Biodegradation of Cyanuric Acid

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Cyanuric acid biodegrades readily under a wide variety of natural conditions, and particularly well in systems of either low or zero dissolved-oxygen level, such as anaerobic activated sludge and sewage, soils, muds, and muddy streams and river waters, as well as ordinary aerated activated sludge systems with typically low (1 to 3 ppm) dissolved-oxygen levels. Degradation also proceeds in 3.5% sodium chloride solution. Consequently, there are degradation pathways widely available for breaking down cyanuric acid discharged in domestic effluents. The overall degradation reaction is merely a hydrolysis; CO₃ and ammonia are the initial hydrolytic breakdown products. Since no net oxidation occurs during this breakdown, biodegradation of cyanuric acid exerts no primary biological oxygen demand. However, eventual nitrification of the ammonia released will exert its usual biological oxygen demand.

The biodegradation of cyanuric acid (s-triazine-2,4,6-triol; see reference 10) is of interest because it rapidly becomes the end product discharged in domestic effluents when its chlorinated derivatives (sodium or potassium dichloroisocyanurate and trichloroisocyanuric acid) are used in swimming-pool treatment, in household cleansers, or in dishwasher detergents (5).

Jensen and Abdel-Ghaffar (7) reported that cyanuric acid could be used as a source of nitrogen by two fungi. They also isolated, but then lost, a bacterium which grew vigorously in a glucose-cyanurate medium. Terman et al. (13) showed that nitrogen from cyanuric acid was available for crop growth. Clark et al. (K. G. Clark, J. Y. Yee, and T. G. Lamont, Abstr. 132nd Meet. Amer. Chem. Soc. New York, No. 22 1957) and Hauck and Stephenson (6) found that alkali cyanurates and cyanuric acid nitrify in soil over a period of generally 6 to 12 weeks. Hauck and Stephenson also reported that less nitrogen was released from cyanuric acid in a "well-aerated solution perfusing through soil in a system more conducive to bacterial activity, than from cyanuric acid incubated in the same soil contained in a bottle." McCormick and Hiltbold (9) reported that the s-triazine ring carbon of atrazine, a herbicide, was evolved as CO₂ during degradation in soil, and that addition of glucose accelerated the decomposition. They concluded that the herbicide was involved only incidentally and non-preferentially in general microbial metabolism that was broadly stimulated by glucose addition. Pfaender and Alexander (11) discussed the bacterial biodegradation of DDT in terms of co-metabolism, defined as "the metabolism of a substance by a microorganism which is unable to use that compound for energy or as a source of any of the elements required for growth."

This paper describes the biodegradation of cyanuric acid in representative anaerobic systems and also in typical aerated activated-sludge treatment systems, which indeed commonly operate at characteristically low (1 to 3 μg/ml) dissolved-oxygen (DO) levels, giving rise to local anaerobic conditions in the biomass.

A discussion of cyanuric acid tautomerism, with literature references, is given in reference 12. Throughout the present paper, the term "cyanuric acid" is meant to apply to the total equilibrium mixture present at any pH.

MATERIALS AND METHODS

Materials. Nutrient broth (Difco Laboratories, Detroit, Mich.) and White Label cyanuric acid and melamine (Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N.Y.) were used. All inorganic reagents were ACS analytical reagent grade. ¹⁴C-labeled cyanuric chloride was purchased from New England Nuclear Corp., Boston, Mass. It had an activity of 7.36 mCi/mmol.

Activated sludge. Activated sludge was maintained in the laboratory in continuous-flow units of the Eckenfelder-Ford (4) type at ~2 g/liter solids content, using either raw or pasteurized domestic primary settled sewage as feed. Mixed liquor was taken from the units and used directly.
Preliminary experiments were done using 2 liters of activated sludge (~2 g/liter) in open beakers with aeration and agitation accomplished by bubbling air through glass tubes (8-mm outer diameter). Every 24 h, the aeration was stopped and the sludge was allowed to settle for 45 min. 1 liter of clear supernatant fluid was withdrawn and analyzed for cyanuric acid, and, finally, 1 liter of supernatant fluid from settled fresh local domestic sewage and cyanuric acid to make the concentration 30 \( \mu \text{g/ml} \) was added. In some experiments, other additions were made at this point. Aeration was then resumed.

Published test methods (2, 8, 14) employing highly aerated systems, commonly used to test for biodegradability, were used.

A 1.5-liter, automated draw-fill, activated sludge unit was operated in the laboratory on the following 8-h cycle: a 7-h period for stirred aeration, a 55-min settling period, a 2-min period for drawing off the upper two-thirds of the volume, and a 3-min period for refilling with refrigerated raw sewage, after which a new aeration cycle was begun. The sewage reservoir was refilled manually every 4 to 6 days. In this unit, air was introduced into the mixed liquor via a fritted-glass dispersion tube.

Cyanuric acid. Cyanuric acid was assayed gravimetrically (analytical method routinely used in FMC Corp. laboratories) as the melamine-cyanuric-acid complex by adding 20 ml (a large excess) of an aqueous solution of melamine (2 g/liter) to 200 ml of filtered test solution adjusted to a pH of 4.4 ± 0.3. On solutions resulting from highly aerobic treatment, the precision of the analysis was satisfactory: ±1.9 mg/liter (standard deviation) in the range 0 to 30 mg of cyanuric acid per ml. However, anaerobic sludge systems sometimes gave unrealistically high cyanuric acid assays, so, although they were useful in preliminary experiments, the gravimetric analyses for these systems were not always completely reliable by themselves. Such results were usually verified by radio-tracer experiments.

\(^{14}\text{C}\)-labeled cyanuric chloride obtained from the New England Nuclear Corp. was hydrolyzed to the acid at pH 4 (~90 C; 2 h) at such concentration to give a final solution of ~1 mCi/ml containing 18 \( \mu \text{g} \) of cyanuric acid per ml, and the solution was then brought to pH 6. To check the radiochemical purity, 5 ml of \(^{14}\text{C}\)-labeled cyanuric acid solution was added to a solution or ordinary cyanuric acid (20 mg) in 10 ml of water adjusted to pH 4.3. Then 20 ml of melamine solution (2 g/liter) was added. After centrifugation, the clear supernatant liquid was assayed for \(^{14}\text{C}\) content, and the non-precipitated \(^{14}\text{C}\) was taken as a measure of the upper limit of radiochemical impurity. The purity was found to be ~99%.

Typical experimental procedure. In radiochemical biodegradation experiments, \(^{14}\text{CO}_2\) evolved was carried by a stream of either \(\text{N}_2\) or air together with any ordinary \(\text{CO}_2\) through an efficient bubble trap containing 0.5 N NaOH solution. This solution, plus rinsings (totaling 75 to 100 ml), was analyzed for \(^{14}\text{C}\) activity by liquid scintillation counting by New England Nuclear Corp. Approximately 2 \( \times 10^4 \) dpm were used per experiment, and, since ~10 dpm/ml could be detected with reasonable certainty over background, a negative result means that less than 0.05% of the cyanuric acid was converted to \(\text{CO}_2\) and that 0.1% is well beyond expected experimental error. Blank control experiments without mud or sewage organisms gave no detectable \(^{14}\text{CO}_2\) yield. The experimental variability for those experiments which gave large \(^{14}\text{CO}_2\) yields was about 10%. The \(^{14}\text{C}\) activity carried over into the traps was 99.8% precipitated with \(\text{BaCO}_3\) when \(\text{BaCl}_2\) was added to the alkaline trap liquid. All incubations were at 23 ± 1 C.

The nitrogen used for deaeration was piped laboratory supply, obtained from evaporation of commercial liquid nitrogen.

DO was measured with a Delta model 85 oxygen meter (Delta Scientific Co.). Kjeldahl, ammonia, and other analyses were done as in Standard Methods (1).

RESULTS

Preliminary experiments. In screening tests using highly aerobic systems (2, 8, 14) cyanuric acid did not biodegrade.

Erratic results were obtained in the preliminary 24-h draw-fill cycle experiments. The quantity of cyanuric acid degraded each day varied from zero to over 10 \( \mu \text{g/ml} \), apparently dependent on the sewage used. It was found that daily addition of 200 \( \mu \text{g} \) of ethanol or 300 \( \mu \text{g} \) of glucose per ml led to dependable degradation, although the quantity of cyanuric acid degraded per day varied in the 10 to 20 \( \mu \text{g/ml\ range} \), building up to the higher value after repeated daily nutrient supplement additions and falling off to the 0 to 10 \( \mu \text{g/ml\ range} \) when the nutrient supplement additions were omitted. The filtered supernatant liquid was inactive even at DO <0.2 \( \mu \text{g/ml} \).

Draw-fill operation, switching between aerobic and anaerobic conditions. The automated laboratory draw-fill unit was used on an 8-h cycle, with ~10 \( \mu \text{g} \) of cyanuric acid per ml added to the fresh raw sewage feed. After several days of normal operation with aeration, the feed analysis was 9.6 \( \mu \text{g} \) of cyanuric acid per ml, and the effluent analysis was 8.3 \( \mu \text{g} \) of cyanuric acid per ml. Continuing operation with the same feed, 16 h after the incoming gas was changed from air to nitrogen, the feed analysis was 7.7 \( \mu \text{g} \) of cyanuric acid per ml, and the effluent analysis was <1 \( \mu \text{g} \) of cyanuric acid per ml. In a repeat experiment, using a new batch of feed but with the same biomass in the unit, after 24 h of normal aerated operation the feed analysis was 10.5 \( \mu \text{g} \) of cyanuric acid per ml and the effluent analysis was 7.4 \( \mu \text{g} \) of cyanuric acid per ml. Continuing operation with the same batch of feed, 16 h after changing the gas from air to nitrogen, the feed analysis was...
9.4 μg of cyanuric acid per ml and the effluent analysis was <1 μg of cyanuric acid per ml. Further continuing with the same batch of feed, and using air for 3 days after the previous sampling, resulted in a feed analysis of 6.8 μg of cyanuric acid per ml and an effluent analysis of 6.7 μg of cyanuric acid per ml.

**Anaerobic sewage.** If primary settled domestic sewage to which 10 μg of cyanuric acid per ml had been added was allowed to become anaerobic or intentionally made anaerobic, the cyanuric acid concentration was reduced by 25 to 50% in 48 h, and complete disappearance of the cyanuric acid was observed within 72 to 96 h.

The nitrogen material balance in this system was checked as follows: cyanuric acid (430 μg/ml) was added to some primary effluent derived from fresh local sewage. A 500-ml Erlenmeyer flask was filled with this solution so that there was no air space in the flask, stoppered, and kept in the dark at 20 ± 1 C for 3 weeks. Analysis showed no detectable cyanuric acid. The sewage-cyanuric acid solutions were analyzed for nitrogen (after adding 3 ml of concentrated H₂SO₄ per 100 ml of sample) at zero time and after the 3-week incubation. The total Kjeldahl nitrogen rose from 65 μg/ml to 209 μg/ml, an increase of 144 μg/ml, and the ammonia nitrogen, determined by distillation, rose from 52 μg/ml (average 49, 54) to 191 μg/ml (average 202, 180), an increase of 139 μg/ml. Since the nitrogen equivalent of 430 μg of cyanuric acid per ml is 140 μg/ml, all of the ammonia and Kjeldahl nitrogen increase is accounted for by conversion of cyanuric acid nitrogen to ammonia nitrogen, within analytical error.

**Anaerobic mixed liquor.** ¹⁴C-labeled cyanuric acid tracer solution (1 ml) was added to 1 liter of mixed liquor from a laboratory unit containing about 2 g of activated sludge and starved by overnight aeration without any feed, and nitrogen was bubbled through the stirred suspension at a rate of 1 liter/h. The ¹⁴C was evolved as ¹⁴CO₂ as follows: 4% within 7 h, 11% (total) in the next 17 h, and 82% (total) in 17 days. Residue from this experiment was dissolved completely in an equal volume of concentrated sulfuric acid containing 15 g of K₂Cr₂O₇ and boiled for 2 h, as if for chemical oxygen demand analysis (1), while a gentle current of air was passed through the system and then through a trap containing 0.5 N sodium hydroxide. The evolved CO₂ from this residue solution contained 1.3% of the ¹⁴C initially added. In a control chromic acid oxidation experiment, ¹⁴C-labeled cyanuric acid evolved about one-half of its ¹⁴C as CO₂ within 2 h under similar conditions. Thus, essentially no ¹⁴C from cyanuric acid was synthesized into biomass. In one repeat of the anaerobic mixed liquor experiment, the ¹⁴CO₂ evolution was as follows: 50% in 8 days, 71% in 13 days; in another repeat, the ¹⁴CO₂ evolution was 93% in 6 h. Mixed liquor activity was clearly very variable.

**Anaerobic nutrient broth.** Nutrient broth was inoculated with mixed inoculum from a sewage plant effluent and incubated aerobically for 48 h at 23 C. A small amount of flocculent biomass was removed by filtration through coarse filter paper. ¹⁴C-labeled cyanuric acid (0.8 ml; 0.8 μCi) was added to 250 ml of this culture in a 500-ml flask, and nitrogen was bubbled through for 48 h. The ¹⁴CO₂ in the effluent gases contained 80% of the radioactivity initially added. A similar experiment to which 10 mg of cyanuric acid (40 μg/ml) was added at the start of the anaerobic period gave the results shown in Table 1.

It should be pointed out that a 48-h mixed population culture in nutrient broth (500 ml of broth magnetically stirred at 23 C in a 2-liter Erlenmeyer flask plugged with a foam stopper) had <0.5 μg of DO per ml, and that 250 ml of such a culture stirred in a 500-ml Erlenmeyer flask through which 1 liter of air per h was bubbled, using an open-ended 8-mm glass tube, also had <0.5 μg of DO per ml. However, when a fritted-glass gas dispersion tube was used, the DO was 5 μg/ml.

**Soils and muds.** The experiments with soils were done by adding 1 ml of ¹⁴C-labeled cyanuric acid solution (18 μg of cyanuric acid, 1 μCi) to 20 g of soil in a stoppered 250-ml Erlenmeyer flask in which an open vial containing 3 ml of 1 N NaOH solution was suspended by a wire hook. After an appropriate time without shaking or agitation, at 23 C, the contents of the vial were assayed for ¹⁴C. Data from experiments with soils, muds, and natural waters are given in Table 2. The 90% yields listed are 100%

| Time (h) | ¹⁴C added evolved as ¹⁴CO₂ (%) |
|---------|-----------------------------|
|         | 0.44 ng of cyanuric acid per ml | 40 μg of cyanuric acid per ml |
| 1.5     | 0.49                        | 0.11                        |
| 3.0     | 1.20                        | 0.47                        |
| 24.0    | 25.0                        | 4.1                         |
| 72.0    | 95.0                        | 98.0                        |
within the experimental error of the method. One of the muds was an Atlantic Ocean coastal mud. The experiments with lake water were stirred occasionally.

Model experiments with soils in the absence of added cyanuric acid showed that the O₂ concentration in the atmosphere inside the flask decreased by 10 to 20% in a 3-week period, indicative of the general level of biological activity in the soil.

Continuous flow aerated laboratory sewage units. The results in Table 3 were obtained using a completely stirred, Eckenfielder-Ford (5) laboratory unit fed with domestic sewage to which 10 µg of cyanuric acid per ml had been added. The low DO values were obtained by reducing the air flow; mechanical stirring was used to agitate the mixed liquor.

NaCl solutions. In order to test whether saline environments would prevent the biodegradation of cyanuric acid, saline (2% NaCl) mixed liquor, containing activated sludge to which 20 µg of cyanuric acid per ml had been added, was made anaerobic by bubbling with nitrogen for 19 h. Tracer ¹⁴C-labeled cyanuric acid was then added, and the nitrogen flow continued for an additional 28 h during which the ¹⁴CO₂ was scrubbed out and then assayed. The yield of ¹⁴CO₂ was 25.1% of the ¹⁴C added. A similar experiment was performed at 3.5% NaCl concentration. In 5 days, 64.3% of the ¹⁴C was evolved as ¹⁴CO₂.

DISCUSSION

The evidence presented here indicates that cyanuric acid and derivatives which revert readily to cyanuric acid in domestic effluents (e.g., chlorinated isocyanurates) will readily break down directly to carbon dioxide and ammonia in conventional sewage collection and treatment facilities, in septic vaults and drainfields, on soils, in lakes or streams when it comes in contact with a soil bed or other local anaerobic condition, and also in saline environments.

Since cyanuric acid exerts no biological oxygen demand in aerobic media, and since all of its carbon and all of its nitrogen are quantitatively released as CO₂ and NH₃ in anaerobic media, a biologically catalyzed hydrolysis is indicated as the overall degradation reaction: C₃H₃N₃O₃ + 3H₂O → 3CO₂ + 3NH₄.

The key to rapid biodegradation of cyanuric acid by mixed cultures of bacteria derived from sewage or soil is the maintenance of an environment with a low DO level. In highly aerobic media cyanuric acid resists biodegradation. The apparent co-metabolism observed in the preliminary draw-fill experiments can in this case

Table 2. ¹⁴CO₂ evolution from ¹⁴C-labeled cyanuric acid* on soils, muds, and in natural waters

| Medium                     | Days at room temp | ¹⁴C added evolved as ¹⁴CO₂ (%) |
|----------------------------|-------------------|-------------------------------|
| Soils                      |                   |                               |
| Garden*                    | 23                | 91                            |
| Chemical plant area*       | 23                | 106                           |
| Farm*                      | 23                | 103                           |
| Barnyard*                  | 23                | 2                             |
| Barnyard*                  | 23                | 10,13                         |
| Barnyard*                  | 15                | 52                            |
| Muds                       |                   |                               |
| Creek bottom*              | 23                | 105, 116                      |
| 1% suspension†             | 8                 | 1                             |
| Lake bottom, 5%*          | 8                 | 26                            |
| Lake bottom, 5%*          | 23                | 41                            |
| Lake water control*        | 29                | 5                             |
| Saline mud*                | 9                 | 73, 55                        |

* One microcurie or 17.6 µg of cyanuric acid per 20 g of soil or mud or 100 g of water.
† Santa Clara, Calif.
‡ From S. Charleston, W.Va.
§ From Princeton, N.J.
¶ New Brunswick, N.J.; air dried in laboratory for >6 months. 20 g of H₂O was added per 100 g of air-dried soil.
∗ Same as footnote e; experiment started 1 month after moistening.
* Same barnyard source; 90 g of H₂O was added per 100 g of air-dried soil.
+ Belcher Creek (near Greenwood Lake, N.J.)
† Same mud, 1% suspension in H₂O.
∗ Carnegie Lake, Princeton, N.J.; 5 g of mud was added to 95 g of lake water.
* Carnegie Lake, Princeton, N.J.
† Carteret, N.J., near shore, polluted Atlantic Ocean water; two samples from different locations.

Table 3. Biodegradation of cyanuric acid by aerated activated sludge

| DO (mg/liter) | Residence time (h) | BOD₅ in effluent | Cyanuric acid degraded (%) |
|--------------|--------------------|------------------|---------------------------|
| 1.0          | 6.5                | 46               | 89                        |
| 1.5          | 6.0                | 75               | 79                        |
| 2.2          | 5.5                | 22               | 62                        |
| 2.5          | 8                  | 78               | 100                       |
| 3.0          | 10                 | 6                | 84                        |
| 5.0          | 5.5                | 6                | 51                        |
| 5.7          | 14                 | 12               | 15                        |
| 7.0          | 6.5                | 23               |                           |
| 8.7          | 5                  | 14               |                           |
| 8.7          | 9                  | 42               |                           |
| 9.0          | 14                 | 12               | 65                        |

* BOD, Biological oxygen demand (1).

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be understood as simply due to lowering of DO.

The ability to degrade cyanuric acid was rapidly gained and lost by bacteria grown in aerated medium when the DO was lowered and raised. Anaerobic growth in sewage also degrades cyanuric acid.

Organisms which degrade cyanuric acid multiply both in aerobic and anaerobic conditions and do not require any acclimatization to be active for cyanuric acid decomposition. The degradation activity is turned on and off with a time lag of not more than a few minutes when the environment is made anaerobic or aerobic.

The experiments with and without 40 μg of cyanuric acid per ml added in addition to the tracer 14C-labeled cyanuric acid showed that 40 μg of cyanuric acid per ml slowed down the biodegradation of the tracer 14C-labeled cyanuric acid. This suggests that the anaerobic biodegradation is kinetically less than first order in cyanuric acid in a gross sense.

Soils taken from locations near an FMC plant (South Charleston, W. Va.) or an FMC laboratory (Princeton, N.J.) where cyanuric acid and derivatives are handled could contain organisms acclimatized to cyanuric acid, so samples were checked from other locations highly unlikely to have been exposed to cyanuric acid. Since all samples were active, organisms commonly present in all were effective without requiring acclimatization. The differences between the soils from different locations are not considered significant, since water and organic matter contents were not controlled. The experiment with the barnyard soil (Table 2) demonstrates that the water content is critical, probably controlling the oxygen level and possibly also the salinity in the immediate environment of actively metabolizing organisms. Biodegradation of cyanuric acid also takes place in systems of considerable salinity. The results in lake and creek water with added mud suggest that the biodegradation takes place in the mud rather than in the liquid phase.

The data reported in Table 3 show that in the realistic range of 1 to 3 μg of DO per ml, good removal of cyanuric acid occurs in continuous-flow, laboratory-aerated sewage units if the residence time is at least 6 hours. At uncharacteristically high DO, the removal is poorer, but nevertheless considerable, if the residence time is longer. These results are consistent with the concept (4, 15) that part of the floc in ordinary activated sludge is anaerobic.

The effect of lowering oxygen availability as demonstrated in this work should be considered in the interpretation of other cases of co-metabolism, e.g., of atrazine (9).

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