negligible proteolysis to NH₄⁺ after 7 h. In further control experiments, the crude extract was desalted on Sephadex G-25 columns and membrane fractions were removed by centrifugation (110 000 *g* for 90 min at 4°C). Neither treatment significantly altered the activities observed in the supernatant fluid; the pellet from centrifugation at 110 000 *g* contained negligible activity. The high molecular weight indicated by elution from the Sephadex G-25 column in the excluded volume, the fact that the activities were heat-labile (no activity after 1 min at 100°C) and the observation that the activities were not sedimented with the membrane fraction showed the activities to be due to soluble enzymes.

To enable identification of the products of these enzymic reactions, enzymes in crude extracts were separated on a DEAE-cellulose column (Fig. 2). The activity catalysing the disappearance of melamine was eluted as a wide band. The activity degrading ammeline was eluted as a sharp peak under the early part of the melamine-degrading activity. The ammelide-degrading activity was eluted as a sharp peak after the bulk of the ammeline-degrading activity. The activity degrading cyanuric acid was eluted still later as a wide band. There were thus four activities to be seen after this separation (disappearance of melamine, ammeline, ammelide or cyanuric acid), each activity with different characteristics. All four reactions can be seen to be quantitative (Fig. 2).

The fractions chosen for further study from the DEAE-cellulose column did not contain activity of the following reaction in the putative degradative pathway. The reaction products from melamine were ammeline and NH₄⁺ in equimolar amounts (Table 3).

Ammeline was degraded stoichiometrically to NH₄⁺ (Table 3) and a product that was detected by h.p.l.c. and that co-chromatographed with authentic ammelide on reversed phase and on the amine-

Table 3. *Stoichiometry of the reactions in s-triazine degradation*
 The amount of product is given as the mean $\pm$ s.d. of n (indicated in parentheses) samples.

Substrate	Products (mol/mol of substrate consumed)	
Melamine (0.4 mM)	Ammeline 1.0 ± 0.08 (15)	NH_4^+ 1.0 ± 0.07 (15)
Ammeline (0.5 mM)	Ammelide 1.0 ± 0.12 (6)	NH_4^+ 1.1 ± 0.02 (6)
Ammelide (0.6 mM)	Cyanuric acid 1.0 ± 0.02 (5)	NH_4^+ 1.0 ± 0.05 (5)
Cyanuric acid (0.8 mM)	No s-triazine	$\text{NH}_4^+ \leq 0.1$ (7)

Fig. 3. *The degradative pathway of melamine*

known and of authentic cyanuric acid on g.l.c. The identification was confirmed by mass spectrometry (not shown; cf. Table 1).

Cyanuric acid was degraded quantitatively (Fig. 2) with negligible release of NH_4^+ (Table 3), and is believed to be metabolized through biuret (and urea; Beilstein & Hütter, 1980; Beilstein, 1982).

We reported four other organisms that were able to utilize intermediates in the melamine degradative pathway (Cook & Hütter, 1981a). The pairs of strains *Pseudomonas* D and F or *Klebsiella* 90 and 99 showed similar growth patterns, so only strains D and 99 were examined further, because of extreme slime formation by strains F and 90. The enzyme activities observed in strains D and 99 (Table 2) were those expected if parts of the same pathway were present as is observed in strain A.

Strains 90 and 99, both facultative anaerobes, were found to grow with equal yield anaerobically and aerobically with ammelide as the sole source of nitrogen (60–62 g of protein/mol of nitrogen), and the organisms were unable to fix dinitrogen aerobically or anaerobically. We thus presume that the degradation of ammelide does not involve molecular oxygen.

Non-growing cells of *Pseudomonas* sp. strain A converted 0.4 mM-melamine anaerobically into 2.3 mM- NH_4^+ and 0.01 mM-ammelide, and non-growing cells of *Pseudomonas* sp. strain D degraded ammeline anaerobically. It would appear that these aerobic organisms also converted, melamine for example, into NH_4^+ without molecular oxygen.

Discussion

The reactions leading to ring cleavage of the s-triazine melamine are seen to be those in Fig. 3. Each of the s-triazine intermediates has been identified by at least three independent methods and the NH_4^+ has been identified previously (Cook & Hütter, 1981a). Each reaction is quantitative (e.g. Fig. 2) and stoichiometric (Table 3), and the specific activity in crude extracts (Table 2) is at least as high as that observed during growth under the appropriate conditions (≥ 0.4 mkat/kg of protein; Cook & Hütter, 1981a). There may be other pathways of

bonded phase. Its u.v. spectrum was identical with that of authentic ammelide ($\lambda_{\text{max.}} = 222$ nm) and the identification was supported by co-chromatography of the tri(trimethylsilyl) derivatives of unknown and authentic ammelide on g.l.c.

Ammelide was degraded stoichiometrically to NH_4^+ (Table 3) and a product that was detected by h.p.l.c. and that co-chromatographed with authentic cyanuric acid. Its u.v. spectrum was identical with that of authentic cyanuric acid ($\lambda_{\text{max.}} = 214$ nm) and the identification was supported by co-chromatography of the tri(trimethylsilyl) derivatives of un-

melamine degradation, but there seems no need to hypothesize them for our organisms.

The mechanism of each of the three deaminations and ring cleavage appears to be hydrolytic. The reactions proceed in the absence of O₂, and the simple reaction mixture is a further argument against (mono-)oxygenases. This hypothesis will have to be tested by experiments with ¹⁸O-labelled water and O₂. Provisionally, we expect the deaminases (catalysing the steps from melamine to cyanuric acid) to belong to class EC 3.5.4.

Data suggesting parts of this pathway have been cited (Reitter & Lihotzky, 1978) for the thermal destruction of melamine and for the degradation of ammeline in soil and mammals (e.g. Esser *et al.*, 1975). Those workers faced considerable analytical problems in their often tentative identifications of intermediates, and only the development of h.p.l.c. analysis (Beilstein *et al.*, 1981) has made routine quantitative determinations possible.

The enzyme assays in this work were not optimized, as the aim was to elucidate the degradative pathway. Nevertheless, the low activity of ammeline degradation relative to melamine degradation (Table 2) is presumably a valid reflection of the activities within the cell, because strain A excretes ammeline when growing with melamine as the sole supplied nitrogen source (Fig. 1b).

We thank J. Seibl for obtaining the mass spectra. This investigation was supported in part by grants from the Swiss Federal Institute of Technology, Zürich, and from Ciba-Geigy AG, Basel.

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