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Biochemical Degradation of Cyanamide and Dicyandiamide**

By *Lydia M. Estermaier*,* *A. Heidemarie Sieber*, *Friedrich Lottspeich*, *Dagmar H. M. Matern*, and *Guido R. Hartmann*

Dedicated to Professor Heinz Harnisch on the occasion of his 65th birthday

Nitrate is the predominant form of nitrogen in the soil available to most plants grown under normal field conditions and is taken up as such as a source of nitrogen. It is produced from ammonium ions by oxidation catalyzed by soil microorganisms (nitrification).[1] Due to the cation exchange properties of soil, NH_4^+ is more easily accumulated than NO_3^- which is rapidly lost by leaching.[2] Most artificial fertilizers contain nitrogen in the form of nitrate or ammonium ions. However, the first synthetic compound used as nitrogen fertilizer was cyanamide, which is synthesized from calcium carbide and atmospheric nitrogen in an exothermic reaction. Despite its relatively high cost, cyanamide is still used in agriculture and horticulture as a nitrogen fertilizer, particularly in the form of its calcium salt, because of its other useful properties. In addition to its ability to provide nitrogen it acts also as a herbicide (defoliating weeds),[3] pesticide, fungicide, and bactericide.[4] In this last function it is also utilized for the deodorization of liquid manure. Cyanamide is also active in halting the dormancy of grape vines[5] and other fruits. This discovery opened a new field of application as plant growth regulator.

Dicyandiamide, the product of dimerization of cyanamide, is applied in agriculture as an inhibitor of nitrification. It prevents the oxidation of NH_4^+ by *Nitrosomonas europea*[6] and thereby stabilizes the supply of nitrogen available in the soil.

The mechanism of the biological degradation of these compounds poses an interesting ecological problem. When applied to fields, cyanamide usually disappears within a couple of days, depending on the soil and its moisture content. It was clear from the beginning that some catalytic mechanism must be involved in the degradation. For a long time it was thought that inorganic catalysts present in the soil are involved in this process, until the experiments of Ernst[7] clearly showed the preponderance of biological mechanisms, although the biochemistry of this particular degradation was not elucidated.

An inducible enzyme, cyanamide hydratase (EC 4.2.1.69), highly specific for the hydration of cyanamide (but not dicyandiamide) to urea, was first detected in the soil fungus *Myrothecium verrucaria*[8] and purified to homogeneity.[9] However, it is unlikely that this enzyme is generally responsible for the biological degradation of cyanamide in the soil, since it is expressed by the fungus only in the complete absence of any other nitrogen source, and expression immediately stops if another nitrogen source becomes available.

Hofmann et al.[10] described another enzymatic activity in extracts of commercial soybean flour which catalyzes the disappearance of cyanamide. We have purified this cyanamide-degrading enzyme from soybean flour (type I, not roasted, Sigma, Munich) in six steps to apparent homogeneity. Its molecular mass is about 600000, and it consists of six identical subunits. The first 23 amino acids at the *N*-terminus of the subunit were sequenced using the methodology described by Eckerskorn et al.[11] The sequence was the same as that at the *N*-terminus of urease from jack bean.[12] These findings suggested that the isolated enzyme may be identical to urease from soybean.

We therefore tested the commercially available, highly purified urease from jack bean (*Canavalia ensiformis*) (type VII, Sigma, Munich) for its ability to degrade cyanamide. Such a comparison disclosed that jack bean urease indeed exhibited the same specific enzymatic activity (310 ± 20 nmol min^{-1}mg^{-1}), and the same K_m value (0.15 ± 0.05 M) with cyanamide as substrate as the enzyme from soybean. Similarly the optimum of pH (7.0) and temperature (70 °C) was the same. In the reaction two moles of ammonia were formed for each mole of cyanamide consumed. Obviously urease catalyzed a cyanamide hydrolase reaction [Eq.(a)].

$$H_2N\text{-}CN + 2H_2O \longrightarrow 2NH_3 + CO_2 \qquad (a)$$

Additional evidence that urease is responsible for the cyanamide hydrolase activity was provided by the effect of specific inhibitors of urease;[13, 14] all of them inhibited the cyanamide hydrolase activity (Table 1).

To determine the substrate specificity we have tested not only cyanamide but also cyanamide derivatives such as *N*-formylcyanamide, acetylcyanamide, and benzoylcyanamide for hydrolysis by urease. But even with a 100-fold greater enzyme concentration than that used in the experiments with unsubstituted cyanamide and with prolonged incubation times up to 24 h, no hydrolysis was detectable.

Since cyanamide is hydrolyzed by urease we investigated also cyanic acid as a substrate. In contrast to cyanamide, cyanic acid is rather unstable at pH 7.0 and decomposes rapidly without addition of a catalyst. Nevertheless, the degradation of this compound is also distinctly enhanced by

[*] Dr. L. M. Estermaier, A. H. Sieber, Dipl.-Chem. D. H. M. Matern, Prof. Dr. G. R. Hartmann (deceased)
Institut für Biochemie der Universität
Karlstrasse 23
D-W-8000 Munich 2 (FRG)
Dr. F. Lottspeich
Laboratorium für Molekulare Biologie – Genzentrum
D-W-8033 Martinsried (FRG)

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Table 1. Effect of inhibitors of jack bean urease on cyanamide hydrolase activity. The conditions of incubation were similar to those of the cyanamide hydratase assay [9], but with 10 mM Tris/HCl buffer 7.4 at 50 °C (Tris: tris(hydroxymethyl)aminomethane). Cyanamide or urea were determined as described [9,23]. *N*-isopentenoylphosphoric trisamide [14] was obtained from Professor K. Kobashi, Toyama. *N*-phenylphosphoric trisamide was synthesized according to Kobayashi [24].

Inhibitor	Concentration [μM]	Cyanamide hydrolase activity [a] [%]	Urease activity [b] [%]
sodium fluoride	20000	48	34
acetohydroxamic acid	20	45	48
hydroquinone	40	45	48
N-isopentanoylphosphoric trisamide	5	60	4
N-phenylphosphoric trisamide	50	34	34

[a] 530 urease units $\times mL^{-1}$ were applied for the cyanamide hydrolase reaction; [b] 3 urease units $\times mL^{-1}$ were applied for the urease reaction.

urease. One mole of ammonia is formed per mole of depleted CNO^-, which suggests reaction (b).

$$HO\text{-}CN + 2HOH \longrightarrow H_2CO_3 + NH_3 \quad (b)$$

Obviously urease exhibits cyanase (EC 3.5.5.3) activity.[15] As in the case of cyanamide (Table 1), low concentrations (5–30 μM) of phenylbisamidophosphate and phenylphosphoric trisamide inhibited the enzymatic hydrolysis of cyanate although only by 30 to 55%.

A relatively large number of compounds structurally related to urea or cyanamide are hydrolyzed by urease from jack bean, although the rate of hydrolysis is 100 to 1000 times less lower than with urease (Table 2). Obviously, at a low level of activity the substrate specificity of urease is rather broad but not unlimited, since derivatives such as guanylurea and cyanourea were not degraded at all, even when we used a urease concentration 100 times higher. Related in structure to cyanourea is dicyandiamide. Thus we have tested also dicyandiamide as a possible substrate of urease.

Table 2. Compounds related to urea or cyanamide as substrate of jack bean urease. In our experiments the incubation conditions at 38 °C of Dixon et al. [16] were applied. Cyanamide and dicyandiamide were determined according to [9, 25].

Substrate	k_{cat} [s^{-1}]	K_m [M]	Ref.
urea (H_2N-CO-NH_2)	3500	0.003	[a]
semicarbazide (H_2N-CO-NH-NH_2)	30	0.06	[16]
hydroxyurea (H_2N-CO-NH-OH)	12	0.002	[16]
methylurea (H_2N-CO-NH-CH_3)	0.075	0.22	[16]
dicyandiamide (H_2N-C(NH)-NH-CN)	1.0	0.25	[a]
cyanamide (H_2N-CN)	8.6	0.2	[a]
acetamide (H_2N-CO-CH_3)	0.55	0.75	[16]

[a] This work.

Unexpectedly, this compound hydrolyzed. The K_m value was almost the same and the k_{cat} value was only nine times less than that for cyanamide (Table 2). Furthermore, the reaction showed a similar dependence on pH and temperature as the enzymatic hydrolysis of cyanamide suggesting that both hydrolytic reactions are catalyzed by the same enzyme.

Analysis of the reaction products revealed that the enzymatic hydrolysis of dicyandiamide led to approximately equal amounts of cyanourea and ammonia. This suggests the following hydrolytic reaction [Eq.(c)].

$$H_2N\text{-}C(NH)\text{-}NH\text{-}CN + HOH \longrightarrow H_2N\text{-}CO\text{-}NH\text{-}CN + NH_3 \quad (c)$$

This hydrolysis is rather substrate-specific, since guanidine, for example, is not hydrolyzed by urease. Recently, a *Rhodococcus* species was discovered[17] which also degrades dicyandiamide to cyanourea. However, the stoichiometry of this reaction was not determined. The catalysis of the conversion of dicyandiamide to cyanourea belongs to a type of enzymatic reaction that is different from the other reactions catalyzed by urease (urea aminohydrolase) such as the hydrolysis of cyanate or cyanamide. With dicyandiamide as substrate, the hydrolysis of an imino group is catalyzed. Arginine deiminase (EC 3.5.3.6) is another enzyme that catalyzes such a reaction. In contrast to dicyandiamide, hydroxyurea is hydrolyzed by urease to NH_3, CO_2, and H_2NOH.[18]

These findings demonstrate the broad range of reactions that can be catalyzed by the enzyme urease. With respect to the mechanism of catalysis, the question arises of whether all these reactions are catalyzed at the same active center or if urease belongs to the class of "double-headed" enzymes with separate active centers for the various catalytic activities. In this context it may be of interest to note that neither hydroxyurea, hydroquinone, acetohydroxamic acid, nor *N*-isopentenoyl phosphoric trisamide inhibit the hydrolysis of dicyandiamide at a concentration (50 μM) which inhibits the cyanamide hydrolase or urea amido hydrolase activity of urease.

Urease is a widely distributed enzyme found also in plants,[19] and a particularly rich source is the seeds of *Leguminosae*. Therefore it was not surprising that extracts of all such seeds (soybean, mung bean, bush bean, lentil, pea) and of leaves from lentil, pea, and lupine exhibited cyanamide hydrolase activity.

Most of cyanamide and dicyandiamide used in agriculture is applied to the soil. Therefore it is likely that microorganisms in the soil are mainly responsible for the biological conversion and degradation of these compounds. Urease is widely distributed among microorganisms.[19] The question arises whether urease from bacteria in the soil also degrades cyanamide. The answer is not obvious, because bacterial ureases differ from each other and from the plant enzymes in parameters such as size, number of subunits, and nickel content.[20] (Nickel is an essential cofactor for urease activity.) *Bacillus pasteurii* is a gram-positive eubacterium with a particularly high urease activity which occurs in the soil. Its urease consists of four identical subunits (M_r 65000) with a total mass of about 230000,[21] whereas the urease from jack bean consists of six identical subunits (M_r 96600) with a holoenzyme molecular mass of 590000.[13] We compared the substrate-degrading activity of a commercially available preparation of urease from *Bacillus pasteurii* (type X, partially purified powder, 100–200 units per mg, Sigma, Munich) with that of the enzyme from jack bean using urea, cyanamide, and dicyandiamide (Table 3). The plant enzyme was more active than the bacterial urease. This may have been due to the higher purity or due to the fact that both enzymes were not tested under saturating substrate concentrations for technical reasons. Dicyandiamide was metabolized to equal amounts of cyanourea and ammonia by the bacterial urease in an identical manner to the plant enzyme.

Table 3. Hydrolysis of cyanamide and dicyandiamide by urease from jack bean or from *Bacillus pasteurii*. The assay was carried out with 20 mM substrate in 10 mM Tris/HCl pH 8.0 at 50 °C. The disappearance of urea, cyanamide, and dicyandiamide was measured by colorimetric assays as in [23, 9, 25].

Substrate	nmol substrate metabolized at pH 8.0 and 50 °C per min and mg protein by urease from	
	jack beam	*B. pasteurii*
urea	366000 [a]	55000 [d]
cyanamide	176 [b]	9 [e]
dicyandiamide	8 [c]	0.2 [f]

[a] 1.2, [b] 104, [c] 520, [d] 1.2, [e] 436, [f] 8720 units of urease used in the assay.

Inhibitors of urease activity reduced the cyanamide hydrolase activity of the bacterial enzyme (Table 4). This supports the conclusion that the bacterial urease is also responsible for the catalysis of cyanamide degradation. In agreement with the observations made with plant urease, the degradation of dicyandiamide by the bacterial enzyme is not susceptible to inhibition by 15 mM NaF, 100 μM hydroquinone, 50 μM isopentenoylphosphoric trisamide, or 50 μM *N*-phenylphosphoric trisamide.

Table 4. Inhibition of cyanamide hydrolase activity of urease from *Bacillus pasteurii* by urease inhibitors. The enzyme was preincubated with the inhibitor in 10 mM Tris/HCl pH 7.4 for 15 min at 50 °C before addition of substrate.

Inhibitor	Inhibitor concentration [μM]	Cyanamide hydrolysis [a] [%]	Inhibitor concentration [μM]	Urea hydrolysis [b] [%]
sodium fluoride	20000	37	40000	42
acetohydroxamic acid	20	27	20	44
hydroquinone	100	40	40	31
N-phenylphosphoric trisamide	50	46	50	63
N-isopentenoyl-phosphoric trisamide	20	76	0.5	19

[a] 1092 urease units per mL (2 h incubation). [b] 4.4 urease units per mL (15 min incubation).

Cyanamide hydrolase activity was also detected at pH 7.4 and 50 °C in urease containing crude extracts of other soil bacteria such as *Proteus vulgaris, Proteus mirabilis, Klebsiella oxytoca*, and *Mycobacterium phlei* in the range of 0.5–4 nmol cyanamide degradation per min and mg protein.

The fertilizers urea and cyanamide must both be degraded in the soil before they can fulfill their physiological role as suppliers of nitrogen for plants. Urea is degraded efficiently by urease, which is ubiquitous in microorganisms in the soil. Thus in the application of urea as a fertilizer, the abundance of urease may lead to a transitory massive formation of toxic ammonia (ammonium ions) with adverse effects on seed germination, seedling growth, and early plant growth. To eliminate these effects the addition of small amounts of urease inhibitors has been suggested.[22]

For the fertilizer cyanamide the situation is different. Enzymes that degrade cyanamide specifically seem to be much less abundant in the soil. So far only one, cyanamide hydratase, has been discovered[8] and characterized.[9] Its high substrate-specificity for cyanamide poses the interesting question of how this enzyme provides the microorganism with a selective advantage for survival and reproduction, since cyanamide is not found in nature.

An alternative cyanamide-hydrolyzing enzyme is the ubiquitous urease, as we demonstrated above. It catalyzes this reaction much more slowly than the hydrolysis of urea, with the consequence that a massive and instantaneous production of ammonia in the soil is prevented. Thereby the rate of the subsequent nitrification and leakage of nitrate formed from the soil is also diminished.

Because cyanamide and dicyandiamide are not found in nature, the question of how such chemicals are metabolized when they are purposely or inadvertantly released into the environment is raised. One might expect that a biological degradation would not occur, because there has been insufficient time for natural selection and development of suitable degrading microorganisms. However, many such chemicals, including cyanamide, disappear relatively rapidly in nature. In this context one should not overlook the fact that in contrast to the usual conditions in the laboratory the exposure time for degradative processes in natural ecosystems is much longer. It follows that enzymatic processes with much lower reaction rates, like that of the hydrolysis of cyanamide or dicyandiamide by urease from plants and bacteria, may contribute to the removal of toxic synthetic chemicals in the natural environment. For the investigation of enzymes that could be relevant for the metabolism of such chemicals it is therefore important that measurements of substrate specificity should not be limited too narrowly to compounds closely related in structure to the natural substrate and those metabolized with high rates. As was shown here, the substrate specificity of enzymes at low catalytic activity is apparently much broader than previously assumed. It suggests the necessity for further work in this area to develop enzyme assays with much higher sensitivity.

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