STUDIES ON THE MICROBIAL DEGRADATION OF NITRILE COMPOUNDS

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INTRODUCTION

Most organic compounds on earth are decomposed to inorganic materials by a tremendous number of soil microorganisms as a part of geochemical recycling. However, due to the recent advances in synthetic chemistry and the urbanization of our life, a huge quantity of unnatural organic chemicals have been produced and widely spread out in our environment. Unlike naturally occurring organic compounds, the synthetic chemicals tend to persist in the environment and they finally casued a severe environmental pollution. They include synthetic surfactants, polymers and plastics, *etc.* (1,2).

Lately, much works on the microbiological processes, including activated sludge method, spray filter bed method and methane fermentation, have been carried out to remove the pollutants (3). Many kinds of microorganisms of different genera were newly isolated and successfully put into practice. Polyvinylalcohol (4) and phthalate esters (5) are typical examples that could be removed from sewage water by the microbiological treatment.

Nitrile compounds are of general structure of R-CN and synthesized on a large scale as solvents, plastics, synthetic rubbers, dyes, pharmaceuticals, herbicides and starting materials of other industrially important compounds (6). They are also found in nature, especially in higher plants (7), insects (8,9) and microorganisms (10). Recent study has shown that acetonitrile is present in automobile exhaust gas (11). Nitrile compounds are notorious for the poisonous character. The acute toxicity of aliphatic nitrile on animals is considered to be brought about by hydrogen cyanide formed as a result of *in vivo* hydroxylation at α-position to form cyanohydrin (12,13). They also cause severe teratologic effect (14,15) and cancer upon adminstration. Acrylonitrile is one of the most important nitriles and is produced as a precursor of acrylic fibers, plastics and antioxidants, *etc.* Acrylonitrile

is not only toxic to the central nervous system of animals but also mutagenic and cause lung cancer among workers in acrylonitrile plant (16,17). In rats, acrylonitrile is postulated to either react with glutathione or be oxidized to an epoxide at the vinyl moiety, which subsequently reacts with glutathione (18-21). The mutagenicity is believed to be caused by the epoxide formed during the oxidation. A direct discharge of nitrile from a factory could cause an environmental pollution due to their poisonous characters mentioned above.

Many higher plants synthesize compounds which liberate hydrogen cyanide (7). The ability is known as cyanogenesis. They are classified in two types of compounds, cyanogenic glycosides and cyanolipids. Some fungi fix cyanide as α-aminopropionitrile, 4-cyano-4-aminobutyric acid (22) or β-cyanoalanine (23-27), followed by hydrolysis to yield alanine, glutamic acid or asparagine, respectively. Among the enzymes of plant origin acting on nitrile compounds, mandelonitrile lyase (EC 4.1.2.10) is distributed in almond and is known to catalyze the liberation of hydrogen cyanide and benzaldehyde from mandelonitrile (28-34). The enzyme is used for the large scale production of optically active α-hydroxynitriles from hydrogen cyanide and aldehydes (35). Another one is nitrilase (EC 3.5.5.1), which was partially purified from barley leaves by Thimann and Mahadevan (36,37). The enzyme hydrolyzed indoleacetonitrile to the corresponding carboxylic acid and ammonia. The enzyme was soluble and susceptible to SH-reagents. The enzyme required neither oxygen nor any cofactors. The striking feature was that the enzyme did not yield a free amide as an intermediate. The same kind of enzyme was later found in bacteria and fungi as ricinine nitrilase (EC 3.5.5.2) and nitrilase. The enzyme was quite unique by its character since the chemical hydrolysis of nitriles exclusively yields free amide as an intermediate (38-41).

It is not long since the microbial degradation of nitrile

compounds was first reported. Consequently, the kind of nitriles as objects of study has been narrowly limited. Ludzack *et al.* (42) first described a practical use of activated sludge to remove several toxic nitriles. However, no isolation of microorganisms was attempted. An acetonitrile-utilizing *Corynebacterium nitrilophilus* C-42 was isolated for the purpose of an application of microorganisms to petrochemical industry (43). The organism accumulated acetamide in the culture broth and it was successively hydrolyzed to acetic acid and ammonia, although the enzyme system was not made clear. Polyacrylonitrile had been considered to be biologically inert (44). Recently, triacrylonitrile-utilizing bacteria were isolated by Hosoya *et al.* (45). However, nothing was noted on the degradation products and mechanisms. More recently, Kuwahara *et al.* reported the degradation of a dinitrile, succinonitrile. They identified two nitrile-utilizers of *Aeromonas* sp. BN 7013 (46) and *Fusarium solani* MN 7030 (47) and proposed a degradation pathway including a simultaneous hydrolysis of two terminal nitrile groups with a formation of amides as intermediate and then to succinic acid.

The first enzymological study on the microbial degradation of nitrile compounds appeared in 1964 by Robinson and Hook (48-50). They partially purified ricinine nitrilase (EC 3.5.5.2) from a ricinine-utilizing pseudomonad. Interestingly, the enzyme produced a mixture of its corresponding carboxylic acid (91%) and amide (9%) as reaction product. However, the amide could not serve as a substrate for the enzyme. They concluded that the enzyme was a kind of nitrilase. Toyocamycin nitrile hydrolase, catalyzing a biosynthesis of an antibiotic, sangivamycin, was reported by Uematsu and Suhadolnik (51). But the enzyme was not purified to homogeneity and the characterization was insufficient. Harper reported the purification and characterization of nitrilase from benzonitrile-grown *Nocardia rhodochrous* and *Fusarium solani* (52-54). Both the enzyme catalyzed the direct hydrolysis of benzo-

nitrile to benzoic acid and ammonia, without accompanying benzamide as an intermediate. The result showed that the enzyme was similar to that found in barley leaves. This work was the first study done with the homogeneous enzyme preparation in the history of the research in nitrile metabolism. On the enzyme responsible for the hydrolysis of acetonitrile, the presence of acetonitrilase and acetamidase was indicated by Arnaud *et al.* (55-57). However, no clear enzymological studies such as the identification of the reaction products has been made (58,59). It remains unsolved whether the formation of amide (60-62) during the hydrolysis of nitriles is catalyzed by a single enzyme analogous to ricinine nitrilase or by more than one enzymes. The use of nitrilase as a catalyst was examined because the hydrolysis of nitrile to carboxylic acid and ammonia is an important chemical reaction of practical use in organic synthesis. Fukuda *et al.* (63) isolated *Tolulopsis candida* GN 405 and successfully applied to the hydrolysis of DL-α-hydroxynitrile to yield L-α-hydroxyacid. They also tried to hydrolyze α-aminonitrile to α-amino acid (64). Amidase (EC 3.5.1.4) has been intensively investigated by Clarke *et al.* (65,66). They isolated a variety of mutants with altered substrate specificity under appropriate selection pressure. In the series of the studies, amidase was isolated in pure form and characterized (67). Asparaginase of *Escherichia coli* was shown to catalyze the direct hydrolysis of β-cyanoalanine to give aspartic acid (68,69). The catalytic property of the enzyme gives an interest in enzymology by its similarity to nitrilase.

In this thesis, the author studied to establish a new microbiological system to degrade nitrile compound and to clarify the mechanism of the degradation. Furthermore, the production of an useful compound, acrylamide, was studied.

In CHAPTER I, the author has isolated and identified a number of nitrile-utilizing microorganisms. The products and conditions of degradation of acrylonitrile, glutaronitrile, diacrylonitrile

and triacrylonitrile were described.

In CHAPTER II, a new pathway of nitrile degradation was clarified. A new enzyme, "aliphatic nitrile hydratase" and amidase were purified to homogeneities and characterized.

In CHAPTER III, a novel method to produce acrylamide from acrylonitrile by the use of microbial nitrile hydratase was established. The cultural and reaction conditions for the production of acrylamide with *Pseudomonas chlororaphis* B23 was described.

CHAPTER I Degradation of Mono-, Di- and Trinitriles by Microorganisms

Section 1 Microbial Utilization of Acrylonitrile[a)]

Acrylonitrile is widely used as a precursor of various synthetic polymers and fibers. The direct discharge of waste water from an acrylonitrile plant could cause severe environmental pollution due to its toxicity and mutagenicity (16,17). Although several workers have succeeded in romoving organic nitriles from waste water with activated sludge systems (42), none of them has isolated microorganisms utilizing acrylonitrile as a sole source of carbon and nitrogen.

In this section, the author describes the degradation of acrylonitrile by both acetonitrile- and acrylonitrile-utilizing bacteria. The identification of degradation products of acrylonitrile is also described.

MATERIALS AND METHODS

Isolation of acrylonitrile-utilizing microorganisms. About 50% SV_{30} (sludge volume after standing for 30 min) of activated sludge, obtained from Kyoto municipal sewage disposal facilities, was suspended in the acclimation medium containing 0.008 g acrylonitrile, 0.5 g glucose, 1 g peptone, 0.1 g K_2HPO_4, and 0.1 g $MgSO_4 \cdot 7H_2O$ in 1 liter of tap water, pH 7.0. The suspension (150 ml) in a 300-ml Erlenmeyer flask was incubated at 28°C on a rotary shaker. Half of the supernatant was removed daily by decantation and the same volume of fresh medium was added. The concentration of acrylonitrile in the fresh medium was raised stepwise: 0.008 g (per liter) in the first week, 0.016 g in the second week, and 0.04 g in the third week and thereafter. After one month's acclimation, glucose was omitted from the fresh medium. The acclimated acti-

vated sludge was directly streaked on an agar plate of culture medium containing 1.6 g acrylonitrile, 2 g K_2HPO_4, 1 g NaCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.002 mg biotin, 0.4 mg calcium pantothenate, 2 mg inositol, 0.4 mg nicotinic acid, 0.4 mg thiamin·HCl, 0.2 mg *p*-aminobenzoic acid, 0.2 mg riboflavin, and 0.01 mg folic acid in 1 liter of tap water, pH 7.0.

Isolation of acetonitrile-utilizing microorganisms. Acetonitrile-utilizing bacteria were isolated from soil samples by an enrichment culture technique using the medium used for the cultivation of acrylonitrile-utilizing microorganisms in which acrylonitrile was replaced by 7.8 g acetonitrile.

Analyses. Concentrations of acrylonitrile, acrylic acid and acetic acid were estimated with a Yanagimoto gas-liquid chromatography, Model GCG-550F, equipped with a flame ionization detector. The column was a stainless column of 3 mm in inside diameter and 2 m in length, packed with Porapack Q (80 to 100 mesh). Conditions for gas-liquid chromatography were: injection temperature, 230°C; column temperature, 200°C; detector temperature, 245°C. The carrier gas was He at 14 cm^3/min.

Gas chromatoraphy-mass spectrometry was carried out with a Hitachi RMS-4 mass spectrometer-Hitachi M-5201 gas chromatograph equipped with an electron impact detector. Operational conditions were: injection temperature, 273°C; column temperature, 158°C; interface temperature, 238°C; carrier gas, He; chamber volatge, 20 eV; total emmission, 80 μA; target current, 34 μA. The column used was a glass column of 3 mm in inside diameter and 1 m in length, packed with Polyester FFAP

Ammonia was estimated with Nessler's reagent in a Conway microdiffusion apparatus.

Chemicals. Polyacrylonitrile ($M\nu$ = 8,000) was prepared as follows: to a solution of acrylonitrile (100 ml; purified by distillation prior to use) and acetaldehyde (20 g) in dimethylsulfoxide (200 ml) was added benzoyl peroxide (0.5 g), and the mixture

was refluxed for 10 hr at 70 to 80°C with stirring under nitrogen atmosphere. After 6.5 hr, further benzoyl peroxide (0.5 g) was added. Then the reaction mixture was added dropwise to 2 liters of water. The precipitate formed was washed three times with the same volume of water (70°C) and dried for 3 days under reduced pressure at 70°C. Nine grams of polyacrylonitrile was obtained. The intrinsic viscosity [η] of the polyacrylonitrile was determined at 25°C in an Ostwald viscometer. The average molecular weight was calculated to be about 8,000 from the equation

$$[\eta] = 1.75 \times 10^{-3} M^{0.66}$$

where M is molecular weight (70). Polyacrylonitrile (Mn = 92,000) was purchased from Aldrich Chemicals Co., Inc. Polyacrylonitrile trimer (1,3,6-hexanetricarbonitrile) was a kind gift from Asahi Kasei Co., Ltd. Other chemicals were usual commercial products and were used without further purification.

RESULTS

Identification of microorganisms

A bacterial strain, I-9, which could utilize acrylonitrile as a sole source of carbon and nitrogen was isolated after a 3-month acclimation. An electron micrograph of strain I-9 is shown in Fig. 1. The shape of the strain changed during growth: fresh cultures (less than 1 day) are composed entirely or largely of irregular rods which vary in

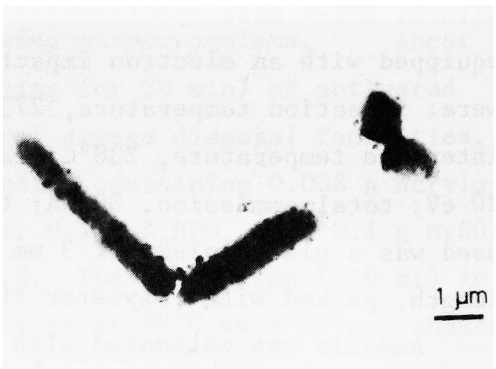


Fig. 1. Electron Micrograph of Strain I-9.

Strain I-9 was grown on a nutrient agar slant for 2 days at 28°C. Washed cells were dried *in vacuo* and shadowed with Cr.

size (0.9 to 1.0 μm by 3.0 to 10.0 μm) and in shape (straight, bent or curved); older cultures (more than 3 days) are composed entirely or largely of coccoid cells (1.0 to 1.1 μm in diameter). The other taxonomical characteristics are as follows.

Non-spore-forming, Gram-positive. Pale yellow orange in fresh cultures and dark orange in older cultures.

Growth on nutrient agar: smooth, entire, convex, glistening. Gelatin liquefaction: negative.

Litmus milk: negative. Nitrate reduction: positive. Denitrification: negative. Indole and hydrogen sulfide formations: negative. Catalase: positive. Oxidase: negative. Urease: negative. Hugh-Leifson: aerobic. Pigment formation on King's A and B media: negative. Growth facter requirement: none. No acid and gas from glycerol, fructose, glucose, galactose, mannose, mannitol, xylose, sucrose, lactose, maltose, trehalose, raffinose, dextrin, starch, inulin and glycogen. No growth on cellulose. No survival heating at 63°C for 30 min in skim milk. Growth temperature: between 24°C and 42°C, with the optimum around 37°C. Growth pH: between 5.6 and 9.3, with the optimum around 7.6.

In "Bergey's Manual of Determinative Bacteriology" 8th ed. (71), the "Coryneform group of bacteria" is divided into four genera: *Corynebacterium*, *Arthrobacter*, *Cellulomonas* and *Kurtia*. The taxonomical studies of strain I-9 indicate that it belongs to the genus *Arthrobacter*, because it is non-motile, never fermentative and shows a marked change in morphology during growth: fresh cultures are composed of irregular rods; older ones of coccoid cells, and it does not utilize cellulose. The strain was thus classified in the genus *Arthrobacter* and was tentatively named *Arthrobacter* sp. I-9.

A bacterial strain, J-1, which could utilize acetonitrile as a sole source of carbon and nitrogen was isolated from soil samples. The strain was classified in the genus *Arthrobacter* by

similar taxonomical studies. *Arthrobacter* sp. J-1 grew slightly on a medium containing acrylonitrile and glucose, but could not utilize acrylonitrile as a sole source of carbon and nitrogen.

Growth of Arthrobacter sp. I-9 on acrylonitrile and related compounds

Table I shows the growth of *Arthrobacter* sp. I-9 in the media containing acrylonitrile or related compounds as a sole source of carbon and nitrogen or as a sole source of nitrogen. Propionitrile, acrylonitrile, crotonitrile and glutaronitrile were utilized as a sole source of carbon and nitrogen. In the presence of glucose, the organism could grow on almost all nitriles, includ-

Table I. Growth of *Arthrobacter* sp. I-9 on Nitriles and Its Related Compounds.

Washed cells (30 μg as dry weight) grown on the culture medium containing 0.2% of acrylonitrile were inoculated into 5 ml of the culture medium containing 0.2% (v/v, except for polyacrylonitrile, w/v) nitriles with or without 0.5% of glucose. Cultivation was carried out in 16.5 mm test tube on a reciprocal shaker for 7 days.

Substrate		Growth (OD_{610})	
		Glucose	
		-	+
Acetonitrile		0.18	2.45
Propionitrile		0.42	2.85
n-Butyronitrile		0.24	1.92
Acrylonitrile		0.47	0.78
Crotonitrile		0.43	4.25
Methacrylonitrile		0.18	0.80
Lactonitrile		0.25	0.61
Glutaronitrile		0.49	3.52
Succinonitrile		0.26	3.50
Benzonitrile		0.16	0.16
Polyacrylonitrile trimer		0.12	2.57
Polyacrylonitrile (*Mv*= 8,000)		0	+[a]
Polyacrylonitrile (*Mn*=92,000)		0	0
Acrylamide	0.2%	1.26	-[b]
	0.5%	2.02	
	1.0%	0.24	-
Acrylic acid[c]	0.2%	1.27	-
	0.5%	1.84	-
	1.0%	0.03	-
None		0.05	0.33
Glucose[c]		3.89	

a, The growth could bot be determined turbidimetrically because of emulsification.
b, Not tested.
c, Ammonium sulfate (0.2%) was added.

ing polyacrylonitrile (Mv = 8,000). Benzonitrile and polyacrylonitrile (Mn = 92,000) were inert as the carbon or nitrogen source. Acrylamide and acrylic acid, the hydrolysis products of acrylonitrile, were well utilized by the strain.

Effect of acrylonitrile concentration on the growth of Arthrobacter sp. I-9

Figure 2 shows the effect of acrylonitrile concentration on the growth of *Arthrobacter* sp. I-9. Increasing the concentration prolonged the lag phase of the growth and concentration over 0.24% decreased the maximum growth.

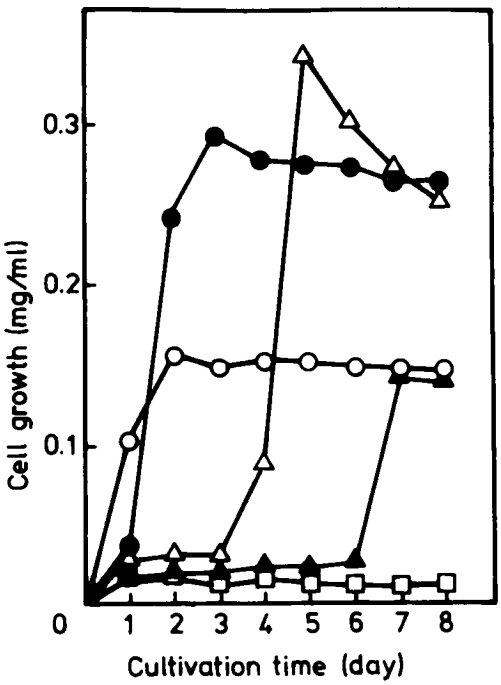


Fig. 2. Effect of Acrylonitrile Concentration on the Growth of *Arthrobacter* sp. I-9.

Washed cells (1.8 mg as dry cell weight) grown on the culture medium containing 0.008% of acrylonitrile were inoculated into 100 ml of the culture medium containig acrylonitrile in a concentration of 0.04% (○), 0.08% (●), 0.16% (△), 0.24% (▲) and 0.4% (□). Cultivation was carried out in 500-ml shaking flasks at 28°C on a reciprocal shaker.

Identification of the product from acrylonitrile

To identify metabolites of acrylonitrile, the supernatant solution of the culture broth of *Arthrobacter* sp. I-9 was acidified to pH 2.0 with HCl and extracted with the same volume of ethyl ether. The ether layer was concentrated about 10 times and analyzed by gas chromatography-mass spectrometry. The spectrum revealed parent peak at m/z 72, which was consistent with the molecular weight of acrylic acid. The fragmentation showed ion peaks at m/z 55 [M^{+} - OH], 45 [M^{+} - CH_2=CH·] and 27 [M^{+} - COOH]. Thus, one of the degradation products of acrylonitrile

was identified as acrylic acid.

Figure 3 shows the change of acrylonitrile in the culture broth during the growth of *Arthrobacter* sp. I-9, as analyzed by gas-liquid chromatography. The supernatant solution of 24-hr cultivation contained both acrylonitrile and acrylic acid. After 48 hr, only a strong peak of acrylic acid was detected. After 96 hr, neither acrylonitrile nor acrylic acid could be found.

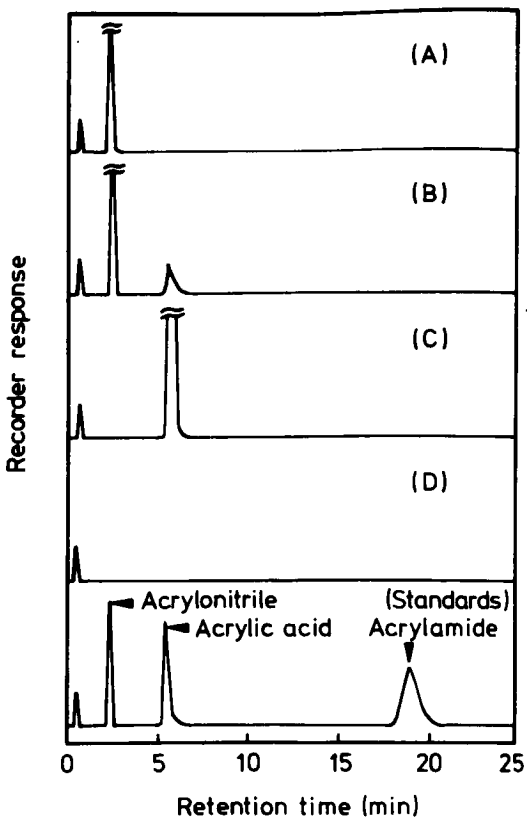


Fig. 3. Gas-liquid Chromatograms of the Products in the Culture Broth of *Arthrobacter* sp. I-9 Grown on Acrylonitrile.

Cultural conditions were as described in Fig. 2, except for that cultivation times were 0 hr (A), 24 hr (B), 48 hr (C) and 96 hr (D). Cells were removed by centrifugation and the supernatant solution was analyzed by gas-liquid chromatography.

Time course of the growth of Arthrobacter sp. I-9 on acrylonitrile

A typical time course of the growth of *Arthrobacter* sp. I-9 on 0.08% acrylonitrile medium is shown in Fig. 4. When acrylonitrile completely had disappeared after 42-hr cultivation, forming ammonia and acrylic acid, the strain began to grow exponentially. The formation of ammonia and acrylic acid in parallel with the decrease in acrylonitrile in the early cultivation time indicates that ammonia was released from acrylonitrile with the formation of acrylic acid. Acrylamide could not be detected at any time of cultivation under the conditions employed.

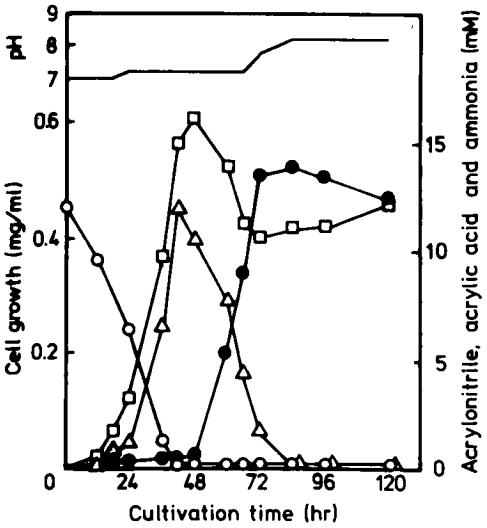


Fig. 4. Time Course of the Cultivation of *Arthrobacter* sp. I-9.

Cultural conditions were as described in Fig. 2 except for cultivation time. Acrylonitrile (○), acrylic acid (△), ammonia (□) and growth (●).

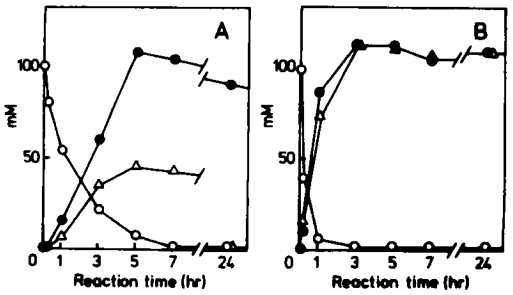


Fig. 5. Time Course of Degradation of Acetonitrile (A) and Acrylonitrile (B), by *Arthrobacter* sp. J-1 Cells.

Arthrobacter sp. J-1 was grown in 500 ml of the culture medium containing 0.77% of acetonitrile in a 2-liter shaking flask at 28°C for 3 days. The cells were harvested by centrifugation and washed 3 times with 0.01 M potassium phosphate buffer, pH 7.4. Washed cells (200 mg as dry weight) were suspended in 20 ml of the same buffer of 0.1 M containing 82 mg of acetonitrile or 106 mg of acrylonitrile. The reaction was carried out aerobically in 21-mm test tubes at 30°C. (A): acetonitrile (○), acetic acid (△) and ammonia (●); (B): acrylonitrile (○), acrylic acid (△) and ammonia (●).

Degradation of acrylonitrile by Arthrobacter sp. J-1

The degradation of acrylonitrile by the resting cells of *Arthrobacter* sp. J-1, which could utilize acetonitrile but not acrylonitrile as the sole source of carbon, was examined. Figure 5 (A) shows the degradation of acetonitrile. The disappearance of acetonitrile resulted in the formation of ammonia and acetic acid. Acetic acid formed was consumed after 24 hr. Acrylonitrile was rapidly degraded when it was incubated under the same conditions (Fig. 5(B)). Ammonia and acrylic acid were formed in the reaction mixture, in proportion to the decrease in the amount of acrylonitrile. This result shows that *Arthrobacter* sp. J-1 has an enzyme system which catalyzes the hydrolysis of acrylonitrile to acrylic acid and ammonia but not one for metabolizing acrylic

acid as cellular materials. Acrylamide could not be detected gas chromatographically.

DISCUSSION

Although DiGeronimo and Antoine (61) have reported that *Nocardia rhodochrous* LL100-21 could grow on a medium containing acrylonitrile as a nitrogen source, the utilization of acrylonitrile as a sole source of carbon and nitrogen by the pure culture of microorganism has not been reported. Recent work by Hosoya *et al.* (45) showed the presence of bacterial strains growing on the medium containing polyacrylonitrile trimer as a carbon source. The metabolism of acrylonitrile and its polymer, however, has not been studied. *Arthrobacter* sp. I-9 isolated in the present study could utilize acrylonitrile as a sole source of carbon and nitrogen, and polyacrylonitrile (M_v = 8,000) as a sole source of nitrogen. Acrylic acid in the culture filtrate was identified as a metabolite of acrylonitrile. Another strain, *Arthrobacter* sp. J-1, hydrolyzed acrylonitrile to acrylic acid in a resting cell system. Acetonitrile was shown to be hydrolyzed to acetic acid through acetamide (43,61,62). Acetamide was also detected in the culture filtrate of *Arthrobacter* sp. J-1 growing on acetonitrile. Recently, the participation of acetonitrilase and amidase in the metabolism of acetonitrile was reported (56, 57). Ricinine nitrilase was also shown to form both amide and carboxylic acid (48-50) On the other hand, a nitrilase purified from benzonitrile-utilizing microorganisms formed only benzo-

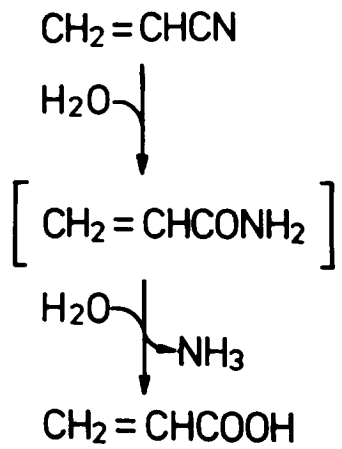


Fig. 6. Degradation of Acrylonitrile by *Arthrobacter* sp. I-9.

ic acid and ammonia without benzamide (52-54).

Nitrilase activity was found in the cell-free extracts of *Arthrobacter* sp. I-9 and J-1. The first step of the degradation of acrylonitrile can be illustrated as shown in Fig. 6. Further investigation is necessary to ascertain whether nitrilase alone or both nitrilase and amidase function in the degradation of acrylonitrile by *Arthrobacter* sp. I-9.

SUMMARY

A bacterium utilizing acrylonitrile as a sole source of carbon and nitrogen was isolated from an acclimated activated sludge and classified in the genus *Arthrobacter*. The strain could utilize propionitrile, acrylonitrile, crotonitrile, or glutaronitrile as s sole source of carbon and nitrogen. Many nitriles, including polyacrylonitrile (Mv = 8,000), were utilizable as the nitrogen source. The strain could utilize acrylamide and acrylic acid as a sole source of carbon and nitrogen or as a sole carbon source. A metabolite of acrylonitrile was identified as acrylic acid. Acrylic acid was formed in the early exponential phase of the growth and completely disappeared in the stationary phase. The resting cells of an acetonitrile-utilizing bacterium formed acrylic acid and ammonia from acrylonitrile.

Section 2 Microbial Utilization of Glutaronitrile[b)]

Various kinds of unnatural nitriles, including mono- and dinitriles are widely used as solvents, plastics and for other purposes. In the previous section, the author described the degradation of acrylonitrile and acetonitrile by isolated strains, *Arthrobacter* sp. I-9 and J-1, respectively, to the corresponding carboxylic acid and ammonia with or without formation of amide.

As for di- and trinitriles, succinonitrile (46,47) and polyacrylonitrile trimer (45) were reported to be utilized as the sole source of nitrogen and carbon, respectively. However, the degradation mechanism has not been described in detail.

In investigating microbial degradation of nitrile compounds, the author aimed to obtain glutaronitrile-utilizing microorganisms, because this unnatural nitrile is the simplest model of polyacrylonitrile.

This section describes the degradation of glutaronitrile by *Pseudomonas* sp. K-9, which was isolated from soil. Identification of degradation products of glutaronitrile is also described.

MATERIALS AND METHODS

Isolation of glutaronitrile-utilizing microorganisms. Glutaronitrile-utilizing microorganisms were isolated from soil samples by an enrichment culture technique with the culture medium of 2 g K_2HPO_4, 1 g NaCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 2 μg biotin, 0.4 mg calcium pantothenate, 2 mg inositol, 0.4 mg nicotinic acid, 0.4 mg thiamin·HCl, 0.4 mg pyridoxine·HCl, 0.2 mg *p*-aminobenzoic acid, 0.2 mg riboflavin and 0.01 mg folic acid in 1 liter of tap water, pH 7.0.

Analyses. Glutaronitrile was estimated with a Shimadzu gas-liquid chromatograph, Model GC-4CM, equipped with a flame ioniza-

tion detector. The carrier gas was N_2 at 40 cm^3/min.

Gas chromatography-mass spectrometry was carried out with a Hitachi RMS-4 mass spectrometer-Hitachi M-5201 gas chromatograph equipped with an electron impact ionization system and gas chromatograms were detected by total ion monitoring.

Ammonia was estimated as described in the previous section. Cell growth was estimated by measuring optical density at 610 nm (OD_{610}) or expressed as dry cell weight (mg/ml): 0.69 mg of dry cell weight/ml was equivalent to 1.0 unit of OD_{610}.

Chemicals. Polyacrylonitrile (trimer, Mv = 8,000 and Mn = 92,000) were as described in the previous section. Diacrylonitrile (1,3-dicyanobutane) was synthesized according to the method of Franke and Kohn (72). Diazomethane was prepared according to the method of DeBoer and Backer (73). Poly-β-hydroxybutyrate was kindly given by Prof. K. Tomita, Kyoto University. Other chemicals were commercial products and were used without further purification.

Preparation of cell-free extract. Cells were aerobically grown on the basal medium containing 0.5% glutaronitrile for 3 days at 28°C. Washed cells from 10 liters of the culture were suspended in 50 ml of 0.1 M potassium phosphate buffer, pH 7.4, and disrupted for 20 min on ice with a Kaijo-denki 19-KHz ultrasonic oscillator. The disrupted cells were centrifuged at 17,000 x *g* for 20 min at 5°C. The supernatant solution was dialyzed against 7 liters of 0.01 M potassium phosphate buffer, pH 7.4 for 12 hr.

RESULTS

Identification of microorganisms

A bacterial strain, K-9, was selected as a glutaronitrile-utilizing microorganism. The taxonomical characteristics of the

strain are as follows.

Rods, measuring 0.6 to 0.8 by 1.4 to 1.7 μm, occurring singly, non-spore-forming. Motile with a single polar flagellum. Gram-negative.

Growth on nutrient agar: circular, convex, glistening and translucent. Gelatin liquefaction and starch hydrolysis: negative.

Litmus milk: negative. Nitrate reduction: positive. Denitrification: positive. Indole and hydrogen sulfide formations: negative. Catalase and oxidase: positive. Urease: negative. Hugh-Leifson: no action on glucose. Pigment production on King's A and B media: negative. Growth factor requirement: none. No acid and gas from glycerol, fructose, glucose, galactose, mannose, mannitol, xylose, sucrose, lactose, maltose, trehalose, raffinose, dextrin, starch, inulin and glycogen. Assimilation of carbon compounds: acetate, propionate, butyrate, valerate, succinate, glutarate, pyruvate, malate, α-ketoglutarate, β-hydroxybutyrate, ethanol, L-alanine and glutaronitrile are assimilated; malonate, lactate, glycollate, glucose, fructose, arabinose, sucrose, *p*-hydroxybenzoate, L-arginine, betaine, testosterone and poly-β-hydroxybutyrate are not assimilated. Growth temperature: between 20°C and 42°C, with the optimum between 28°C and 34°C. Growth pH: between 5.1 and 9.3 with the optimum between 6.3 and 8.4.

According to "Bergey's Manual of Determinative Bacteriology" 8th ed. (71), the strain was classified in the genus *Pseudomonas* and tentatively named *Pseudomonas* sp. K-9. The strain does not appear to be identical with any described in the manual.

Growth of Pseudomonas sp. K-9 on various nitriles and glutaric acid

Table I shows growth of *Pseudomonas* sp. K-9 on the media containing glutaronitrile, mono-, di- and polynitrile compounds, and glutaric acid as the sole source of carbon and nitrogen or of carbon. It utilized only glutaronitrile among 17 nitriles tested. It grew well on a medium containing up to 2% glutaronitrile. Glu-

Table I. Growth of *Pseudomonas* sp. K-9 on Various Nitriles and Glutaric Acid.

Washed cells (70 μg as dry weight) grown on the basal medium containing 0.2% glutaronitrile were inoculated into 5 ml of the basal medium containing 0.2% (v/v, except for polyacrylonitrile, w/v) nitriles. Cultivation was carried out in 16.5-mm test tubes on a reciprocal shaker at 28°C for 7 days.

Substrate (0.2%)	Growth (OD_{610})
Acetonitrile	0
Propionitrile	0
n-Butyronitrile	0
Acrylonitrile	0
Crotonitrile	0
Methacrylonitrile	0
Isobutyronitrile	0
Succinonitrile	0
Glutaronitrile	0.32
Adiponitrile	0
2,4-Dicyano-1-butene	0
Benzonitrile	0
Lactonitrile	0
Polyacrylonitrile,	0
dimer	0
trimer	0
M_v= 8,000	0
M_n= 92,000	0
Glutaric acid* 0.2%	0.44
0.5%	0.31

* Ammonium sulfate (0.2%) was added.

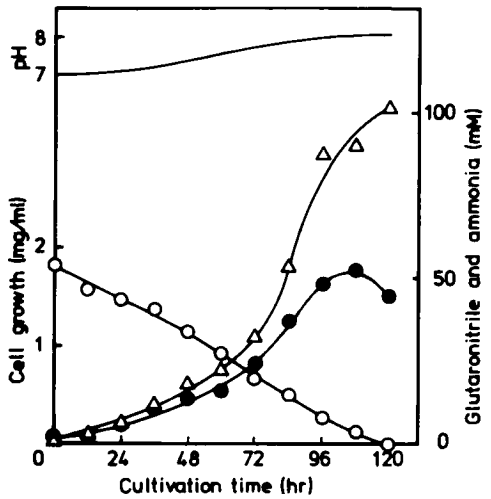


Fig. 1. Time Course of Growth of *Pseudomonas* sp. K-9.

Washed cells (1.62 mg as dry weight) grown on the basal medium containing 0.5% glutaronitrile were inoculated into 500 ml of the same medium in a 2-liter shaking flask. Cultivation was carried out at 28°C on a reciprocal shaker. Glutaronitrile was estimated with gas-liquid chromatography. The column was a glass column of 3 mm inside diameter and 0.5 m length, packed with Porapack Q (80 to 100 mesh). Conditions for gas-liquid chromatography were: column temperature, 240°C; injection and detector temperature, 280 °C. Glutaronitrile (○), ammonia (△) and growth (●).

taric acid was utilized.

Time course of growth of Pseudomonas sp. K-9 on glutaronitrile

A typical time course of growth of *Pseudomonas* sp. K-9 on glutaronitrile is shown in Fig. 1. Formation of ammonia paralleled growth. When glutaronitrile had completely disappeared, after 120 hr of cultivation, the concentration of ammonia in the culture broth was nearly twice that of glutaronitrile consumed.

Identification of products from glutaronitrile

Figure 2 shows gas-liquid chromatograms of metabolites extracted from the culture broth during the grwoth of *Pseudomonas* sp.

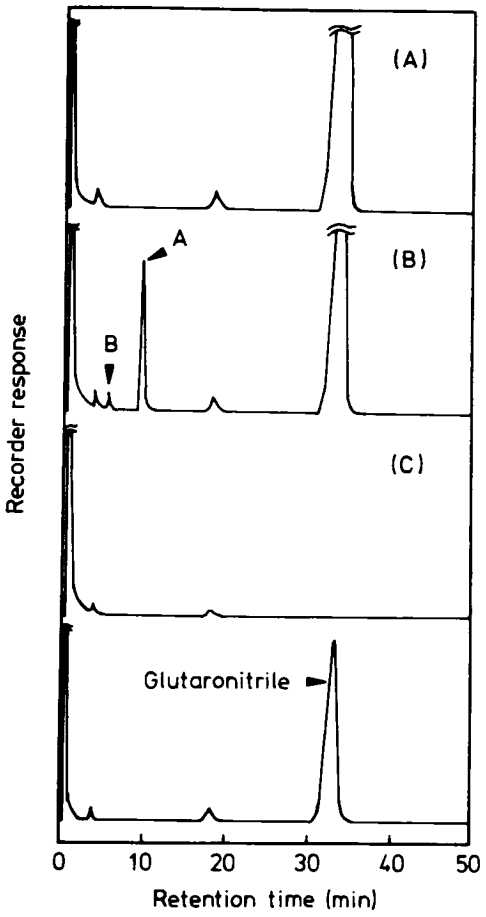


Fig. 2. Gas-liquid Chromatograms of Methyl Esters of the Products of *Pseudomonas* sp. K-9 Grown on Glutaronitrile.

Cultural conditions were as described in Fig. 1, except for that cultivation times were 0 hr (A), 60 hr (B) and 120 hr (C). Cells were removed by centrifugation and the supernatant solution (10 ml) was acidified to pH 1-2 with HCl. The solution was extracted twice with 5 ml of ethyl ether and methylated with diazomethane. This solution was concentrated to dryness, and the residue was taken up in 0.5 ml of ethyl ether and analyzed by gas-liquid chromatography on a glass column of 3 mm inside diameter and 2 m length,

K-9. Peaks A and B, which were not detected at 0 hr, appeared during growth and reached their maximum areas after 60 hr of cultivation. After 120 hr of cultivation, glutaronitrile and peaks A and B were not found. Compounds A and B, corresponding peaks A and B on gas-liquid chromatography, were isolated and characterized as follows. Ten liters of culture filtrate of 60-hr cultivation was mixed with Amberlite IR-120 A (H^+), then filtered. The filtrate was put onto a column (2.1 x 66 cm) of Amberlite IRA-410 (OH^-). Compounds A and B were eluted with 0.5 M formic acid. To detect the compounds, 0.2 ml-aliquots of fractions were acidified to pH 1-2 with HCl and extracted with 0.5 ml of ethyl ether. The extract was methylated with an ethyl ether solution of diazomethane, then analyzed by gas-liquid chromatography, under the conditions described in Fig. 2, except that column temperature was 160°C, and injection and detector temperature were 190°C. The fractions which gave a peak at retention time of 5.0 min were collected and concen-

trated *in vacuo*. The concentrate was applied onto a silica gel column (2.9 x 40 cm) and eluted with benzene-methanol (25:4, v/v). Aliquots of fractions were methylated with an ethyl ether solution of diazomethane. Fractions were combined and evaporated to dryness *in vacuo*. The mixture of compound A and B was again loaded on a column (2.5 x 75 cm) of Amberlite IRA-410 (OH^-) and eluted with 0.1 M formic acid. Yellow oil (965 mg) was obtained.

Compound A. Compound A (colorless oil, 111 mg) was obtained by distillation of the yellow oil. bp. 144-146°C (7.0 mmHg). *Anal.* found: C, 52.76; H, 6.18; N, 12.04: calcd. for $C_5H_7O_2N$: C, 53.10, H. 6.19; N, 12.39%. IR spectrum of compound A is shown in Fig. 3. The compound had a broad absorption band between 3,300 and 2,600 cm^{-1} and a band between 1,750 and 1,700 cm^{-1}, indicating the presence of a carboxylic group. The absorption of a typical cyano group was also detected at 2,240 cm^{-1}. Results of ^1H-NMR spectrum of compound A were as follows: $\delta^{CDCl_3}_{Me_4Si}$ ppm: 1.97 (2H, quintet, J = 7Hz, $NCCH_2\underline{CH_2}CH_2COOH$), 2.38-2.56 (4H, overlap of two methylenes), 10.93 (1H, singlet, carboxylic, disappeared on shaking with D_2O). Mass spectrum of methyl ester of compound A is shown in Fig. 4. The spectrum revealed ion peaks at *m/z* 129 $[M + 2]^{+\cdot}$, 128 $[M + 1]^+$ and prominent peaks of fragmentation at *m/z* 100 $[M^{+\cdot} - HCN]$, 96 $[NCCH_2CH_2CH_2C{\equiv}O]^+$, 74 $[CH_2{=}C(OH)OCH_3]^{+\cdot}$, 68 $[NCCH_2CH_2CH_2]^+$,

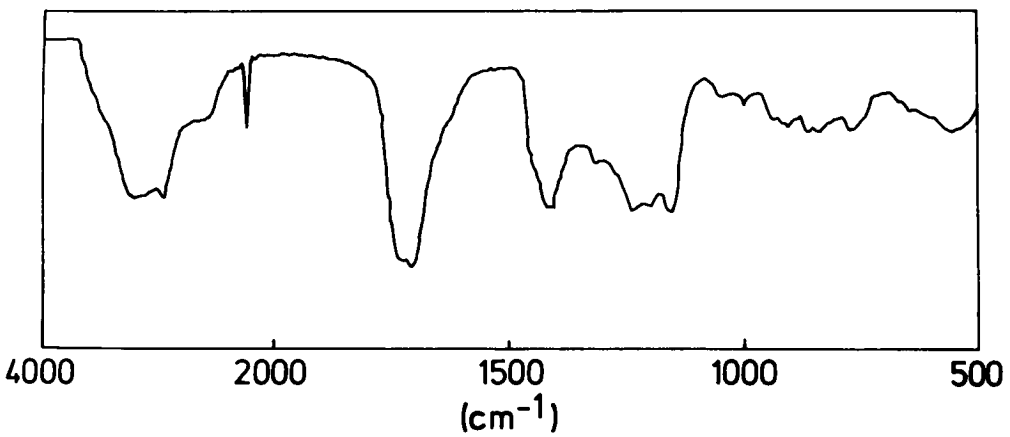


Fig. 3. IR Spectrum of Compound A.

59 $[COOCH_3]^+$, 55 $[HCNCH_2CH_2]^{+\cdot}$, 41 $[HCNCH_2]^{+\cdot}$. From these results, compound A was determined to be 4-cyanobutyric acid.

Compound B. The yellow oil obtanied after the second Amberlite IRA-410 (OH^-) column chromatography. The yellow oil gave two spots on a paper chromatogram developed with a solvent system of ethanol-28% ammonia water-water (16:1:3, by vol) and visualized with Bromphenol Blue. One of the spots coincided with that of 4-cyanobutyric acid at an R_f value of 0.90. The other spot (R_f, 0.7, compound B) was extracted and subjected to gas-liquid chromatographic analysis. As shown in Table II, retention times of the methyl ester of the compound B were identical with those of authentic dimethyl glutarate. Further, the mass spectrum of the methyl ester of compound B showed

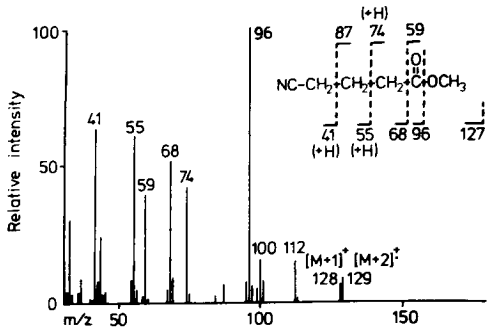


Fig. 4. Mass Spectrum of Methyl Ester of Compound A.

Compound A was methylated with diazomethane. The column was a glass column of 3 mm inside diameter and 1 m length, packed with Silicone OV-61. Operational conditions were: injection temperature, 240°C; column temperature, 210°C; interface temperature, 225°C; ionization voltage 20 eV; total emission, 80 μA, target current, 52 μA. The carrier gas was He.

Table II. Gas-liquid Chromatographies of Methyl Ester of Compound B.

Fifty microliters of the yellow oil was developed with a solvent system of ethanol-28% ammonia water-water (16:1:3, by vol). The zone between *Rf* 0.65 and 0.75 was cut and extracted with 10 ml of hot water. The solution was acidified to pH 1-2 with HCl and extracted with the same volume of ethyl ether. Then solution was methylated with an ethyl ether solution of diazomethane.

Column*	Retention time (min)		
	Methyl ester of compound B	Dimethyl glutarate	Methyl 4-cyanobutyrate
A	6.3	6.3	10.9
B	14.3	14.3	10.1
C	19.0	19.0	15.8

* Column and operational conditions were:
A, as described in Fig. 2.
B, Porapack Q (80 to 100 mesh), 3 mm x 1 m; column temperature, 240°C; injection and detector temperature, 270°C.
C, Tenax GC (80 to 100 mesh), 3 mm x 2 m; column temperature, 190°C; injection and detector temperature, 220°C.

no molecular ion and prominent fragment ion peaks were at *m/z* 129 $[CH_3OOCCH_2CH_2CH_2C{\equiv}O]^+$, 100 $[CH_3OOCCH_2CH_2CH_2]^+$, 87 $[CH_3OOCCH_2CH_2]^+$, 74 $[CH_2{=}C(OH)OCH_3]^+$, 59 $[COOCH_3]^+$. It was identical with the mass spectrum of authentic dimethyl glutarate. Therefore, compound B was concluded to be glutaric acid.

Compound C. The neutral fraction which passed through both Amberlite IR-120 A (H^+) and Amberlite IRA-410 (OH^-) was concentrated about 100 times and subjected to paper chromatography with a solvent system of *n*-butanol-acetic acid-water (4:1:1, by vol). The zone between R_f 0.5 and 0.6, where amide substance was detected with hydroxylamine and $FeCl_3$ (74). was cut out, extracted with hot water and concentrated *in vacuo*. A single peak, designated compond C, was detected by gas-liquid chromatographic analysis (retention time, 6.2 min on Porapack Q: length, 0.5 m; inside diameter, 3 mm; column temperature, 250°C; injection and detector temperature, 280°C). The mass spectrum is shown in Fig. 5. A molecular ion peak appeared at *m/z* 112, which was consistent with the molecular weight of 4-cyanobutyramide. The fragmentation showed ion peaks at *m/z* 96 $[NCCH_2CH_2CH_2C{\equiv}O]^+$, 68 $[NCCH_2CH_2CH_2]^+$, 59 $[CH_2{=}C(OH)NH_2]^+$, 44 $[CONH_2]^+$, 41 $[HCNCH_2]^+$. Thus, compound C was determined to be 4-cyanobutyramide.

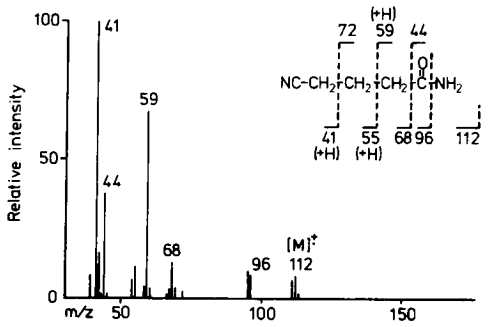


Fig. 5. Mass Spectrum of Compound C.
The column was a glass column of 3 mm inside diameter, 1 m length, packed with Tenax GC. Other operational conditions were as described in Fig. 4.

Degradation of glutaronitrile by cell-free extract of Pseudomonas sp. K-9

Extract of *Pseudomonas* sp. K-9 showed strong activity to degrade glutaronitrile. Figure 6 (A) shows the stoichiometry of glutaronitrile consumption and ammonia formation. Figure 6 (B) is the time course of the reaction. Both the results show that the amount

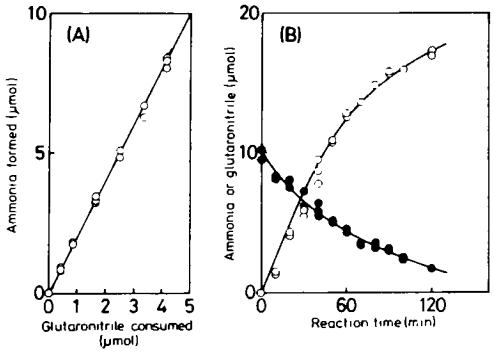


Fig. 6. Degradation of Glutaronitrile by Cell-free Extract of *Pseudomonas* sp. K-9.

(A) Stoichiometry of degradation of glutaronitrile. The reaction was carried out at 30°C in a system of final volume 2.0 ml containing 20 μmol potassium phosphate buffer, pH 7.4, 0 to 20 μmol glutaronitrile and the crude extract (21.6 mg as protein). After 120 min, the reaction was stopped by addition of 0.2 ml of 12% trichloroacetic acid. After removing denatured protein, the supernatant was used for analysis of ammonia and glutaronitrile. (B) Time course of degradation of glutaronitrile. Conditions were as described in Fig. 6 (A) except that glutaronitrile concentration was 20 μmol. glutaonitrile (●) and ammonia (○).

of ammonia formed was twice that of glutaronitrile consumed. The reaction products were determined by a similar procedure to that used for culture broth. The 120-min reaction mixture in Fig. 6 (B) was acidified to pH 1-2 with HCl and extracted with the same volume of ethyl ether. The extract was concentrated and methylated with diazomethane solution. The mixture was concentrated by gas chromatography-mass spectrometry. The chromatogram gave a single peak. No peak of methyl 4-cyanobutyrate was detected. Mass spectrum of the peak was identical with that of dimethyl glutarate, the fragment ions of which are described in the text. Thus, the degradation products were determined to be glutaric acid and ammonia.

DISCUSSION

4-Cyanobutyramide, 4-cyanobutyric acid, glutaric acid and ammonia were identified in the culture filtrate of *Pseudomonas* sp. K-9 grown on glutaronitrile as the sole source of carbon and nitrogen. These results suggest that initial steps of glutaronitrile degradation are as shown in Fig. 7. Glutaronitrile is hydrated to 4-cyanobutyramide, which is successively hydrolyzed to 4-cyanobutyric acid and ammonia. 4-Cyanobutyric acid is again hy-

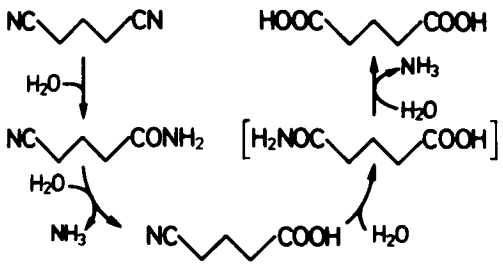
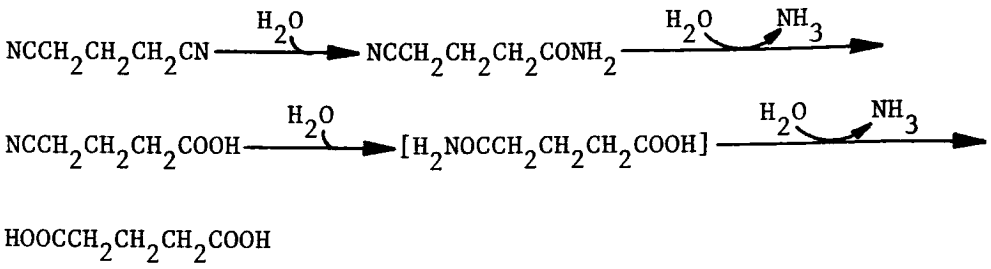


Fig. 7. Proposed Pathway of Glutaronitrile Degradation by *Pseudomonas* sp. K-9.

drolyzed to glutaric acid and ammonia. Kuwahara *et al.* (46,47) have shown the microbial degradation of another dinitrile, succinonitrile. They proposed a degradation pathway through simultaneous hydrolysis of two terminal cyano groups. In this section, the author proved the presence of 4-cyanobutyramide and 4-cyanobutyric acid as intermediates in the degradation of glutaronitrile to glutaric acid and ammonia. The concentrations of 4-cyanobutyric acid and glutaric acid were 0.37 and 0.04 mg/ml, respectively, after 60 hr of cultivation, as estimated by gas-liquid chromatography. The experiment with cell-free extract confirmed the overall reaction.

SUMMARY

A bacterium which utilized glutaronitrile as the sole source of carbon and nitrogen was isolated and classified in the genus *Pseudomonas*. The strain could utilize only glutaronitrile among 17 nitriles tested. Glutaric acid served as a carbon source. Metabolites of glutaronitrile in the culture broth were determined to be 4-cyanobutyramide, 4-cyanobutyric acid, glutaric acid and ammonia. In the cell-free extract system, ammonia was formed in twice the amount of glutaronitrile consumed, together with glutaric acid. From these results, the following metabolic pathway of glutaronitrile was proposed:



$HOOCCH_2CH_2CH_2COOH$

Section 3 Fungal Degradation of Di- and Trinitriles[c,d)]

In the previous sections, the author described the degradation of acrylonitrile and glutaronitrile, and their degradation pathways.

On the biodegradation of synthetic polymers, polyvinylalcohol (75), polyethylene glycol (76), polypropylene glycol (77) and polyesters (78) have been known to be oxidized or hydrolyzed by microorganisms. However, polyacrylonitrile has been considered to be biologically inert. Recently, microorganisms degrading polystyrene oilgomers (79), polybutadiene oligomers (80) and polyamide oligomers (45) have been also reported. Triacrylonitrile (1,3,6-hexanetricarbonitrile, TAN) is produced as a by-product of electrohydrodimerization of acrylonitrile to yield adiponitrile. Although the degradation of TAN was reported (45), the detail of the degradation has not been described. The author aimed to obtain triacrylonitrile-utilizing microorganisms, because it is not only one of toxic nitrile compounds, but also an oligomer of non-biodegradable polyacrylonitrile.

This section describes the isolation and identification of TAN-degrading fungi. Identification of the degradation products of TAN and two dinitriles are also described.

MATERIALS AND METHODS

Isolation of TAN-utilizing microorganisms. The screening of TAN-utilizing microorganisms was performed against about 200 soil samples, stock cultures of 95 bacteria, 48 molds and 34 yeasts, and activated sludge which were obtained from 6 sewage disposal facilities in Kyoto and Osaka and then acclimated for more than 40 days on TAN. The screening medium contained 0.2% (w/v) TAN with or without 0.5% (w/v) glycerol in the basal medium described in

the previous section.

Analyses. TAN was estimated quantitatively as follows. Samples containing TAN was extracted with the equal volume of chloroform. The chloroform layer was subjected to a Shimadzu gas-liquid chromatograph, Model GC-4CM, equipped with a flame ionization detector. The column used was a glass column of 3 mm in inside diameter and 2 m in length, packed with Silicone OV-17, 3% on Chromosorb W (80 to 100 mesh). Operational conditions were: column temperature, 250°C; injection and detector temperature, 280 °C. The carrier gas was N_2 at 40 cm^3/min. Detection of degradation products by gas-liquid chromatography was carried out as described in the previous section.

Gas chromatography-mass spectrometry was carried out as described in the previous section.

High resolution mass spectra were measured with a Hitachi M-80 gas chromatograph-mass spectrometer, with ionization voltage at 70 eV.

Cultivation of microorganisms. Cultivation of microorganisms was carried out in 2-liter shaking flasks at 28°C unless otherwise stated.

Chemicals. Polyacrylonitrile (dimer, trimer, polymer (Mv = 8,000) and polymer (Mn = 92,000)) and diazomethane were obtained or prepared as described in the previous sections. 1,3,6-Hexanetricarboxylic acid was prepared from TAN as follows. Five grams of TAN in 50 ml of conc. HCl was refluxed for 24 hr in a 200-ml flask. After standing at room temperature for 12 hr, salts formed were separated. The filtrate was then stood overnight. Crude crystals formed were dissolved in hot water, treated with active chrcoal, and filtered. Recrystallization from hot water gave colorless crystals (1.7 g) mp, 112-113°C; *Anal.* Found: C, 49.54; H, 6.42; Calcd. for $C_9H_{14}O_6$: C, 49.41; H, 6.56%. IR spectrum ν_{max}^{film} cm^{-1}; 2,930, 1,710, 1,470, 1,265, 950. ^{1}H-NMR $\delta_{Me_4Si}^{DMSO\text{-}d_6}$ ppm:

1.32-1.73 (6H, multiplet), 2.08-2.29 (5H, multiplet). 12.01 (3H, singlet, carboxylic, disappeared on shaking with D_2O). Mass spectrum of its trimethylester esterified by diazomethane revealed ion peaks at *m/z* 229 [M^{+} - $\cdot OCH_3$], 200 [M^{+} - $\cdot COOCH_3$], 155, 137, 128, 100.

RESULTS

Identification of microorganisms

Microorganisms which can grow on TAN as the sole source of carbon and nitrogen could not be isolated, but 35 strains which can grow on TAN as the sole source of nitrogen were obtained. Among them, 2 well-grown fungal strains, TG-1 and TG-2, were identified taxonomically and chosen for further experiments.

Characteristics of the strain TG-1 are as follows: Colonies on potato sucrose agar attained a diameter of 1.1 cm within 4 days at 25°C; they are pale yellow orange or ivory. Sparce felt-like mycelium with a white fringe at the growing front is seen. Erumpent funiculose central area surrounded by an outer slimy region is formed. The color of the reverse side is pale yellow orange or pale yellowish brown. Only macroconidia are formed. Typical macroconidia are long, slender, falcate, with 3 to 5 transverse septa, 45 to 60 by 2.5 to 4 μm. Chlamydospores are not observed. A Photomicrograph of macroconidia of the strain is shown in Fig. 1. No perithecial state has been seen.

According to the Booth's classification (81), the strain was identified as *Fusarium merismoides* Corda var. *merismoides*. The type strain IFO 30040 of *F. merismoides* Corda var. *acetilereum* Tubaki, Booth *et* Harada (82). could not utilize TAN as the sole nitrogen source.

Characteristics of the strain TG-2 are as follows: Colonies on potato-sucrose agar attained a diameter of 2.8 cm within 4

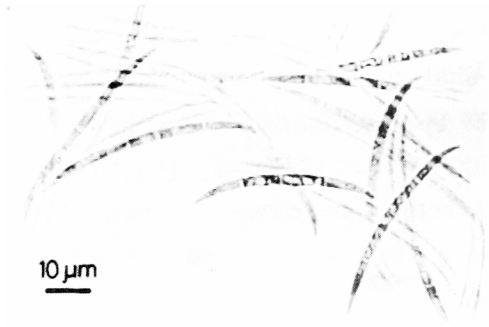


Fig. 1. Macroconidia of *F. merismoides* TG-1.

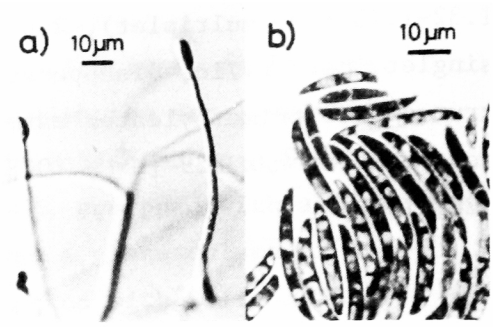


Fig. 2. a) Microconidia of *F. solani* TG-2.
b) Macroconidia of *F. solani* TG-2.

days at 25°C. Aerial mycelium is dense, floccose and greyish white. Colonies are not pigmented. Microconidia develop abunduntly. They are formed from lateral conidiophores which are merely elongated lateral phialides. Branching of conidiophores is not observed. They terminate in a single cylindrical phialide which measures 28 to 45 by 2.5 to 3 µm. Microconidia are ellipsoidal or oval. They are continuous or with 1 septum, measuring 8 to 13 by 3 to 4 µm. Typical macroconidia are curved falcate, with 1 to 3 septa, 15 to 45 by 3 to 5 µm. Chlamydospores are not observed. Photomicrographs of microconidia and macroconidia are shown in Fig. 2 (a) and 2 (b), respectively. No perithecial state has been seen.

According to a modern classification given by Booth (81), the strain TG-2 was identified as *Fusarium solani* (Mart.) Sacc. emend. Snyder *et* Hansen var. *solani*. The strain, *F. solani* IFO 5232 could utilize TAN as the sole source of nitrogen, but the growth was much less than that of *F. solani* TG-2.

Growth of F. merismoides TG-1 on various nitriles

Table I shows the growth of *F. merismoides* TG-1 on various nitriles as the sole source of carbon and nitrogen. TAN, adiponitrile, glutaronitrile and 2,4-dicyano-1-butene were utilized as the sole source of nitrogen. In the absence of glycerol, the

strain could not grow on any nitriles tested.

Cultural conditions of F. merismoides TG-1

1) Carbon source. The effect of carbon source on growth of *F. merismoides* TG-1 was examined with a medium containing 0.2 % TAN as the nitrogen source. Among sugars, organic acids and alcohols, glycerol and succinate were effective for the growth. In the range of 0.5-2% glycerol, the growth was proportional to glycerol concentration. The growth reached the maximum (10-11 mg dry weight/ml) around 2-3% glycerol.

2) Nitrogen source. The effect of nitrogen source on growth of *F. merismoides* TG-1 was examined with a medium containing 0.5% glycerol as the carbon source. In addition to TAN, inorganic compound such as $NaNO_3$ and $NaNO_2$ were effective for the growth.

3) TAN concentration. The effect of TAN concentration on growth of *F. merismoides* TG-1 was studied. As shown in Fig. 3, the maximum growth was obtained at 0.1% TAN in 0.5% glycerol medium.

Table I. Growth of *F. merismoides* TG-1 on Various Nitriles.

Washed cells (0.1 mg as dry weight) grown on the basal medium containing 0.2% TAN and 0.5% glycerol were inoculated into 5 ml of the basal medium containing each 0.2% (v/v, except for polyacrylonitrile, w/v) nitrile with 0.5% glycerol. Cultivation was carried out in 16.5-mm test tubes on a reciprocal shaker for 5 days.

Nitrile	Growth (mg dry weight /ml)
TAN	2.81
Adiponitrile	2.69
Glutaronitrile	2.39
Diacrylonitrile	1.36
2,4-Dicyano-1-butene	0.73
Isobutyronitrile	0.14
Succinonitrile	0.12

* The following compounds were not utilized: acetonitrile, propionitrile, acrylonitrile, *n*-butyronitrile, methacrylonitrile, crotonitrile, lactonitrile, benzonitrile, polyacrylonitrile (*Mv*= 8,000) and polyacrylonitrile (*Mn*= 92,000).

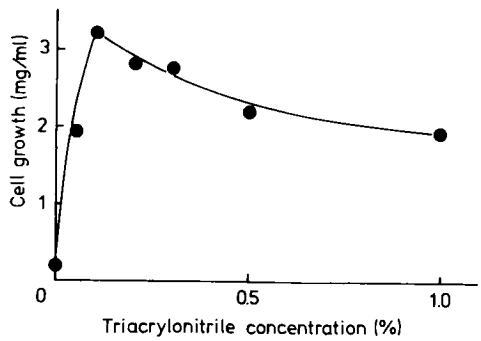


Fig. 3. Effect of Concentration of Triacrylonitrile on the Growth of *F. merismoides* TG-1.

Washed cells (12 mg as dry weight) grown on the basal medium containig 0.2% TAN and 0.5% glycerol were inoculated into 100 ml of the basal medium containing various concentration of TAN and 0.5% glycerol. Cultivation was carried out in 500-ml shaking flasks for 7 days.

As TAN concentration was increased over 0.2%, the growth was gradually inhibited.

4) pH of medium. Initial pH of the medium was varied between pH 1 to 12. The growth was not seen below pH 3. Maximum growth was obtained between pH 6.5 and 7.5.

Isolation and determination of products from TAN by F. merismoides TG-1

In order to examine the degradation products of TAN, *F. merismoides* TG-1 was grown on 0.2% TAN and 0.5% glycerol medium for 4 days. The culture broth was acidified to pH 1 with conc. HCl and extracted with the same volume of ethyl ether. The ethyl ether layer was treated with ether solution of diazomethane. The gas-liquid chromatogram of the sample gave peaks A and B and C (retention time: 12.3, 13.2 and 20.3 min, respectively, by gas-liquid chromatography, column temperature, 180°C; injection and detector temperature, 210°C). Although the retention times of peak C was identical with that of TAN, the retention times of peaks A and B were not identical with that of trimethyl 1,3,6-hexanetricarboxylate (retention time: 10.8 min). When the extract was not treated with diazomethane, only peak C, without peaks A or B, was detected on gas-liquid chromatorgaphy. These results strongly suggest that these products have carboxylic group.

Compound A and B, corresponding peaks A and B, respectively, on gas-liquid chromatography, were isolated and characterized as follows. Six liters of the culture filtrate of 4-days cultivation was mixed with 200 ml of Amberlite IR-120B (H^+). The filtrate was put onto a column (3.5 x 52 cm) of Amberlite IRA-410 (OH^-). Compound A and B were eluted with 0.5 M formic acid. They were detected with gas-liquid chromatography (column temperature, 230°C; injection and detector temperature, 260°C). The fractions which gave peaks (2.6 min) were collected and evaporated. The crude oily products obtained were applied on a silica gel column (3.6 x 70 cm) and eluted with benzene-methanol (25:4, v/v). Fractions

were combined and evaporated to dryness *in vacuo*. Brown oil (9.62 g) was obtained. When the sample was analyzed with gas-liquid chromatography after diazomethane esterification, peaks A and B were detected. The other properties of the product were as follows. IR spectrum, ν_{max}^{film} cm^{-1}: 3,200 (C-OH), 2,940, 2,230 (C≡N), 1,710-1,740 (C=O). ^{1}H-NMR ppm: 1.70-2.15 (6H, multiplet), 10.33 (1H, singlet, carboxylic, disappeared on shaking with D_2O). *Anal.* Found: C, 59.82; H, 6.85; N, 15.26%. From these data, compound

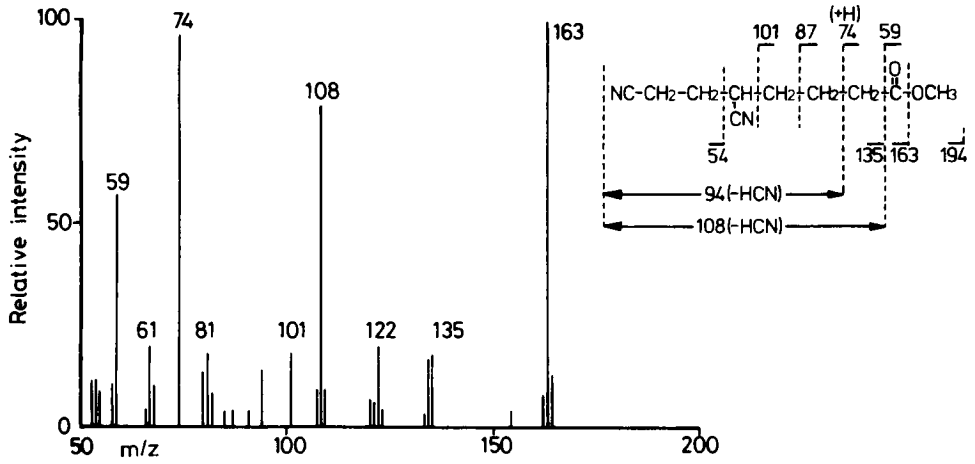


Fig. 4. Mass Spectrum of Peak A.

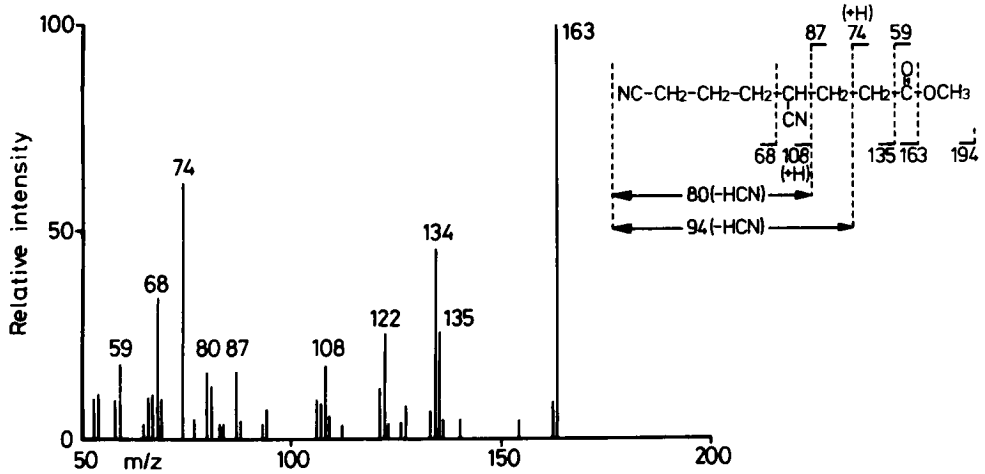
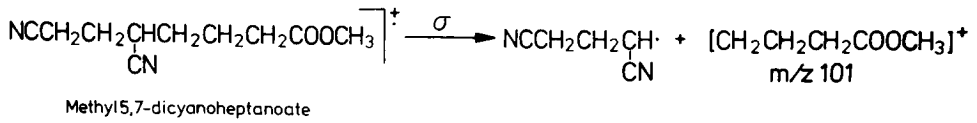
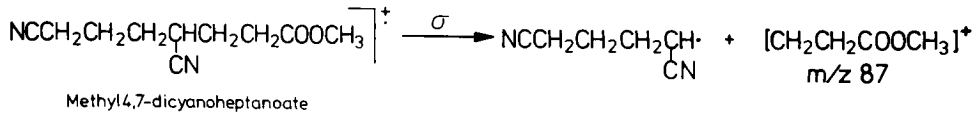


Fig. 5. Mass Spectrum of Peak B.

A and B could be thought to be two of 5,7-dicyanoheptanoic acid, 4,7-dicyanoheptanoic acid or 5-cyano-2-(2-cyanoethyl)pentanoic acid. The high resolution mass spectra of the methylated products (Figs. 4 and 5) were analyzed. In comparison of the mass spectra of peaks A and B, peaks *m/z* 163, 135, 134, 122, 74 and 59 were common ions in both spectra. It is clear that these compounds are methyl esters of carboxylic acid whose α-positions are not substituted. If α-position of a carboxylic acid is substituted like 5-cyano-2-(2-cyanoethyl)pentanoic acid, its methyl ester whould not yield ion peaks at *m/z* 74, but at *m/z* 141 $[NCCH_2CH_2CH_2CH{=}C(OH)OCH_3]^{+\cdot}$, 127 $[NCCH_2CH_2CH{=}C(OH)OCH_3]^{+\cdot}$ and 87 $[CH_2{=}CHC(OH)OCH_3]^{+}$ by McLafferty rearrangement (83,84). Accordingly, *m/z* 163 shows a removal of methoxy radical from the molecular ion of methyl 4,7-dicyanoheptanoate. *m/z* 101 ($C_5H_9O_2$, $[CH_2CH_2CH_2COOCH_3]^{+}$) in the mass spectrum of peak A is absent in that of peak B. The peak would only be derived by a fission between 4- and 5-carbons of methyl 5,7-dicyanoheptanoate as follows:



Methyl 5,7-dicyanoheptanoate



Methyl 4,7-dicyanoheptanoate

σ · breakage of σ bond.

The information with high resolution mass spectra, together with other analytical data, the products which corresponded peaks A and B, were determined to be 5,7-dicyanoheptanoic acid and 4,7-dicyanohepanoic acid, respectively. The molecular ration of A and B was estimated to be 62:38 from the area of peaks on gas-liquid chromatography.

Isolation and determination of products from TAN by F. solani TG-2

F. solani TG-2 was cultivated for 5 days on the screening medium. Brown oil (3.34 g) was obtained from 5 liters of the culture filtrate with the same procedures as with *F. merismoides* TG-1. Gas-liquid chromatography of methylated products gave peaks in the same retention times as methyl 5,7-dicyanoheptanoate and methyl 4,7-dicyanoheptanoate. The ratio of the peaks was 11:89. The other properties of the product were as follows. *Anal.* Found: C, 59.74; H, 6.67; N, 15.28%. IR spectrum, ν_{max}^{film} cm^{-1}: 3,200 (C-OH). 2,940, 2,230 (C≡N), 1,710-1,740 (C=O). ^{1}H-NMR $\delta_{Me_4Si}^{CDCl_3}$ ppm: 1.65-2.08 (6H, multiplet), 2.35-2.88 (5H, multiplet), 10.61 (1H, carboxylic, disappeared on shaking with D_2O). Further, mass spectrum of the latter peak gave exactly the same spectrum as that of methyl 4,7-dicyanoheptanoate. Thus, the degradation products of TAN by *F. solani* TG-2 were determined to be a mixture of 5,7-dicyanoheptanoic acid (11%) and 4,7-dicyanoheptanoic acid (89%).

Isolation and determination of products from glutaronitrile and diacrylonitrile by F. merismoides TG-1

F. merismoides TG-1 was cultured for 4 days on the screening medium containing 0.3% glutaronitrile and 2.5% glycerol. A yellow oil (7.41 g) was obtained from 6 liters of the culture filtrate with the same procedures as described above. The properties of the product were as follows. *Anal.* Found: C, 52.88; H, 6.23; N, 12.22: Calcd. for $C_5H_7O_2N$: C, 53.10; H, 6.19; N, 12.39%. The IR and ^{1}H-NMR spectra and mass spectrum of its methyl ester were identical with those of 4-cyanobutyric acid. 4-Cyanobutyric acid was previously isolated from the culture filtrate of *Pseudomonas* sp. K-9 by the author. *F. merismoides* TG-1 could accumulate it with a good yield without any accompanying glutaric acid.

F. merismoides TG-1 was cultured for 3 days on the basal medium containing 0.2% diacrylonitrile and 0.8% glycerol. The degradation product was isolated from 7 liters of the culture fil-

trate by a similar procedure to that described above involving Amberlite IRA-120B (H^+) treatment and Amberlite IRA-410 (OH^-) and silica gel column chromatographies. A yellow oil (8.25 g) was obtained. bp, 136-138°C (7.0 mm Hg). *Anal.* Found: C, 56.58; H, 7.27; N, 10.80: Calcd. for $C_6H_9O_2N$: C, 56.69; H, 7.09; N, 11.02. IR spectrum ν_{max}^{film} cm^{-1}: 3,200 (C-OH), 2,950, 2,230 (C≡N). 1,710 (C=O). ^{1}H-NMR $\delta_{Me_4Si}^{CDCl_3}$ ppm; 1.36 (3H, doublet, J = 7 Hz, methyl), 2.45-2.98 (3H, overlap of methylene and methine), 11.13 (1H, singlet, carboxylic, disappeared on shaking with D_2O). Figure 6 is the mass spectrum of the methylated derivative of the product. The molecular ion peak is not detectable. The spectrum revealed ion peaks at *m/z* 126 $[M^+ - \cdot CH_3]$, 110 $[M^+ - \cdot OCH_3]$, 82 $[CH_3CH(CN)CH_2CH_2]^+$, 74 $[CH_2{=}C(OH)OCH_3]^+$, 68 $[CH_3CH(CN)CH_2]^+$, 59 $[COOCH_3]^+$, and 55 $[CH_3CHCNH]^+$. It is clear that this compound is methyl 4-cyanopentanoate, because in the mass spectrum of 4-cyano-2-methylbutyrate, *m/z* 88 $[CH_3CH{=}C(OH)OCH_3]^+$ appeares instead of *m/z* 74 on McLafferty rearrangement (83,84).

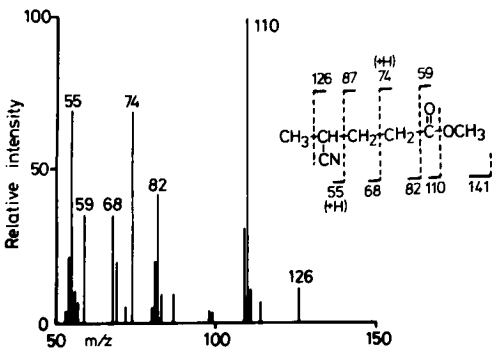


Fig. 6. Mass spectrum of Methyl 4-Cyanopentanoate.

Gass-chromatography-mass spectrometry was carried out with a Hitachi RMS-4 mass spectrometer-Hitachi M-5201 gas chromatograph equipped with an electron impact ionization system and gas chromatograms were detected by a total ion monitor. Operational conditions were: injection temperature, 235°C; column temperature, 115°C; interface temperature, 185°C; ionization voltage, 20 eV; carrier gas, He. The column used was a glass column of 3 mm inside diameter and 1 m in length, packed with Silicone OV-17.

DISCUSSION

Although there has been a report on TAN-utilizing microorganisms (45), their growth conditions or degradation products were not described at all. The author obtained microorganisms

which can utilize TAN as a sole nitrogen source. TAN disappearance and a simultaneous formation of ammonia courred when the cells of *F. merismoides* TG-1, grown on TAN and glycerol, were incubated with TAN. A mixture of 5,7-dicyanoheptanoic acid and 4,7-dicyanoheptanoic acid was isolated from the culture broth of *F. merismoides* TG-1 and *F. solani* TG-2, indicating that the degradation of TAN proceed as shown in Fig. 7. Any metabolites such as 1,3,6-hexanetricarboxylic acid was not acculmultaed in the culture broth under any culture conditions tested. When the mixture of the products were used as the nitrogen source, both the fungi could not grow. Further studies on their enzyme systems responsible for the hydrolysis of cyano group may prove the reason. The difference between the ratio of two hydrolysis products by the two strains may be ascribed to the differences in the substrate specificities of the enzyme systems. The degradation of dinitriles by *F. merismoides* TG-1 is also summarized in Fig. 7.

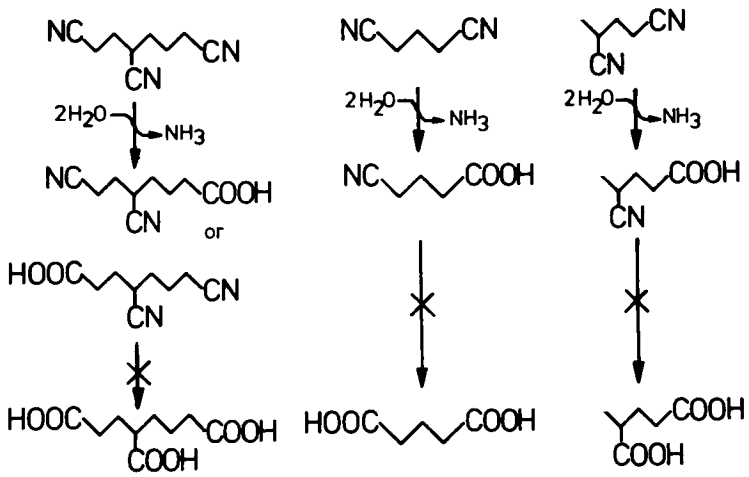


Fig. 7. Fungal Degradation of Di- and Trinitriles.

SUMMARY

Two fungal strains, TG-1 and TG-2, utilizing triacrylonitrile (1,3,6-hexanetricarbonitrile, TAN) as nitrogen source, were isolated from soil and identified as *Fusarium merismoides* Corda var. *merismoides* and *Fusarium solani* (Mart.) Sacc. emend. Snyder *et* Hansen var. *solani*, respectively. *F. merismoides* TG-1 could utilize TAN, adiponitrile, glutaronitrile, diacrylonitrile and 2,4-dicyano-1-butene as nitrogen source. Conditions for cultivation of the strain were studied. Degradation products of TAN were isolated from the culture broth and determined to be a mixture of 5,7-dicyanoheptanoic acid (62%) and 4,7-dicyanoheptanoic acid (38%). A mixture of 5,7-dicyanoheptanoic acid (11%) and 4,7-dicyanoheptanoic acid (89%) was also obtained from the culture broth of *F. solani* TG-2. The degradation products of glutaronitrile and diacrylonitrile by *F. merismoides* TG-1 were determined to be 4-cyanobutyric acid and 4-cyanopentanoic acid, respectively.

CHAPTER II Enzymatic Studies on the Degradation of Acetonitrile by *Arthrobacter* sp. J-1

Section 1 Aliphatic Nitrile Hydratase from *Arthrobacter* sp. J-1 -Purification and Characterization-[e,f)]

Since Thimann and Mahadevan demonstrated that nitrilase (EC 3.5.5.1) purified from barley leaves catalyzed the hydrolysis of indoleacetonitrile to indoleacetic acid and ammonia, several nitrilases have been found and characterized (36,37.49-51,52-54). Nitrilase has been defined to hydrolyze nitrile to the corresponding carboxylic acid and ammonia without forming amide as an intermediate, and does not use amide as substrate. However, a small but a constant amount of amide was detected in a reaction mixture of partially purified ricinine nitrilase (EC 3.5.5.2) (49-51). On the other hand, the formation of amides during the growth of microorganisms on aliphatic nitriles has been reported by several workers (46,47,61,62). A question remained unsolved whether the enzymatic hydrolysis of an aliphatic nitrile is catalyzed by more than one enzyme (56,57).

In the course of investigation on the microbial degradation of nitrile compounds, the author found a new enzyme, which catalyzed the hydrolysis of acetonitrile to form ammonia through acetamide in cooperation of amidase, in *Arthrobacter* sp. J-1. The enzyme was apparently different from known nitrilases.

This section describes the formation of nitrile hydratase and amidase, and purification and some properties of the former enzyme from *Arthrobacter* sp. J-1.

MATERIALS AND METHODS

Materials. Hydroxyapatite was prepared according to the method of Tiselius *et al.* (85). Dimethylsuberimidate dihydrochlo-

ride was purchased from Wako Chemicals Ltd., Osaka. Other chemicals were usual commercial products.

Microorganisms and cultivation. *Arthrobacter* sp. J-1, which could grow on acetonitrile as the sole source of carbon and nitrogen, was used. The culture medium contained 3.9 g acetonitrile, 13.4 g K_2HPO_4, 6.5 g KH_2PO_4, 1 g NaCl, 0.2 g NaCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 2 μg biotin, 0.4 mg calcium pantothenate, 2 mg inositol, 0.4 mg nicotinic acid, 0.4 mg thiamin·HCl, 0.4 mg pyridoxine·HCl, 0.2 mg *p*-aminobenzoic acid, 0.2 mg riboflavin and 0.01 mg folic acid in 1 liter of tap water, pH 7.0. Cells were aerobically grown at 28°C. Cell growth was esimated turbidimetrically: 0.65 mg dry cell weight per ml was equivalent to 1.0 unit of OD_{610}.

Assay methods. Nitrile hydratase activity was routinely assayed by measuring the production of ammonia from nitriles in the presence of amidase. The standard reaction mixture contained 50 μmol of potassium phosphate buffer, pH 7.0, 5 μmol of acetonitrile, 0.11 units of amidase of *Arthrobacter* sp. J-1 from step 5 of purification procedure (Section 3 of this chapter) and enzyme solution in a total volume of 0.5 ml. The reaction was started by an addition of acetonitrile and carried out at 30°C for 20 min. The amount of ammonia was measured by indophenol method (86). In this assay system, the enzyme activity was evaluated properly in proportion to the protein concentration of nitrile hydratase, when more than 0.1 units of amidase was added to the reaction mixture (Fig. 1A). The reaction was found to be linear against time and enzyme concentration (Fig. 1B).

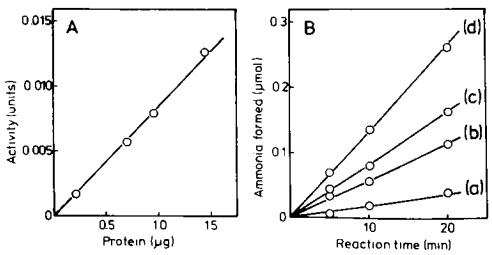


Fig. 1. Effects of Enzyme Concentration and Incubation Time on Nitrile Hydratase Activity.

The activity was assayed by measuring ammonia formation. The purified enzyme (9.17 units/mg) was used. Amidase (0.15 units) was added to the reaction mixture. In Fig. 1A, the incubation was carried out for 20 min. In Fig. 1B, the amount of the enzyme was (a), 0.21; (b), 0.70; (c), 0.94 and (d), 1.41 μg.

To estimate the amount of

acetamide, the reaction was carried out in the absence of amidase, terminated by an addition of 0.05 ml of 1 *N* HCl. The reaction mixture was applied to a Shimadzu gas-liquid chromatograph, Model GC-4CM, equipped with a flame ionization detector. The column used was a glass column of 3 mm in inside diameter, 1 m in length, packed with Porapack Q (80 to 100 mesh). Column, injection and detector temperatures were 210°C, 240°C and 240°C, respectively. The carrier gas was N_2 at 40 cm^3/min. The integration and calibration of peak area were carried out by a Shimadzu Chromatopack C-R1A.

Amidase activity was measured by the production of ammonia in the reaction mixture containing 50 μmol of potassium phosphate beffer, pH 7.0, 5 μmol of acetamide and enzyme solution in a total volume of 0.5 ml. The reaction was carried out at 30°C for 20 min.

One unit of enzyme activity was defined as the amount of enzyme which catalyzed the formation of 1 μmol of ammonia or amide.

Analytical methods. Protein was assayed by the method of Lowry *et al.* (87) or from the absorbance at 280 nm. The purity and sedimentation coefficient measurement was performed with a Spinco Model E analytical ultracentrifuge.

RESULTS

Formation of nitrile hydratase and amidase by Arthrobacter sp. J-1

Figure 2 shows the change in the specific activities of nitrile hydratase and amidase in *Arthrobacter* sp. J-1 during growth on acetonitrile. The specific activity of nitrile hydratase was high in the early logarithmic phase, around 12-hr cultivation, and thereafter rapidly decreased. The specific activity of amidase reached its maximum in the middle logarithmic phase. The successive formation of nitrile hydratase and amidase coincides well with the result that the concentration of acetamide in the culture broth was maximum (about 10 mM) around 12-hr cultivation and then

decreased to zero by 18 hr.

The effect of carbon and nitrogen sources on the activities of nitrile hydratase and amidase were examined. Table I shows that nitrile hydratase was inducibly formed when the strain was grown on acetonitrile, acetamide, and *n*-butyronitrile. Amidase was also induced when grown on acetonitrile, acetamide and acetate.

Purification of nitrile hydratase

All enzyme purification procedures were performed at 0-5°C and using potassium phosphate buffer, pH 7.0.

Step. 1. Preparation of cell-free extract. Washed cells

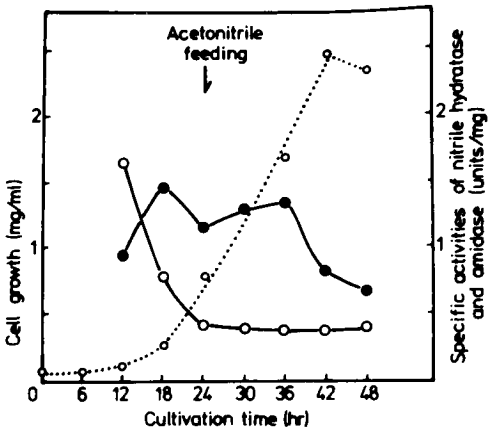


Fig. 2. Formation of Nitrile Hydratase and Amidase During Cultivation of *Arthrobacter* sp. J-1 on Acetonitrile.

The cultivation was carried out at 28 °C in 100-liter jar fermentor with 70 liters of the basal medium, under aeration of 35 liter/min and agitation at 280 rpm. Initial concentration of acetonitrile was 0.4% and 0.4% of acetonitrile was added to the medium at 24-hr cultivation time. The activity was assayed by ammonia formation. Nitrile hydratase (○); amidase (●) and growth (··○··).

Table I. Specific Activities of Nitrile Hydratase and Amidase in Cell-extract of *Arthrobacter* sp. J-1.

Washed cells of 2-days cultivation (33 mg) were inoculated into 500 ml of the basal medium containing growth substrates at the indicated concentrations (w/v). Cultivation was carried out in 2-liter shaking flasks. The activities in cell-free extract were assayed by measuring ammonia formation.

Growth substrate	Cultivation time (hr)	Specific activity (milli-units/mg)	
		Nitrile hydratase	Amidase
Acetonitrile (0.4%)	24	100	300
Acetamide (0.5%)	24	170	210
Sodium acetate (0.5%) + ammonium sulfate (0.2%)	48	13	89
n-Butyronitrile (0.4%)	36	96	7.2
Benzonitrile (0.1%)	48	0.30	2.0
Glucose (0.5%) + ammonium sulfate (0.2%)	48	9.0	1.0
Sodium succinate (0.5%) + ammonium sulfate (0.2%)	48 24	3.0	5.4

from 60 liters of culture were suppended in 0.1 M buffer and disrupted for 120 min on ice with a Kaijo-denki 19-KHz ultrasonic oscillator. The disrupted cells were centrifuged at 14,000 x *g* for 20 min.

Step. 2. Protamine sulfate treatment. To the resultant supernatant was added 5% protamine solution, to a final concentration of 0.1 g protamine per 1 g protein. After 30 min, the precipitate formed was removed by centrifugation at 14,000 x *g* for 20 min. The supernatant was dialyzed against 0.01 M buffer.

Step. 3. DEAE-cellulose column chromatography. The dialyzed solution was applied on a DEAE-cellulose column (10.5 x 20 cm) equilibrated with 0.01 M buffer. After washing the column with 0.05 M buffer containing 0.2 M NaCl, the enzyme was eluted with the same buffer containing 0.35 M NaCl.

Step. 4. Ammonium sulfate fractionation. Solid ammonium sulfate was added to the eluate (760 ml) to 40% saturation (185 g). After stirring for 30 min, the mixture was centrifuged at 14,000 x *g* for 30 min, and then solid ammonium sulfate was added to the supernatant to 60% saturation. The active precipitate was dissolved in a small volume of 0.01 M buffer and dialyzed against the same buffer.

Table II. Summary of Purification of Nitrile Hydratase from *Arthrobacter* sp. J-1.

The activity was assayed by measuring ammonia formation. The amount of protein was determined from the absorbance at 280 nm, except for the steps of cell-free extract and protamine sulfate.

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)
Cell-free extract	19,945	638	0.032
Protamine	10,230	528	0.052
DEAE-cellulose	377	423	1.12
Ammonium sulfate (40-60%)	232	513	2.21
1st Hydroxyapatite	52	298	5.70
2nd Hydroxyapatite	29	166	5.71
1st Sephadex G-200	12	84	7.28
2nd Sephadex G-200	7	63	9.17

Step. 5. First hydroxyapatite column chromatography. The dialyzed enzyme solution was placed on a hydroxyapatite column (5.2 x 13.5 cm) equilibrated with 0.01 M buffer. The enzyme was eluted with a linear concentration of buffer from 0.01 M to 0.06 M. The active fractions (519 ml) were concentrated by adding solid ammonium sulfate to 80% saturation. The precipitate collected by centrifugation was dissolved in a small volume of 0.01 M buffer and dialyzed against the same buffer.

Step. 6. Second hydroxyapatite column chromatography. The dialyzed enzyme solution was again applied on a hydroxyapatite column (4.9 x 6 cm), and eluted with a similar manner. The active fractions (414 ml) were concentrated by ultrafiltration.

Steps 7 and 8. Sephadex G-200 column chromatography. The concentrated enzyme solution was placed on a column of Sephadex G-200 (3.5 x 110 cm) equilibrated with 0.05 M buffer containing 0.1 M NaCl. The active fractions eluted with the same buffer were pooled and concentrated by ultrafiltration. This step was carried out twice.

The summary of purification of nitrile hydratase shows that about 290-fold purification with 10% yield from the cell-free extract was achieved. (Table II).

Purity of nitrile hydratatase

The purified enzyme was sedimented as a single symmetric peak

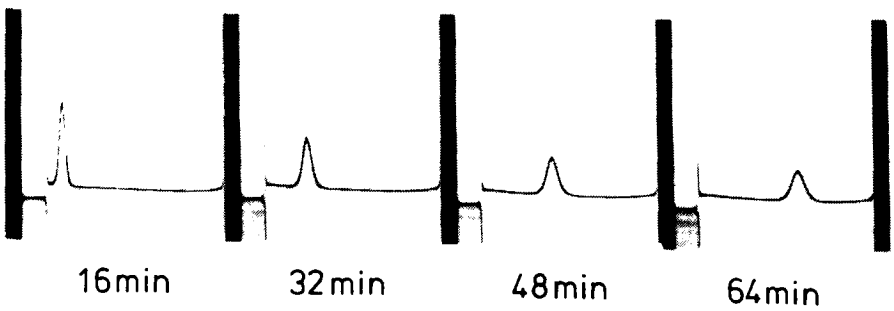


Fig. 3. Sedimentation Boundaries of Purified Enzyme.

Sedimentation was run at 42,040 rpm in sodium phosphate buffer, pH 7.0. Sedimentation is from left to right.

in the ultracentrifuge as shown in Fig. 3. Assuming a partial specific volume of 0.75, the sedimentation coefficient ($s_{20,w}$) was estimated to be 15.4 S at the concentration of 0.25%. Figure 4 shows the result of disc gel electrophoresis of the purified enzyme after a treatment with dimethylsuberimidate, which is a specific cross-linking reagent between protomers of a protein (88)

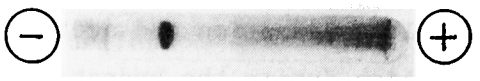


Fig. 4. Polyacrylamide Gel Electrophoresis of Purified Enzyme.

Purified enzyme (150 μg) was mixed with 36 μmol of Triethanolamine-NaOH buffer, pH 8.2 and 900 μg of dimethylsuberimidate dihydrochloride in a total volume of 240 μl and incubated at room temperature for 12 hr. The treated enzyme (6 μg) was applied to the electrophoresis at a current of 5 mA.

Identification of reaction product

Identification of reaction product by nitrile hydratase was performed with acetonitrile as substrate by gas-liquid chromatographies. As shown in Table III, the retention times of the reaction product were identical with those of authentic acetamide.

Table III. Identification of Reaction Product by Gas-liquid Chromatography.

The reaction was performed under the standard conditions. Nitrile hydratase (2.91 μg) was used.

Sample	t_R (min)			
	Column*			
	A	B	C	D
Reaction product	20.5	10.9	17.8	14.2
Acetamide	20.1	10.9	17.7	14.3

*, columns used and other operational conditions were:
A, Porapack Q (80 to 100 mesh), 3 mm x 2 m, column temperature, 170°C, injection and detector temperature, 200°C.
B, Tenax GC (80 to 100 mesh), 3 mm x 2 m, column temperature, 150°C, injection and detector temperature, 200°C.
C, Thermon-1000 + H_3PO_4 (5 + 0.5%) on Chromosorb W (80 to 100 mesh), 3 mm x 2 m, column temperature, 120°C, injection and detector temperature, 150°C.
D, Thermon-1000 + H_3PO_4 (10 + 0.5%) on Chromosorb W (80 to 100 mesh), 3 mm x 2 m, column temperature, 140°C, injection and detector temperature, 170°C.

Stoichiometry of acetonitrile hydration

The stoichiometry of acetonitrile consumption and acetamide formation during the hydration of acetonitrile was examined by gas-liqiud chromatography. As shown in Fig. 5, it was found that 1 mol of acetamide was produced per 1 mol of acetonitrile consumed. The enzyme did not hydrolyze acetonitrile directly to acetic acid and ammonia and did not have amidase activity. Based on the results, the enzyme was proved to catalyze the following reaction:

$$CH_3CN + H_2O \longrightarrow CH_3CONH_2.$$

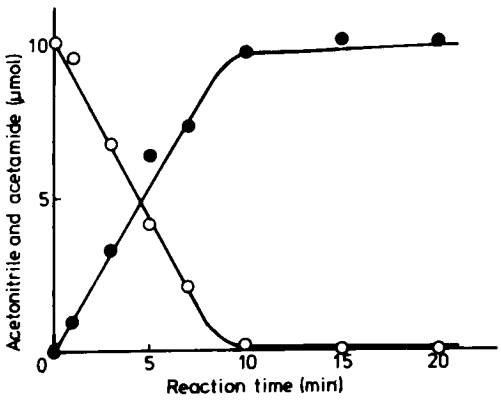


Fig. 5. Time Course of Acetonitrile Hydration by the Enzyme.

The reaction mixture contained 1 unit of the enzyme, 10 μmol of acetonitrile and 100 μmol of potassium phosphate buffer, pH 7.0. The concentration of acetonitrile and acetamide was determined by gas-liquid chromatography. Acetonitrile (○) and acetamide (●).

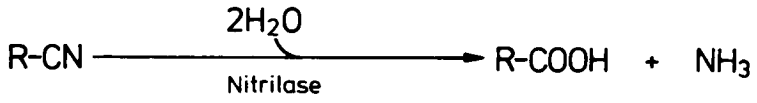
DISCUSSION

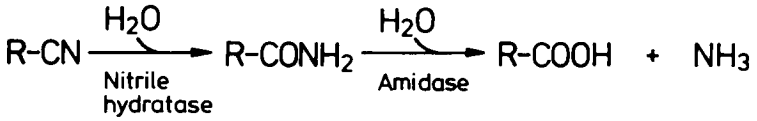
Nitrile hydratase which was inducibly formed in the cells of acetonitrile-grown *Arthrobacter* sp. J-1 was purified. The enzyme was characterized to catalyze the formation of acetamide from acetonitrile. No activity to hydrolyze acetonitrile directly to acetic acid and no amidase activity were detected.

Nitrilases which degrade nitrile to the corresponding carboxylic acid and ammonia have been well-characterized (36,37,49-51,52-54). However, the enzyme to form amide by the hydration of nitrile has not been known except for a few instances of conversion of specific nitriles. Enzymes which catalyzed the hydration of β-cyanoalanine and toyocamycin were found in blue lupin (24) and in *Streptomyces rimous* (51) producing the antibiotic, respectively.

The hydration of potassium cyanide was also shown by formamide hydro-lyase (74). These enzymes have not been purified to homogeneity and not characterized well. None of them were active toward low molecular weight aliphatic nitriles.

The enzyme in the present section was active on low molecular weight aliphatic nitriles to form corresponding amides, and no carboxylic acid and ammonia have been detected. The enzyme was completely different from known nitrilases or other hydrolases (51,74) acting on nitrile group in the reaction mode and substrate specificity. Therefore, the author proposes that the new enzyme should be called "aliphatic nitrile hydratase", because nitrilase is defined to catalyze the direct hydrolysis of nitrile to carboxylic acid and ammonia:





Details of the substrate specificities and the other properties of nitrile hydratase and amidase will be described in the following sections.

SUMMARY

A new enzyme, nitrile hydratase which hydrates acetonitrile to form acetamide, was purified from the cell-free extract of acetonitrile-grown *Arthrobacter* sp. J-1 by a procedure involving protamine sulfate precipitation, ammonium sulfate fractionation, column chromatographies on DEAE-cellulose, hydroxyapatite and Sephadex G-200. The overall purification was about 290-fold with a yield of 10%. The purified enzyme was homogeneous as judged by ultracentrifugation and disc gel electrophoresis. The enzyme catalyzed the stoichiometric hydration of acetonitrile to form acetamide according to the following scheme: $CH_3CN + H_2O \longrightarrow CH_3CONH_2$. Nitrile hydratase was inducibly formed and then amidase which hydrolyzed acetamide was formed.

Section 2 Properties of Aliphatic Nitrile Hydratase from *Arthrobacter* sp. J-1[g)]

In the previous section, the author found a new enzyme "aliphatic nitrile hydratase" which formed stoichiometrically acetamide from acetonitrile without accompanying acetic acid or ammonia as the following scheme: $CH_3CN + H_2O \longrightarrow CH_3CONH_2$. The enzyme was purified from the cell-free extract of acetonitrile-grown *Arthrobacter* sp. J-1 to a homogeneous state by a procedure involving protamine sulfate precipitation, ammonium sulfate fractionation, column chromatographies on DEAE-cellulose, hydroxyapatite and Sephadex G-200. The enzyme was quite different from known nitrilases (36,37.48-50,52-54) or other hydrolases acting on nitrile group (24,51) in the reaction product and the substrate specificity.

This section describes the physicochemical and enzymological properties of the nitrile hydratase from *Arthrobacter* sp. J-1.

MATERIALS AND METHODS

Materials. Nitrile hydratase was purified from the cell-free extract of acetonitrile-grown *Arthrobacter* sp. J-1 as described in the previous section. The standard proteins for the determination were purchased from Boehringer Mannheim GmbH and Pharmacia Fine Chemicals. Other chemicals are usual commercial products.

Assay methods. Nitrile hydratase and amidase activities were assayed with acetonitrile and acetamide as substrate, respectively, as described in the previous section.

Analytical methods. Protein concentration of nitrile hydratase was determined from the absorbance at 280 nm using the $E^{1\%}_{1cm}$ value of 26.6 which was obtained from dry weight determination.

Mass spectra were measured with a Hitachi M-70 gas chromatography-mass spectrometer (GC-MS). The column used was a glass

column of 3 mm in inside diameter, 1 m in length, packed with Tenax GC. Operational conditions were: injection temperature, 242°C; column temperature, 193°C; interface temperature, 248°C; chamber temperature, 185°C; ionization voltage, 20 eV; carrier gas, He at 14 cm^3/min.

The molecular weight of the enzyme was estimated by the method of Andrews (89), using a 0.9 x 115 cm column of Sepharose 6B in 0.05 M potassium phosphate buffer, pH 7.0, containing 0.1 M NaCl. The molecular weight of the subunit was determined by polyacrylamide gel electrophoresis according to the method of Weber and Osborn (90).

Isoelectric focusing. Isoelectric focusing was performed according to the method of Vesterberg (91). Electrophoresis was carried out at 5°C for 70 hr using Ampholite (LKB produkter) at pH range of 4 to 6.

RESULTS

Molecular weight and isoelectric point

The molecular weight of the native enzyme was calculated to be approximately 420,000 by gel filtration on Sepharose 6B (Fig. 1). The enzyme gave two bands in sodium dodecylsulfate (SDS)-disc gel electrophoresis as shown in Fig. 2. The molecular weight of the bands were calculated to be 24,000 and 27,000, and absorption at 600 nm was 41:59 in the ratio, as estimated by a densitometer. The enzyme had an isoelectric point of pH 3.6 as shown in Fig. 3.

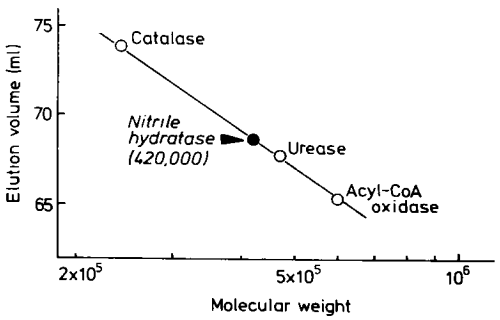


Fig. 1. Determination of Molecular Weight of the Enzyme by Gel Filtration on Sepharose 6B.

Experimental conditions are described in Materials and Methods. The molecular weights of reference proteins were: catalase, 240,000; urease, 480,000 and acyl-CoA oxidase, 600,000.

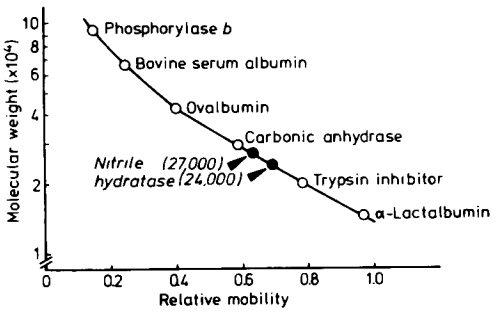


Fig. 2. (A) SDS-Polyacrylamide Gel Electrophoresis of Purified Enzyme.

Purified enzyme was incubated in the presence of 1% SDS and 3% 2-mercaptoethanol at 95°C for 3 min. The enzyme (12 μg) was applied to the electrophoresis in the presence of 0.1% SDS at a current of 8 mA per tube. The gels were stained with Coomasie Brilliant Blue R-250.

(B) Determination of Molecular Weight of Subunits of the Enzyme by SDS-disc Gel Electrophoresis.

The experimental conditions are as described in Fig. 2 (A). The moleculer weights of reference proteins were: α-lactalbumin, 14,400; trypsin inhibitor, 20,100; carbonic anhydrase, 30,000; bovine serum albumin, 68,000 and phosphorylase *b*, 94,000.

Absorption spectrum

The absorption spectrum of the purified enzyme in 0.01 M potassium phosphate buffer, pH 7.0, showed a maximum of absorbance at 280 nm, with a small shoulder at 290 nm.

Effect of temperature and pH on the enzyme activity

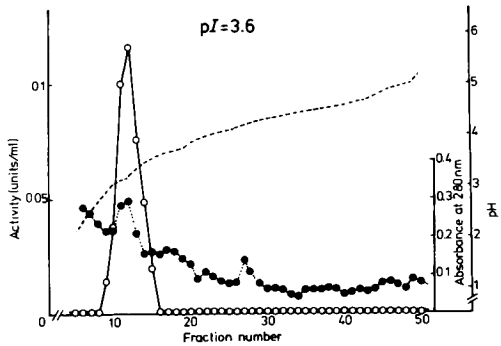


Fig. 3. Isoelectric Focusing of the Enzyme.

The enzyme (0.188 mg) was used. The enzyme activity was measured by amide formation with gas-liquid chromatography. Enzyme activity (○) and absorbance at 280 nm (●).

The optimum temperature of the enzyme was found to be 35°C (Fig. 4A). The activation energy below 35°C was calculated to be 9580 cal/mol. Eighty percent of the activity was retained after incubation at 35°C for 10 min, whereas the enzyme was nearly completely inactivated at 55°C for 10 min (Fig. 4B). The enzyme exhibited the maximal activity at pH 7.0-7.2 (Fig. 5A) and was stable around pH 6 (Fig. 5B). Glycine-NaOH buffer activated the en-

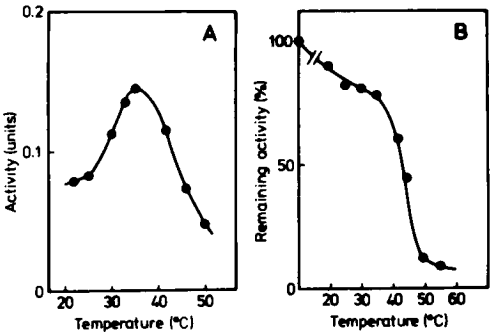


Fig. 4. Effect of Temperature on Activity (A) and Stability (B) on the Enzyme.

(A) The enzyme activity was measured after a termination of the reaction by heating. Formed acetamide was determined by ammonia formation with amidase.
(B) The enzyme in 0.1 M buffer was heated for 10 min at the temperature indicated. The remaining activity was measured by ammonia formation with amidase.

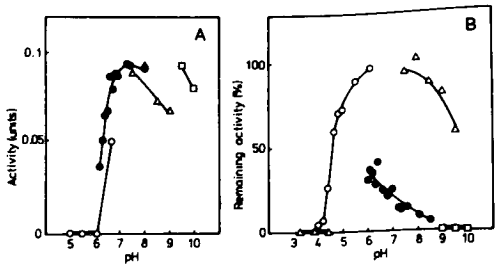


Fig. 5. Effect of pH on Activity (A) and Stability (B) of the Enzyme.

(A) The enzyme activity was measured by amide formation with gas-liquid chromatography.
(B) The enzyme in 0.1 M buffer was incubated at 50°C for 10 min. The remaining activity was measured by ammonia foramtion. As Tris-HCl buffer interfered with the color formation of indophenol, ammonia concentration was measured after diffusing with Conway microdiffusion apparatus. The buffers used were: sodium phosphate-citrate buffer (▲); sodium acetate buffer (○); potassium phosphate buffer (●); Tris-HCl buffer (△); glycine-NaOH buffer (□).

zyme and potassium phosphate buffer had a negative effect on the enzyme stability.

Substrate specificity

The enzyme activity toward various nitriles was examined at a concentration of 10 mM. Table I shows that chloroacetonitrile, acetonitrile, propionitrile, *n*-butyronitrile, hydroxyacetonitrile, acrylonitrile and methacrylonitrile are significantly active as the substrate for the enzyme. Aliphatic nitriles with more than five carbon atoms, aromatic nitriles and various amides tested were inactive as substrate. These include isobutyronitrile, capronitrile, crotonitrile, lactonitrile, succinonitrile, glutaronitrile, adiponitrile, 2,4-dicyano-1-butene, benzonitrile, benzylcyanide, potassium cyanide, α-cyanohydrin, β-cyano-L-alanine, formamide, acetamide, acrylamide, propionamide, *n*-butyramide, isobutyramide, methacrylamide, *n*-valeramide, succinamide, benzamide, phenylacetamide and lactamide. The enzyme did not catalyze the

Table I. Substrate Specificity of the Enzyme.

Substrate	Structure	Relative activity (%)	*Km* (mM)
Chloroacetonitrile	$ClCH_2CN$	130	10.9
Acetonitrile[a]	CH_3CN	100	5.78
Propionitrile[a]	CH_3CH_2CN	81.0	1.90
n-Butyronitrile	$CH_3CH_2CH_2CN$	59.8	10.8
n-Valeronitrile	$CH_3CH_2CH_2CH_2CN$	2.4	-
Hydroxyacetonitrile[b,c]	$HOCH_2CN$	30.0	-
Acrylonitrile[a]	$CH_2=CHCN$	25.5	0.88
Methacrylonitrile	$CH_2=C(CH_3)CN$	15.5	8.77
Methoxyacetonitrile[b,d]	CH_3OCH_2CN	10.0	
α-Cyanoacetamide	H_2NOCH_2CN	5.3	
Malononitrile	$NCCH_2CN$	4.5	

The enzyme activities toward nitriles except for those marked "a" and "b" were assayed by amide formation with gas-liquid chromatography. The enzyme activities toward amides (except for α-cyanoacetamide) were assayed by ammonia formation without an addition of amidase.
a, Assayed by ammonia formation with an addition of amidase.
b, Assayed by substrate consumption with gas-liquid chromatography.
c, The reaction product was identified as hydroxyacetamide by GC-MS analysis. The spectrum revealed ion peaks at m/z 75 $[M]^{+\cdot}$, 73 $[M^{+\cdot} - H_2]$, 44 $[O{\equiv}CNH_2]^+$ (base peak).
d, The reaction product was identified as methoxyacetamide by GC-MS analysis. The spectrum revealed ion peaks at m/z 90 $[M + 1]^+$, 89 $[M]^{+\cdot}$, 70, 59 $[CH_2CONH_2]^+$ (base peak), 44 $[CONH_2]^+$

hydrolysis of nitrile or amide to carboxylic acid and ammonia for all nitriles or amides tested.

Effect of metal ions and inhibitors

The effect of metal ions and inhibitors at 1 mM concentration on the enzyme activity is shown in Table II. The enzyme activity was strongly inhibited by sulfhydryl reagents such as Ag^+, Hg^{2+}, iodoacetate and *p*-chloromercuribenzoate. The inhibition was counteracted by an addition of 2-mercaptoethanol as shown in Table III.

Potassium cyanide competitively inhibited the enzyme activity and the inhibition was recovered by overnight dialysis. As shown in Fig. 6, the inhibition was competitive with acetonitrile as substrate. *Ki* value was 1.5 μM. The enzyme was inhibited by isobutyronitrile and glutaronitrile, which presumably act as substrate

Table II. Effect of Metal Ions and Inhibitors on the Enzyme.

The enzyme activity was measured by amide formation with gas-liquid chromatography, after the enzyme was preincubated at 30°C for 10 min with various metal ions or inhibitors at the concentration indicated.

Metal (1 mM)	Relative rate (%)	Inhibitor (1 mM)	Relative rate (%)
None	100	None	100
LiCl	96	NaN_3	101
NaCl	91	HCN (0.1 mM)	0
$AgNO_3$	0	Hydroxylamine (10 mM)	98
$MgCl_2$	96	*o*-Phenanthroline	96
$CaCl_2$	100	α,α'-Dipyridyl	97
$BaCl_2$	85	8-Oxyquinoline	98
$MnCl_2$	112	EDTA	98
$ZnCl_2$	92	5,5'-Dithio-bis(2-nitrobenzoic acid)	100
$CdCl_2$	92	*N*-Ethylmaleimide	112
$SnCl_2$	97	Iodoacetic acid	23
$PbCl_2$	99	*p*-Chloromercuribenzoate	59
$NiCl_2$	104	Urea	97
$CuCl_2$	99	Acrylamide	118
$HgCl_2$ (0.01 mM)	0		
$FeCl_3$	95		
$AlCl_3$	94		

Table III. Effect of SH-Inhibitors on the Enzyme Activity with or without 2-Mercaptoethanol.

The enzyme activity was measured as described in Table II with or without 2-mercaptoethanol at 1 mM before an addition of substrate.

SH-Inhibitor	Concentration (mM)	2-Mercaptoethanol (1 mM)	
		−	+
None		100%	98%
$HgCl_2$	0.001	34	105
	0.01	0	101
p-Chloromercuri-benzoate	0.1	88	98
	1.0	59	94
	5.0	16	67
Iodoacetate	1.0	23	105
	10.0	0	95

analogs. Urea, acrylamide, propionamide, *n*-butyramide, methacrylamide, benzamide did not inhibit the enzyme activity at 10 mM.

Stability

The enzyme was stable to the storage at -20°C in the presence of 50% glycerol for at least 40 days at pH 7.0 and about 60% of the activity was remained after 6 months storage. Half of initial activity was lost with a storage at 0°C for 40 days, pH 7.0.

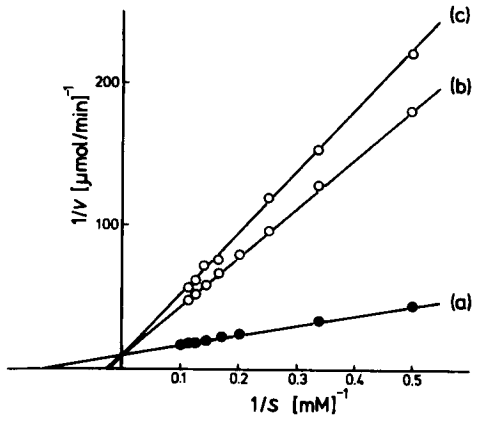


Fig. 6. Inhibition of the Enzyme by Potassium Cyanide.

Potassium cyanide was added to the reaction mixture at the concentrations of 0 mM (A), 0.005 mM (B) and 0.01 mM (C). The enzyme activity was assayed by ammonia formation, acetonitrile as a substrate. Amidase was not inhibited by potassium cyanide at the concentration.

DISCUSSION

A new enzyme, aliphatic nitrile hydratase, which was purified as shown to be completely different from nitrilase (EC 3.5.5.1), ricinine nitrilase (EC 3.5.5.2) and other hydrolase acting on nitrile group or cyanide, was characterized in detail in the present investigation.

The inhibition by potassium cyanide, which was an inhibitor of the enzyme having metal, carbonyl or persulfide group at the active center (92,93), was peculiar in this enzyme. The inhibition was restored by overnight dialysis and was competitive type with Ki value of 1.5 μM. There has been no report to show that cyanide acts as a competitive inhibitor, even with enzymes concerning with nitrile as substrate. The enzyme was strongly inactivated by metal chelating or carbonyl reagents, but strongly inactivated by sulfhydryl reagents. These results indicate that the enzyme has neither metal nor carbonyl group at the active centor but reactive sulfhydryl group.

Considering the mechanisms for nitrile hydrolysis by acid or base (94) and the existence of reactive sulfhydryl group in the

enzyme, proposed reaction mechanisms for nitrilase and nitrile hydratase are shown in Fig. 7. A mechanism of nitrile hydrolysis catalyzed by nitrilase, which was first proposed by Hook and Robinson (49), is presented in the equations (1) and (2). A nucleophilic attack by a reactive sulfhydryl group of the enzyme to the nitrile carbon forms an enzyme-bound imine, which is hydrated to form a tetrahedral intermediate (I). Acyl-enzyme is formed from the intermediate (I), with ammonia as a leaving group. The acyl-enzyme is then hydrolyzed to form a carboxylic acid. The reaction of nitrile hydratase in the present study which makes only amide would proceed as shown in the equations (1) and (3), in which the tetrahedral intermediate (I) decomposes to yield amide, with the enzyme as a leaving group. The enzyme produced only amide with-

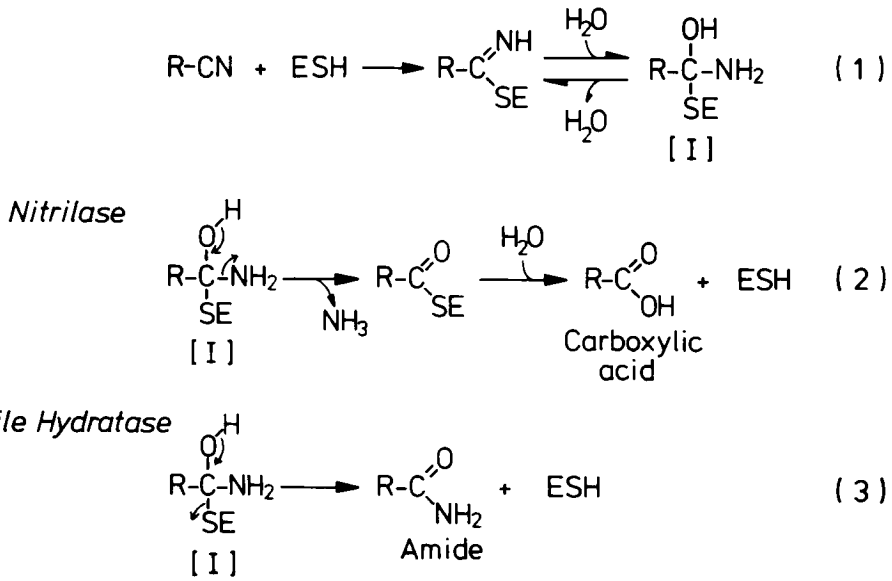


Fig. 7. Proposed Reaction Mechanisms of the Enzyme.

out accompanying carboxylic acid or ammonia from all substrates tested. The small but constant amount of amide was produced through the same pathway by ricinine nitrilase.

Harper showed in *Nocardia rhodochrous* (58,59) and *Fusarium solani* (60) that benzonitrile was directly hydrolyzed to benzoic acid and ammonia by nitrilase. *Arthrobacter* sp. J-1 also decomposed benzonitrile likewise, as it grows on benzonitrile as a sole source of carbon and nitrogen. These results suggest that there are at least two distinct pathways for nitrile hydrolysis, regardless of the species. One is a pathway in which both nitrile hydratase and amidase are involved, and the other is a pathway in which only nitrilase is involved. The chemical hydrolysis of acetonitrile, propionitrile and benzonitrile proceed exclusively *via* amide (38-41). Whether there is a correlation between the kind of nitrile and the hydrolitic enzyme or not would give an interest in enzymology. It may be possible to say that nitriles with saturated alkyl groups, such as acetonitrile, is hydrated with nitrile hydratase followed by hydrolysis of amide by amidase. On the other hand, as acrylonitrile is degraded directly to carboxylic acid and ammonia in *Arthrobacter* sp. I-9, it may be possible to say that nitriles can be directly hydrolyzed to carboxylic acid and ammonia if the nitrile group is conjugated with double bond(s) like acrylonitrile and benzonitrile.

The enzyme was active toward low molecular weight aliphatic nitriles with 2 to 5 carbon atoms. As the alkyl group of nitrile becomes longer, the enzyme becomes less active toward the substrate. Chloroacetonitrile was the best substrate for the enzyme. The electron-withdrawing effect of chlorine atom seems to favor the nucleophilic attack of sulfhydryl group of the enzyme to the nitrile carbon. However, malononitrile, which also has a strong electron-withdrawing group, cyano group, was not so active as chloroacetonitrile.

SUMMARY

The molecular weight of purified nitrile hydratase from *Arthrobacter* sp. J-1 was determined to be about 420,000 by gel filtration. The enzyme was composed of two kinds of subunits, of which molecular weights were 24,000 and 27,000. The isoelectric point was 3.6. The enzyme was active toward low molecular weight aliphatic nitriles of 2 to 5 carbon atoms. The *Km* value for acetonitrile was determined to be 5.78 mM. The enzyme had an optimal pH at 7 to 8. The enzyme was inactivated by sulfhydryl reagents such as Ag^{+}, Hg^{2+}, iodoacetate and *p*-chloromercuribenzoate. The enzyme was competitively inhibited by potassium cyanide: *Ki* value was 1.5 μM.

Section 3 Purification and Characterization of Amidase which Participates in Nitrile Degradation[h)]

In the previous section, the author described that a new enzyme "aliphatic nitrile hydratase" catalyzed the hydration of nitriles to amides in *Arthrobacter* sp. J-1 as follows: R-CN + H_2O ⟶ R-$CONH_2$. The activity of amidase to form acetic acid and ammonia from acetamide was also detected in the cell-free extract of acetonitrile-grown *Arthrobacter* sp. J-1. Both nitrile hydratase and amidase were induced during the exponential phase of cultivation when grown on acetonitrile as the sole source of carbon and nitrogen.

The author aimed to characterize the amidase, because little is known on amidase which participates in nitrile degradation (42, 43), and the substrate specificity of the enzyme is to be clarified for the measurement of nitrile hydratase activity.

This section describes the purification and characterization of amidase formed in *Arthrobacter* sp. J-1.

MATERIALS AND METHODS

Materials. Hydroxyapatite was prepared as described in Section 1 of this chapter.

Microorganisms and cultivation. The microorganisms and culture conditions were the same as described in Section 1 of this chapter.

Assay methods. The assay system was consisted of 50 μmol of acetamide and enzyme solution in a final volume of 0.5 ml. The mixture was incubated at 30°C for 20 min. Formed ammonia was estimated by indophenol method (86). Acyltransferase activity was assayed according to the method of Kelly and Kornberg (95), with acetamide and hydroxylamine as substrates. Acetohydroxamate was measured using succinic anhydride as a standard (96).

Analytical methods. Protein determination and ultracentrifugal analysis were performed as described in Section 1 of this chapter. The molecular weight of native enzyme was determined by polyacrylamide gel electrophoresis according to the method of Hedrick and Smith (97) and by gel filtration as described in Section 2 of this chapter. The molecular weight of the subunits were determined by polyacrylamide gel electrophoresis and isoelectric focusing was performed as described in the Section 2 of this chapter.

Enzyme purification. All the enzyme purification procedures were performed at temperature lower than 5°C. Potassium phosphate buffer, pH 7.0, was used throughout the purification.

Step. 1. Preparation of cell-free extract. Washed cells from 60 liters of culture were suspended in 0.1 M buffer and disrupted for 100 min on ice with a Kaijo-denki 19-KHz ultrasonic oscillator. The purified cells were centrifuged at 14,000 x *g* for 20 min.

Step. 2. Protamine sulfate precipitation. To the supernatant was added 5% protamine sulfate solution, to a final concnetration of 0.1 g protamine to 1 g protein. After 30 min, the precipitate formed was discarded by centrifugation at 14,000 x *g* for 20 min. The supernatant was dialyzed against 0.01 M buffer.

Step. 3. DEAE-cellulose column chromatography. The dialyzed solution was applied on a DEAE-cellulose column (10.5 x 20 cm) which had been equilibrated with 0.01 M buffer. The enzyme was eluted with 0.05 M buffer containing 0.35 M NaCl.

Step. 4. Ammonium sulfate fractionation. To the active fractions (760 ml) was added solid ammonium sulfate to 40% saturation (185 g) under stirring. After standing for 30 min, the precipitate formed was removed by centrifugation and discarded. Ammonium sulfate was added to the supernatant to 60% saturation. The precipitate formed was collected by centrifugation and dissolved in 0.01 M buffer and dialyzed against the same buffer.

Step. 5. Hydroxyapatite column chromatography. The dialyzed enzyme solution was placed on a hydroxyapatite column (5.2 x 13.5 cm) which had been equilibrated with 0.05 M buffer. The enzyme was eluted with a linear concentration of buffer from 0.01 M to 0.06 M. The active fractions (433 ml) were concentrated by adding solid ammonium sulfate to 80% saturation. The precipitate formed was collected by centrifugation and dissolved in 0.01 M buffer.

Steps 6-8. Sephadex G-200 column chromatography. The concentrated enzyme solution was placed on a column of Sephadex G-200 (3.5 x 110 cm), equilibrated with 0.05 M buffer containing 0.1 M NaCl. Active fractions eluted with the same buffer were concentrated by ultrafiltration. This step was repeated three times.

RESULTS

Purification of amidase

Table I shows the summary of purification of amidase. Forty-seven fold purification with 3.3% yield from the cell-free extract was achieved.

Table I. Summary of Purification of Amidase from *Arthrobacter* sp. J-1.

The amount of protein was determined from the absorbance at 280 nm, except for the steps of cell-free extract and protamine sulfate.

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)
Cell-free extract	19,945	3,044	0.153
Protamine sulfate	10,230	2,900	0.284
DEAE-cellulose	732	661	0.903
Ammonium sulfate (40-60%)	451	604	1.34
Hydroxyapatite	104	339	3.26
1st Sephadex G-200	39	178	4.56
2nd Sephadex G-200	22	152	6.91
3rd Sephadex G-200	14	100	7.14

Purity of amidase

The purified enzyme sedimented as a single symmetric peak in the ultracentrifuge in 0.1 M sodium phosphate buffer, pH 7.0. Assuming a partial specific volume of 0.75, the sedimentation coefficient ($s_{20,w}$) was estimated to be 9.3 S at the enzyme concentration of 0.72%. The enzyme was found to be homogeneous by criteria of disc gle electrophoresis (Fig. 1).

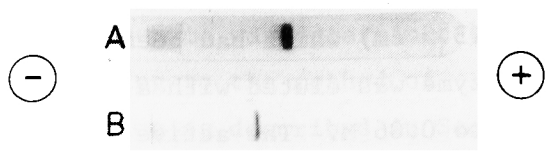


Fig. 1. Polyacrylamide Disc Gel Electrophoresis of Purified Enzyme.
(A) Purified enzyme (2 μg) was applied to the electrophoresis in the absence of SDS at a current of 2 mA.
(B) Purified enzyme was incubated in the presence of 1% SDS and 3% 2-mercaptoethanol at 95°C for 3 min. The enzyme (2 μg) was applied to the electrophoresis in the presence of 0.1% SDS at current of 8 mA per tube. The gels were stained with Coomasie Brilliant Blue R-250.

Molecular weight

As shown in Fig. 2, the molecular weight of purified amidase was estimated to be about 300,000 by disc gel electrophoresis on a series of polyacrylamide gels containing various concentrations of acrylamide. The molecular weight of amidase was also estimated to be approximately 320,000 by gel filtration.

The molecular weight of subunit of the enzyme was estimated to be approximately 39,000 by sodium dodecylsulfate (SDS)-disc gel electrophoresis. The native enzyme has possibly octameric structure consisted of eight identical subunits.

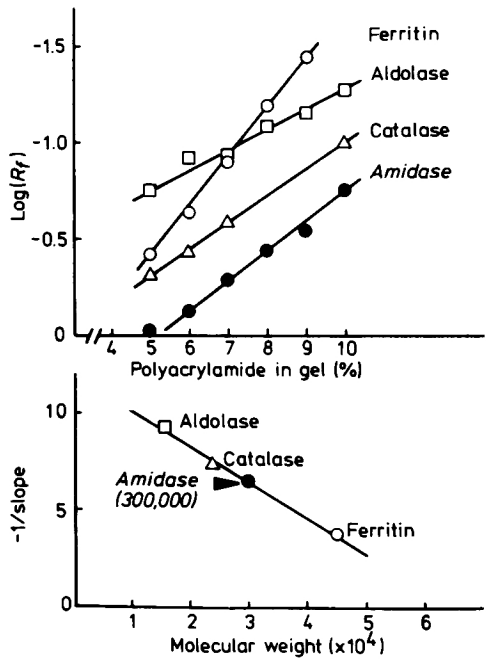


Fig. 2. Determination of Molecular Weight of Subunit of the Enzyme by SDS-disc Gel Electrophoresis.

The molecular weight of reference proteins were: aldolase, 158,000; catalase, 240,000 and ferritin, 450,000.

Isoelectric point

The isoelectric point of amidase was estimated to be pH 3.8 by electric focusing.

Absorption spectrum

The purified enzyme showed absorption maximum at 280 nm in 0.01 M buffer. The extinction coefficient ($E^{1\%}_{1cm}$) was calculated to be 13.7 by dry weight measurement.

Stoichiometry of the enzyme reaction

The stoichiometry of acetamide consumption, and acetic acid and ammonia formation during the hydrolysis of acetamide was examined. As shown in Fig. 3, it was found that 1 mol of acetic acid and ammonia were produced per 1 mol of acetmide consumed. Based on these results, the enzyme was proved to catalyze the following reaction:

$$CH_3CONH_2 + H_2O \longrightarrow CH_3COOH + NH_3$$

Effect of temperature and pH on the enzyme activity

The optimum temperature of the enzyme activity was found to

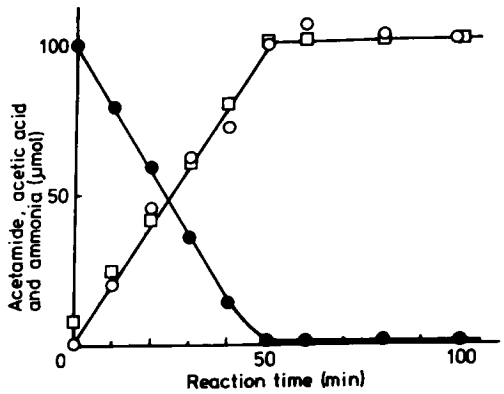


Fig. 3. Time Course of Acetamide Hydrolysis by the Enzyme.

The reaction mixture contained 1 mmol of potassium phosphate buffer, pH 7.0, 100 μmol of acetamide and 2 units of amidase in a total volume of 10 ml at 30°C. The reaction was terminated by HCl. Acetamide (●), acetic acid (□) and ammonia (○).

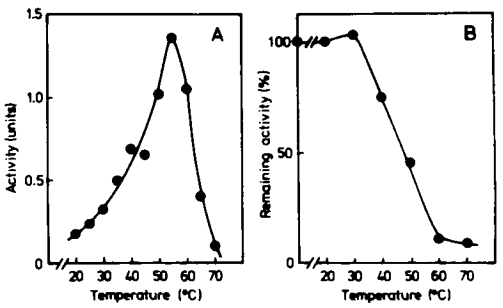


Fig. 4. Effect of Temperature on Activity (A) and Stability (B) of the Enzyme.

(A) The enzyme activity was measured under the standard conditions at various temperatures.
(B) The enzyme in 0.01 M buffer was heated for 10 min at the temperature indicated. The remaining activity was measured under the standard assay conditions.

be 55°C (Fig. 4 (A)). The enzyme activity was stable after incubation at 30°C for 10 min, whereas half of the enzyme activity was lost at 50°C for 10 min (Fig. 4 (B)). The enzyme exhibited an optimum for the activity at pH 7-9 (Fig. 5 (A)) and was stable around pH 7.0 (Fig. 5 (B)).

Substrate specificity

The apparent *Km* values estimated from double reciprocal plots were 0.97, 23.3 and 8.05 mM, for acetamide, acrylamide and propionamide, respectively (Fig. 6). The enzyme activity

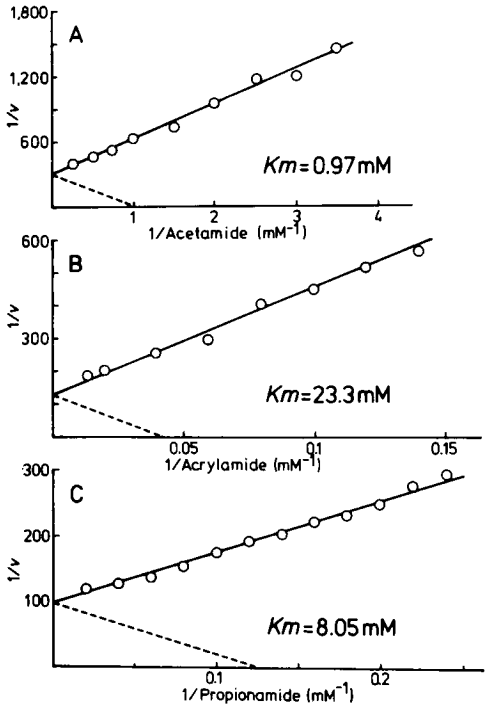


Fig. 6. Double Reciprocal Plots by the Enzyme.

(A) Acatamide, (B) acrylamide and (C) propionamide.

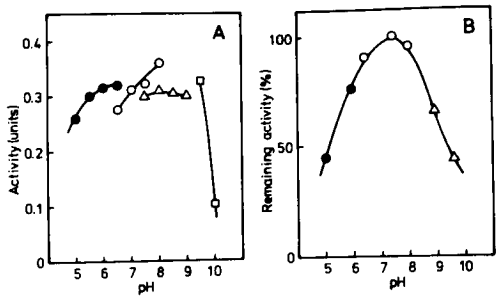


Fig. 5. Effect of pH on Activity (A) and Stability (B) of the Enzyme.

(A) The enzyme activity was measured under the standard assay conditions in 0.1 M buffer.
(B) The enzyme in 0.01 M buffer was incubated at 30°C for 60 min. The remaining activity was measured under the standard assay conditions. The buffers used were: sodium acetate buffer (●); potassium phosphate buffer (○); Tris-HCl buffer (△) and glycine-NaOH buffer (□).

Table II. Substrate Specificity of Amidase.

Substrate	*Km* (mM)	Relative* activity (%)
Acetamide	0.97	100
Acrylamide	23.3	224
Propionamide	8.05	303

*Relative *Vmax* values calculated from Fig. 6. The following compounds did not serve as substrate at the concentration of 10 mM: acetonitrile, acrylonitrile, propionitrile, *n*-butyronitrile, *n*-valeronitrile, *n*-capronitrile, isobutyronitrile, methacrylonitrile, crotonitrile, succinonitrile, glutaronitrile, benzonitrile, benzylcyanide, β-cyano-L-alanine, formamide, *n*-butyramide, *n*-valeramide, isobutyramide, methacrylamide, malonamide, succinamide, lactamide, α-cyanoacetamide, glycinamide, benzamide, phenylacetamide, urea, nicotinamide, L-glutamine and L-asparagine.

toward various nitriles and amides is shown in Table II. It shows that the enzyme hydrolyzed acetamide, acrylamide and propionamide, however, it does not act on other amide or nitriles tested.

Effect of metal ions and inhibitors

Table III. Effect of Metal Ions and Inhibitors on the Enzyme.

The enzyme activity was measured under the standard assay conditions after the enzyme was preincubated for 10 min with the metal ions or inhibitors at a concentration of 1 mM.

Metal (1 mM)	Relative rate (%)	Inhibitor (1 mM)	Relative rate (%)
None	100	None	100
LiCl	96	NaN_3	108
NaCl	98	KCN[a]	81
$AgNO_3$[a]	0	*o*-Phenanthroline	102
$MgCl_2$	102	α,α'-Dipyridyl[a]	96
$CaCl_2$	99	8-Oxyquinoline[a]	99
$BaCl_2$	96	EDTA	99
$MnCl_2$	100	5,5'-Dithio-bis(2-nitrobenzoic acid)	105
$ZnCl_2$[a]	92	*N*-Ethylmaleimide	90
$FeSO_4$	102	Iodoacetic acid	72
$NiCl_2$	97	*p*-Chloromercuribenzoate	101
$CuSO_4$[a]	53	Urea	16
$PbCl_2$	105	Thiourea	9
$HgCl_2$[a]	0		54
$FeCl_2$	101		
$AlCl_3$	98		

a, Formed ammonia was assayed after a diffusion with Conway microdiffusion apparatus because of the reagents marked "a" inhibited the color formation of indophenol.

Table IV. Effect of SH-Inhibitors on the Enzyme Activity with or without 2-Mercaptoethanol.

The enzyme activity was measured as described in Table III with or without 2-mercaptoethanol at 1 mM before an addition of substrate. Formed ammonia was measured after a diffusion with Conway microdiffusion apparatus.

SH-Inhibitor	Concentration (mM)	2-Mercaptoethanol (1 mM). -	+
None		100%	98%
$HgCl_2$	0.001	15	100
	0.01	8	98
	0.1	0	89
	1.0	0	9
$AgNO_3$	0.001	0	113
	0.01	0	103
	0.1	0	109
	1.0	0	5
p-Chloromercuri-benzoate	0.1	47	76
	1.0	8	68

The effect of metal ions and inhibitors on the enzyme activity is shown in Table III. The enzyme activity was markedly inhibited by sulfhydryl reagents such as Ag^{+}, Hg^{2+} and *p*-chloromercuribenzoate. Urea and thiourea also inhibited the enzyme activity. The inhibition was counteracted by an addition of 2-mercaptoethanol as shown in Table IV.

Acyltransferase activity

Amidase is known to catalyze the transfer of acyl moiety of amide to hydroxylamine besides amide hydrolysis (98,99). Acyltransferase activity was measured in the final stage of purification of amidase. Figure 7 shows that the elution pattern of acyltransferase activity on Sephadex G-200 column coincides with amidase activity and protein. It is evident that acyltransferase and amidase activities are shown by the same enzyme protein.

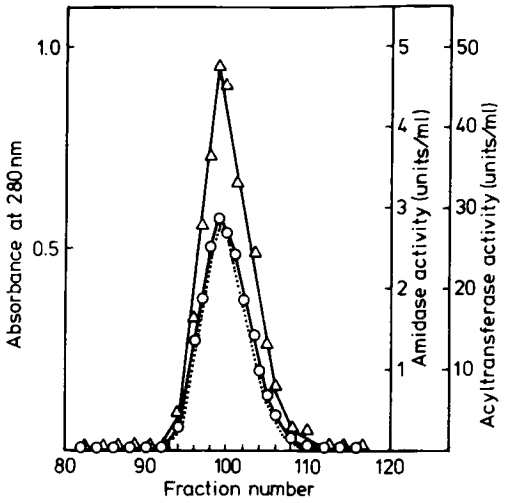


Fig. 7. Gel Filtration of Amidase and Acyltransferase on Sephadex G-200.

Six milliliters fractions were collected. Amidase (○), acyltransferase (△) and protein (····).

DISCUSSION

This section described the purification and properties of an amidase formed in *Arthrobacter* sp. J-1 grown on acetonitrile as the sole source of carbon and nitrogen. Amidase was purified about 47-fold from the cell-free extract to homogeneity.

A number of hydrolytic enzymes acting on linear amide are known. They include asparaginase, glutaminase (100) and amidase *etc*. Among them, *Pseudomonas* amidase has been intensively investigated by Clarke *et al*. (65-67) as an example of artificial evolution

with altered substrate specificities. Table V compares the properties of amidase from *Arthrobacter* sp. J-1 and *Pseudomonas aeruginosa*. The marked differences between them are the molecular weight and the number of subunits. *Pseudomonas* amidase catalyzes the hydrolysis of formamide and butyramide at one tenth and two hundredths of the rate of acetamide, respectively (66). The hydrolysis of formamide and butyramide by *Arthrobacter* amidase could not be detected. However, the enzyme exhibited the similar substrate specificities toward acetamide and propionamide, and the apparent affinities for these substrates were close. *Arthrobacter* amidase was not inhibited at least up to 50 mM of acrylamide, whereas *Pseudomonas* amidase was significantly inhibited (99). Both the enzyme were strongly inhibited by sulfhydryl reagents and urea, and showed acyltransferase activity. Thus, amidase purified from *Arthrobacter* sp. J-1 resembles acylamide

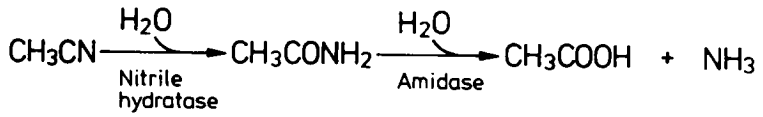
Table V. Comparison of Properties of Amidase from *Arthrobacter* sp. J-1 and *Pseudomonas aeruginosa*.

Property	Unit	*Arthrobacter* sp. J-1	*Pseudomonas aeruginosa*
$s_{20,w}$	S	9.3	10.6
Molecular weight		300,000 to 320,000	200,000
Subunit		8	6
pI		3.8	-
Optimum temperature	°C	55	-
Heat stability[a]	°C	50	-
Optimum pH		8.0	7.2
pH stability		7.4	-
Inhibition			
SH reagent		Yes	Yes
Metal ion		Ag^{+}, Hg^{2+}, Cu^{2+}	
Urea		Yes	Yes
Km, (V)	mM, (%)		
Acetamide		0.97 (100)	1 (100)
Acrylamide		23.3 (224)	(189)
Propionamide		8.05 (303)	21 (274)
Acyltransferase		+	+
Formation		Inducible	Inducible

a, A half of initial activity was lost by an addition at this temperature for 10 min.

amidohydrolase (EC 3.5.1.4) characterized in *P. aeruginosa* and *P. fluorescens* (101). Further, the enzyme had no nitrilase activity though asparaginase of *Escherichia coli* catalyzed the direct hydrolysis of β-cyanoalanine to aspartic acid and ammonia (24,68, 69).

As the author has discussed in Section 1 of this chapter, amidase is induced in the exponential phase of growth of *Arthrobacter* sp. J-1 on acetonitrile shortly after the maximum specific activity of nitrile hydratase. The role of amidase in the metabolism of acetonitrile by *Arthrobacter* sp. J-1 is to hydrolyze acetamide formed from acetonitrile by nitrile hydratase and to yield successively acetic acid and ammonia, which could be used for carbon and nitrogen sources for the synthesis of cell materials. The author proved for the first time with purified enzyme preparations that nitrilase-like activity detected in the cell-free extract of *Arthrobacter* sp. J-1 was a combination of two enzymes, nitrile hydratase and amidase as follows:



SUMMARY

Amidase was purified from the cell-free extract of acetonitrile-grown *Arthrobacter* sp. J-1 by a procedure involving protamine sulfate precipitation, ammonium sulfate fractionation, column chromatographies on DEAE-cellulose, hydroxyapatite and Sephadex G-200.

The overall purification was 47-fold. The purified enzyme was homogeneous as judged by ultracentrifugal analysis and disc gel electrophoresis. The molecular weight of the enzyme was estimated to be about 300,000 and 320,000 by disc gel electrophoresis and gel filtration, respectively. The enzyme was possibly composed of eight identical subunits of molecular weight of 39,000. The isoelectric point was 3.8. The enzyme catalyzed the stoichiometric hydrolysis of acetamide to form acetic acid and ammonia. The enzyme was active toward acetamide, acrylamide and propionamide and the *Km* values were 0.97, 23.3 and 8.05 mM, respectively. The enzyme showed acyltransferase activity.

CHAPTER III A New Enzymatic Method of Acrylamide Production[i)]

Acrylamide is industrially produced as a monomer for synthetic fibers, floculant agent, *etc.* The process involves hydration of acrylonitrile with sulfuric acid and ammonia. The acid hydrolysis of nitrile has difficulty in controlling the reaction which proceeds in a sequential formation of amide, carboxylic acid and ammonia. The process using sulfuric acid also yields ammonium sulfate as by-product. Recently, catalysts of copper salts or paradium complexes (102,103) have been developed for the selective hydration of nitriles to yield amide without using acid or base. However, the preparation of the catalysts is laborious and the process requires high temperature.

In the previous chapter, the author described that acetonitrile is hydrolyzed to acetic acid and ammonia with nitrile hydratase and amidase in *Arthrobacter* sp. J-1. Nitrile hydratase was purified to homogeneity and found to catalyze the stoichiometric hydration of nitrile to yield amide. Therefore, the author attempted to produce acrylamide from acrylonitrile using the microbial enzyme.

This chapter describes the screening of microorganisms which produce acrylamide from acrylonitrile, identification of the strain, and the culture and reaction conditions for the production of acrylamide.

MATERIALS AND METHODS

Isolation of nitrile-utilizing microorganisms. Nitrile-utilizing microorganisms were isolated from soil samples by an enrichment culture technique using medium containing 0.2% (v/v) nitrile with or without 0.5% (w/v) glycerol in the basal medium which was described in Section 2 of CHAPTER I. Microorganisms which utilized acetonitrile, propionitrile, acrylonitrile, isobutyronitrile, suc-

cinonitrile, glutaronitrile, adiponitrile and triacrylonitrile as a sole source of carbon and nitrogen or that of nitrogen were isolated.

Screening and assay method of acrylamide accumulating strain. The isolated strains were cultured aerobically at 28°C for 3 days on the isolation medium. The cells were centrifuged, washed with physiological saline and suspended in 0.1 M potassium phosphate buffer, pH 7.0. The reaction mixture for the screening of acrylamide-accumulating strains contained 100 μmol of potassium phosphate buffer, pH 7.0, 300 μmol of acrylonitrile as substrate, and washed cells from 3 ml of culture broth in a total volume of 1.0 ml. The reaction was carried out at 30°C for 1 hr with moderate shaking and terminated by an addition of 0.2 ml of 1 *N* HCl. The amount of acrylamide formed in the reaction mixture was determined by a Shimadzu gas-liquid chromatograph, Model GC-4CM, equipped with a flame ionization detector. The column used was a glass column of 3 mm in inside diameter, packed with Porapack Q (80 to 100 mesh). Operational conditions were: column temperature, 210°C; injection and detector temperature, 240°C. The carrier gas was N_2 at 40 cm^3/min. The integration and calibration of peak area were carried out by a Shimadzu Chromatopack C-R1A. One unit of acrylamide-forming activity was defined as the activity which catalyzed the formation of 1 μmol of acrylamide per min.

Preparation of cell-free extract and enzyme assay. The suspended cells obtained as described above were disrupted for 10 min on ice with a Kaijo-denki 19-KHz ultrasonic oscillator. The disrupted cells were centrifuged at 17,000 x *g* for 20 min at 5°C. The supernatant solution was dialyzed overnight against 0.01 M potassium phosphate buffer, pH 7.0, containing 1 mM 2-mercaptoethanol.

Nitrile hydratase and amidase activities were measured as described in CHAPTER II.

Isolation of acrylamide. Reaction mixture containing acrylamide was centrifuged to remove the cells. The supernatant was lyophilized and then extracted with methanol. After removing unsoluble residue by centrifugation, the extract was evapoated *in vacuo* at room temperature. Crude crystals were dissolved in warm methanol and filtered. Recrystallization from warm methanol gave colorless crystals.

Chemicals. Triacrylonitrile was prepared as described in Section 1 of CHAPTER I. Other chemicals were usual commercial products and used without further purification.

RESULTS

Screening of acrylamide producing strains

The ability to accumulate acrylamide from acrylonitrile was tested among a hundred and eighty-six strains which were isolated as utilizers of one of acetonitrile, propionitrile, acrylonitrile, isobutyronitrile, succinonitrile, adiponitrile and triacrylonitrile.

Table I shows the distribution of acrylamide producing strains among the nitrile-utilizers. High activity was shown in propionitrile- and isobutyronitrile-utilizing strains. Most of the di- and trinitrile utilizers did not accumulate either acrylamide nor acrylic acid. About ten acetonitrile-utilizing strains accumulated

Table I. Acrylamide Accumulation by Nitrile-utilizing Microorganisms.

Growth substrate	Number of strain tested	Number of acrylamide accumulating strain		
		<0.01	0.01-0.1 (units/ml)	>0.1
Acetonitrile	55	43	12	0
Propionitrile	47	40	4	3
Acrylonitrile	3	3	0	0
Isobutyronitrile	11	6	2	3
Succinonitrile	20	20	0	0
Adiponitrile	26	26	0	0
Triacrylonitrile	24	24	0	0
Total	186	162	18	6

higher amount of acrylic acid than that of acrylamide.

Identification of microorganisms

A bacterial strain, B23, which was isolated as isobutyronitrile-utilizing microorganism and chosen as the best producer of acrylamide, was identified taxonomically as follows.

Rods, measuring 0.8 to 1.1 by 1.6 to 2.7 μm, occurring singly. Non-spore-forming. Motile with one to three polar flagella. Gram-negative. Aminopeptidase-positive (104).

Growth on nutrient agar: circular, convex, glistening, butyrous. Crystals of pigment are produced in older nutrient agar slant. Nitrate reduction: positive. Indole and hydrogen sulfide formations: negative. Catalase and oxidase: positive. Hugh-Leifson: oxidation. Pigment formation on King's A medium: negative; King's B medium: fluorescent green pigment. Acid without gas from glucose, galactose, and mannose. No acid and gas from fructose, sucrose, lactose, maltose, glycerol, raffinose, dextrin, starch, inulin, glycogen and mannitol. Assimilation of carbon compounds: acetate, succinate, pyruvate, lactate, ethanol, glucose, arabinose, sucrose, sorbitol, trehalose, *meso*-inositol, L-alanine, L-valine, β-alanine, propylene glycol, α-ketoglutarate, L-glutamate and betaine are assimilated; DL-β-hydroxybutyrate, propionate, butyrate, methanol and geraniol are not assimilated. Growth temperature: between 5°C and 36.5°C, with the optimum at 31°C. Growth pH: between 6.0 and 9.9 with the optimum between 7.0 and 8.0. The G + C content of DNA was 64.6% by thermal denaturation method.

According to "Bergey's Manual of Determinative Bacteriology" 8th ed. (71), the strain was identical with *Pseudomonas chlororaphis*.

Identification of acrylamide

Acrylamide was isolated in crystalline form by the procedure described in Materials and Methods. mp, 85.0°C, *Anal.* Calcd. for C_3H_5ON: C, 50.69; H, 7.09; N, 19.71. Found: C, 50.68; H, 7.17;

N, 20.01. The infrared spectrum of the crystals coincided well with that of isolated acrylamide as shown in Fig. 1. ^{1}H-NMR spectrum of isolated acrylamide was identical with that of authentic acrylamide.

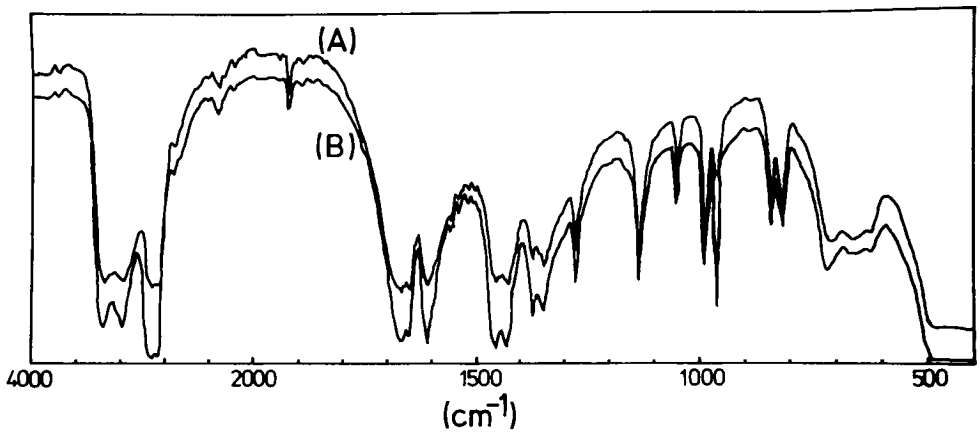


Fig. 1. IR Spectrum of Authentic (A) and Isolated (B) Acrylamide.

Culture conditions for the preparation of cells of P. chlororaphis B23 with high activity

Culture conditions to prepare the cells having the higher activity were investigated as follows.

1) Optimum temperature.

Figure 2 shows that the maximum activity was obtained when the strain was grown below 25°C, whereas the best growth was at 31 °C. The cells had no activity when it was grown above 35°C, although it grew well at the temperature.

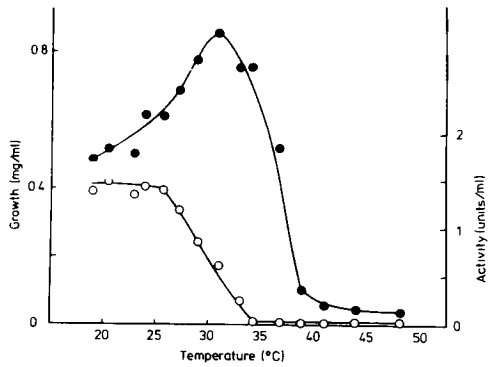


Fig. 2. Effect of Temperature on the Growth and Enzyme Formation of *P. chlororaphis* B23.

Washed cells (0.43 mg as dry weight) grown on the culture medium containing 0.15% isobutyronitrile and 0.5% glycerol were inoculated into 5 ml of the same medium. The cultivation was carried out for 2 days. The activity was measured as described in Materials and Methods. Growth (●) and enzyme activity (○).

2) Effect of carbon and nitrogen source. Table II shows that the enzyme activity was the highest when dextrin was used as carbon source. As shown in Table III, isobutyronitrile was the

Table II. Effect of Carbon Source on the Production of Acrylamide.

Washed cells (24 μg as dry weight) grown on the culture medium containing 0.15% (w/v) isobutyronitrile and 0.5% glycerol were inoculated into 5 ml of the culture medium containing 0.15% isobutyronitrile and 0.5% carbon compounds. The cultivation was carried out as described in Materials and Methods.

Carbon source	Cell growth (mg dry weight/ ml culture broth)	Enzyme activity (units/ml)	Specific activity (units/mg)
Glycerol	0.90	0.13	0.14
Glucose	0.22	0	0
Fructose	0.03	-	-
Sucrose	0.21	0.05	0.21
Maltose	0.16	0.13	0.77
Galactose	0.38	0	0
Dextrin	0.55	0.40	0.72
Succinate·Na_2	0.11	0.09	0.14
Acetate·Na	0.11	0.03	0.07
Ethanol	0.40	0.03	0.07
None	0.20	0.17	0.85

-, not tested

Table III. Effect of Nitrogen Source on the Production of Acrylamide.

Washed cells (41 μg as dry weight) grown on the culture medium containing 0.15% isobutyronitrile and 0.5% glycerol were inoculated into 5 ml of the culture medium containing 0.2% (v/v) nitriles with 0.5% glycerol. The cultivation and assay were carried out as described in Table II.

Nitrogen source	Cell growth (mg dry weight/ ml culture broth)	Enzyme activity (units/ml)	Specific activity (units/mg)
Acetonitrile	0.70	0	0
Propionitrile	1.46	0.08	0.05
n-Butyronitrile	1.78	0.11	0.07
n-Capronitrile	0.23	0.11	0.38
Methacrylonitrile	0.65	0.02	0.03
Isobutyronitrile	1.98	0.25	0.13
Glutaronitrile	0.73	0	0
Triacrylonitrile	0.18	0	0
Yeast extract	2.84	0.04	0.01
Malt extract	1.61	0.01	0.01
Polypepton	2.90	0.02	0.01
$(NH_4)_2SO_4$	1.07	0	0
$NaNO_3$	0.76	0	0

most favorable nitrogen source for the production. The activity was induced when grown on nitriles. The strain did not grow on acrylonitrile, *n*-valeronitrile, crotonitrile, lactonitrile, malononitrile, succinonitrile, benzonitrile and triacrylonitrile as the nitrogen source. It grew well on a medium containing up to 0.23% isobutyronitrile.

The total activity of acrylamide production was growth-associated and it was not lost even at the stationary phase of the growth. The cells of 3-days cultivation had high activity and was suitable for the production of acrylamide.

Reaction conditions for the acrylamide production with P. chlororaphis B23

1) Optimum pH and temperature. Figures 3 and 4 show that

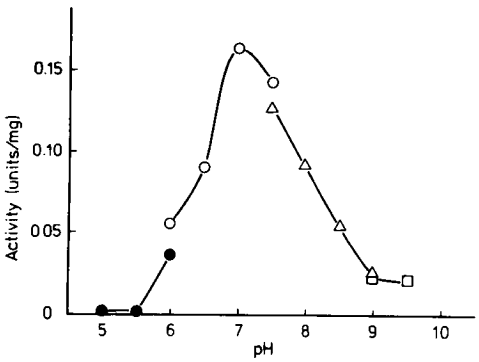


Fig. 3. Effect of pH on the Production of Acrylamide.

The reaction mixture contained 300 μmol of acrylonitrile, 100 μmol buffers and 10 mg cells (as dry weight) in a total volume of 1.0 ml. The reaction was carried out as described in Materials and Methods, except for that the reaction time was 30 min. The buffer used were: sodium acetate buffer (●), potassium phosphate buffer (○), Tris-HCl buffer (△) and glycine-NaOH buffer (□).

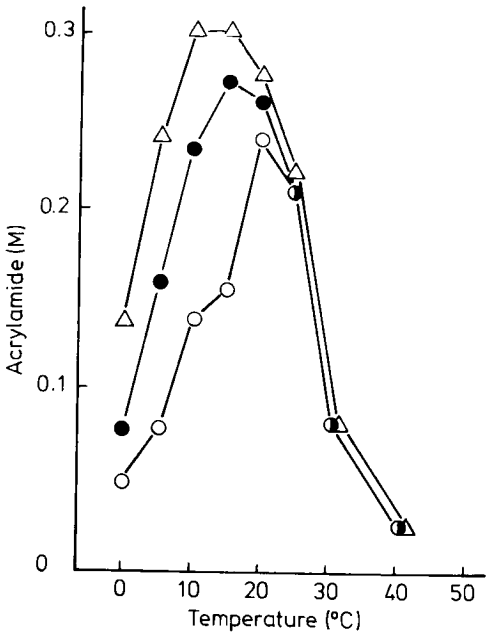


Fig. 4. Effect of Temperature on the Production of Acrylamide.

Washed cells (0.8 mg as dry weight) were used. The activity was measured at various temperatures as described in Materials and Methods. The reaction time was: 1 hr (○), 2 hr (●) and 3 hr (△).

maximum activity was obtained at pH 7.0 and between 10°C and 20°C. At the temperature higher than 30°C, the activity was inactivated rapidly and the reaction did not proceed for long time.

2) Effect of detergent. Twenty-one kinds of cationic, anionic and nonionic detergents were tested for the ability to increase the activity. However, none of them was effective for the production, and most of them inhibited the reaction.

Substrate specificity

The substrate specificity of the activity of nitrile hydratase of the strain was investigated in intact cell system. Table IV shows the result obtained by the cells grown on dextrin and isobutyronitrile, Propionitrile was hydrated faster than isobutyronitrile. The enzyme acted on acetonitrile, propionitrile, acrylonitrile, *n*-butyronitrile, methacrylonitrile and isobutyronitrile. *n*-Valeronitrile and benzonitrile were not attacked by the enzyme.

Table IV. Substrate Specificity of Nitrile Hydratase of *P. chlororaphis* B23

The reaction mixture contained 300 μmol nitrile, 100 μmol potassium phosphate buffer, pH 7.0 and 10 mg cells (as dry weight) in a total volume of 1.0 ml. The reaction was carried out as described in Materials and Methods. Amides formed were assayed by gas-liquid chromatography.

Nitrile	Nitrile hydratase activity	
	(units/mg)	(%)
Acetonitrile	0.11	31
Propionitrile	0.41	117
Acrylonitrile	0.35	100
n-Butyronitrile	0.20	57
Isobutyronitrile	0.18	51
Methacrylonitrile	0.08	23
n-Valeronitrile	0	0
Benzonitrile	0	0

Production of acrylamide by intact cells

Production of acrylamide through the hydration of acrylonitrile by the cells of *P. chlororaphis* B23 was carried out. After the strain was cultured under the optimum conditions, the culture broth was directly used as an enzyme source and incubated at 5°C with

moderate shaking. Acrylonitrile was added successively so as not to exceed a concentration of 0.4 M. Thus, 100 gram/liter of acrylamide was produced in 84 hr. Figure 5 shows a typical time course of the reaction using washed cells of the strain. The reaction mixture contained 2 g (as dry weight) of washed cells of the strain, 10 mmol of potassium phosphate buffer, pH 7.0 and 560 mmol of acrylonitrile which were added by a portion of 40 mmol at 30 min intervals, in a total volume of 100 ml. About 400 gram/liter of acrylamide was produced after 7.5 hr of reaction. The yield of acrylamide was more than 99% with a trace amount of acrylic acid (0.7%). Although the reaction seemed to proceed further on, it was stopped because the reaction mixture became gradually viscous presumably by polyacrylamide formed.

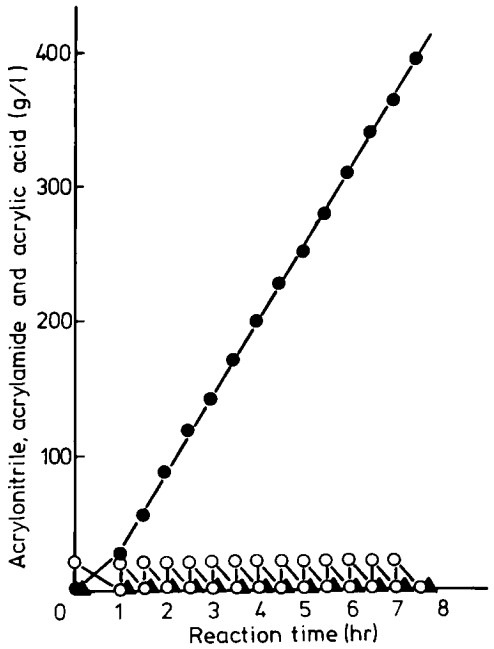


Fig. 5. Time course of Acrylamide Production with *P. chlororaphis* B23 Cells.

The reaction was carried out at 0°C to 4°C with stirring. Acrylonitrile (○), acrylamide (●) and acrylic acid (▲).

Degradation of isobutyronitrile by P. chlororaphis B23

When the strain was grown on isobutyronitrile and glycerol, the successive formation of isobutyramide and isobutyric acid was observed with the decrease of isobutyronitrile in concentration. The maximum concentration of isobutyramide and isobutyric acid in the culture broth were 11 and 19 mM, respectively, when the concentration of isobutyronitrile at start was 38 mM (0.15%, w/v).

Enzyme activities of nitrile hydratase and amidase in the cell-free extract of P. chlororaphis B23

To investigate the mechanism of the accumulation of acrylamide, activities of nitrile hydratase and amidase of isobutyronitrile-grown *P. chlororaphis* B23 and acetonitrile-grown *Arthrobacter* sp. J-1 were measured (Table V). Nitrile hydratase of *P. chlororaphis* B23 was not specific on isobutyronitrile. It also acted on acrylonitrile about 60% the rate of isobutyronitrile. On the other hand, amidase of the strain was active to isobutyramide, but scarecely active to acrylamide. The spectrum of the substrate specificity did not change at 0°C, indicating the accumulation of acrylamide was not caused by the effect of the low reaction temperature. Amidase activity of *Arthrobacter* sp. J-1 was much higher than that of nitrile hydratase. The enzyme acted on acrylonitrile and acrylamide, respectively. In the other acrylamide producing strains which were found in the screening test, the specific activities of amidase toward acrylamide was also much lower than that of nitrile hydratase toward acrylonitrile.

Table V. Substrate Specificity of Nitrile Hydratase and Amidase in Cell Extract of *P. chlororaphis* B23 and *Arthrobacter* sp. J-1.

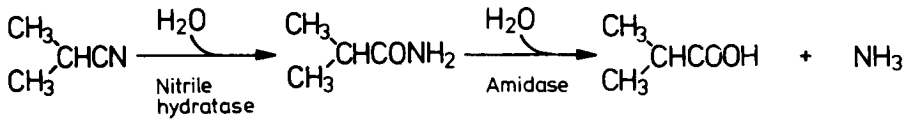
Specific activity (units/mg) of					
	Nitrile hydratase			Amidase	
Substrate	*P. chlororaphis* B23	*Arthrobacter* sp. J-1	Substrate	*P. chlororaphis* B23	*Arthrobacter* sp. J-1
Acetonitrile	0.006	0.003	Acetamide	0.005	0.088
Acrylonitrile	0.076	0.011	Acrylamide	0.006	0.167
Isobutyronitrile	0.130	0	Isobutyramide	0.100	0

DISCUSSION

This is the first report on the production of acrylamide by microbial enzyme system catalyzed by nitrile hydratase which was found and characterized by the author. He has established the cultural and reaction conditions for the production of acrylamide with

Pseudomonas chlororaphis B23, which was isolated as an isobutyronitrile-utilizing strain. Nearly 100% of acrylonitrile was converted to acrylamide with only a trace amount of acrylic acid. The optimum temperature of the reaction was between 10°C and 20°C. This would be a promissing method for the production of acrylamide as compared with the chemical hydration of acrylonitrile in the respects of selectivity, easiness in the preparation of the enzyme, and in the reaction temperature.

There are two pathways in the microbial degradation of nitrile compounds. One is the direct hydrolysis of nitrile to carboxylic acid and ammonia, catalyzed by nitrilase (36,37,48-50,52-54). The other is the two step degradation pathways of nitrile which involves nitrile hydratase and amidase, amide as an intermediate. When *P chlororaphis* B23 was grown on isobutyronitrile, isobutyramide, isobutyric acid and ammonia were successively formed in the culture broth as isobutyronitrile was consumed. This shows that *P. chlororaphis* B23 degraded isobutyronitrile as follows:



The specific activity of nitrile hydratase and amidase toward isobutyronitrile and isobutyramide in the cell-free extract of *P. chlororaphis* B23 grown on isobutyronitrile were almost the same levels, in agreement with the result that isobutyramide was not much accumulated in the culture broth. On the other hand, the latter enzyme scarcely attacked acrylamide which was formed from acrylonitrile by the former enzyme. The accumulation of large amount of acrylamide might be due to the difference in the rate of hydration of acrylonitrile and hydrolysis of acrylamide. There is a good evidence which explains the phenomenon, when acetonitrile-

grown *Arthrobacter* sp. J-1 were incubated with acrylonitrile, acrylic acid was accumulated instead of acrylamide, because the specific activity of amidase was higher than that of nitrile hydratase. With a successive feeding of acrylonitrile, more than hundred gram/liter of acrylic acid was accumulated in a short time (data not shown).

The low reaction temperature would be one of the advantageous characteristics of this enzymatic method, as compared with the chemical processes. The low optimum temperature for acrylamide production was also shown in 3 other acrylamide producing strains. As acrylamide is a specific modificator of protein sulfhydryl group (105,106), the active site of nitrile hydratase was possibly masked with acrylonitrile at higher temperatures.

SUMMARY

To produce acrylamide from acrylonitrile by use of a new enzyme, nitrile hydratase, a number of nitrile-utilizing microorganisms were screened for the enzyme activity by intact cell system. An isobutyronitrile-utilizing bacterium, strain B23, showed the best productivity among 186 strains tested. The strain was identified taxonomically as *Pseudomonas chlororaphis*. The culture and reaction conditions for the production were studied with the strain. Under the optimum conditions, 400 gram/liter of acrylamide was produced in 7.5 hr. The yield was nearly 100% with a trace amount of acrylic acid. The cell-free extract of the strain had a strong activity of nitrile hydratase toward acrylonitrile and extremely low activity of amidase toward acrylamide.

CONCLUSION

In this thesis, the author investigated the microbial degradation of nitrile compounds.

In CHAPTER I, a number of microorganisms which could utilize various nitriles as the sole source of carbon and nitrogen or as that of nitrogen were isolated from natural sources by the enrichment culture or the acclimation technique. All the strain hydrolyzed nitriles to their corresponding carboxylic acids and ammonia with or without amide as an intermediate.

Acrylonitrile is believed to be one of the most toxic organic nitriles. No microorganism had been known to utilize acrylonitrile as the sole source of carbon and nitrogen. The author could isolate *Arthrobacter* sp. I-9 for the first time from an activated sludge which had been acclimated to acrylonitrile for 3 months. The growth of the strain occurred after acrylonitrile had been degraded to acrylic acid and ammonia. Acrylamide could not be detected either in the culture broth nor in the reaction mixture with resting cells. *Arthrobacter* sp. I-9 could grow not only on a variety of nitriles but also on acrylamide.

Arthrobacter sp. J-1, which could utilize acetonitrile as a sole source of carbon and nitrogen, was also isolated from soil. Acetonitrile was degraded to acetic acid and ammonia with acetamide as an intermediate.

A bacterial strain utilizing glutaronitrile as a sole source of carbon and nitrogen was isolated from soil and classified in the genus *Pseudomonas* and tentatively named as *Pseudomonas* sp. K-9. Metabolites of glutaronitrile in the culture broth were determined to be 4-cyanobutyramide, 4-cyanobutyric acid, glutaric acid and ammonia. In the cell-free extract system, ammonia was formed in twice the amount of glutaronitrile consumed, together with glutaric acid.

Fusarium merismoides TG-1 and *Fusarium solani* TG-2 were isolated and identified as triacrylonitrile (1,3,6-hexanetricarbonitrile)-utilizing fungi. The degradation products were isolated from the acidic fraction of the culture broth and determined to be a mixture of 4,7-dicyanoheptanoic acid and 5,7-dicyanoheptanoic acid. When the mixture of the products were used as the nitrogen source, both the fungi could not grow. *F. merismoides* TG-1 could also grow on glutaronitrile and diacrylonitrile (1,3-dicyanobutane) as nitrogen sources. The degradation products were determined to be 4-cyanobutyric acid and 4-cyanopentanoic acid, respectively.

Figure 1 shows the degradation of nitrile compounds by these microorganisms isolated by the author.

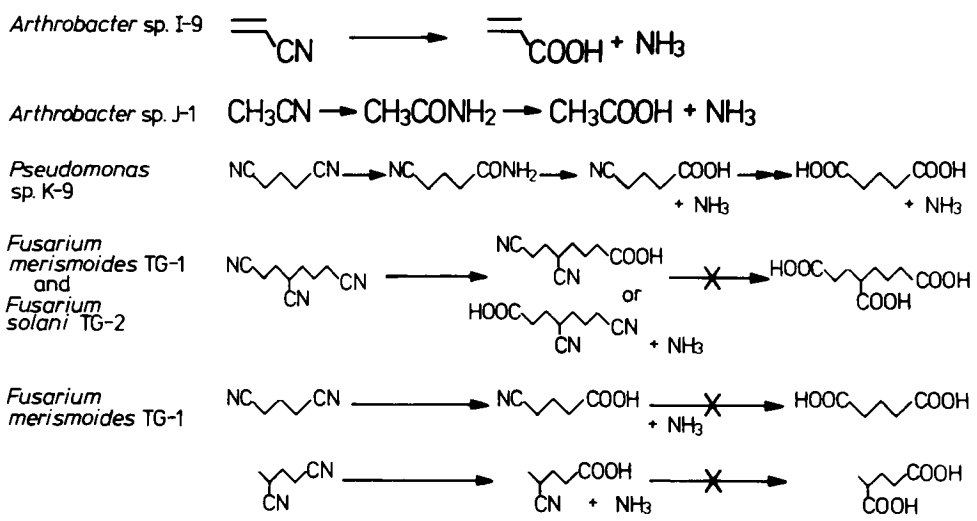


Fig. 1. Degradation of Nitriles by Microorganisms.

In CHAPTER II, the enzyme system responsible for the hydrolysis of acetonitrile was investigated. Although nitrilase (EC 3.5.5.1 and 3.5.5.2) had been defined to catalyze the hydrolysis of a nitrile to carboxylic acid and ammonia, nitrilase of microbial origin had not been characterized well. On the other hand, it had been noted of the existence of nitrile-hydrating enzymes in toyocamycin biosynthesis and asparagine formation from β-cyanoalanine.

Although activities of nitrilase and amidase were detected in the cell-free extract of acetonitrile-grown *Arthrobacter* sp. J-1, the activity of the former enzyme was lost as it was highly purified. Instead, ammonia-forming activity from acetonitrile with an addition of amidase was detected. The ammonia-forming activity from acetonitrile in the presence of amidase was purified about 290-fold from the cell-free extract of acetonitrile-grown *Arthrobacter* sp. J-1 to homogeneity. The enzyme catalyzed the hydration of low molecular weight aliphatic nitriles of 2 to 5 carbon atoms to yield amides. The enzyme was characterized as a new enzyme "aliphatic nitrile hydratase". The molecular weight of the enzyme was determined to be 420,000. The enzyme was composed of two subunits, of which molecular weights were 24,000 and 27,000. The *Km* value for acetonitrile was determined to be 5.78 mM. The enzyme was inactivated by sulfhydryl reagents. Potassium cyanide competitively inhibited the enzyme: *Ki* value was 1.5 μM.

Amidase (EC 3.5.1.4) has been intensively investigated in *Pseudomonas aeruginosa* as a model of mutation with altered substrate specificities. However, little has been known on amidase which participates in nitrile degradation. Amidase was purified to homogeneity from the cell-free extract of acetonitrile-grown *Arthrobacter* sp. J-1. The molecular weight of the enzyme was determined to be from 300,000 to 320,000. The enzyme was composed of 8 identical subunits of molecular weight of 39,000. The enzyme acted on acetamide (*Km*: 0.97 mM), acrylamide and propionamide and produced the corresponding carboxylic acid and ammonia.

The author has been the first to demonstrate a new pathway of nitrile degradation which involves nitrile hydratase and amidase:

$$R\text{-}CN \xrightarrow[\text{Nitrile hydratase}]{H_2O} R\text{-}CONH_2 \xrightarrow[\text{Amidase}]{H_2O} R\text{-}COOH + NH_3$$

In CHAPTER III, the microbial production of acrylamide was investigated. Acrylamide has been produced by the chemical hydration of acrylonitrile using copper salt or paradium complex as catalysts. However, the preparation of the catalysis has been laborious and the processes required high temperature. The author tried to utilize microbial nitrile hydratase as a catalyst, and screened a number of nitrile-utilizing microorganisms and tested for the ability to produce acrylamide from acrylonitrile. An isobutyronitrile-utilizing strain, *Pseudomonas chlororaphis* B23 which was isolated from soil, was chosen as the best producer. The strain could produce 400 gram/liter of acrylamide in a resting cell system. The yield was nearly 100% with a trace amount of acrylic acid. The cell-free extract of the strain had a strong activity of nitrile hydratase toward acrylonitrile and extremely low activity toward acrylamide.

In the present study, the author obtained a number of nitrile-utilizing microorganisms from nature. All of them hydrolyzed nitrile to carboxylic acid and ammonia. The author purified a new enzyme, aliphatic nitrile hydratase, and amidase to homogeneous states and clarified a new degradation pathway of nitrile compounds. Furthermore, he established a novel method to produce acrylamide by the use of microbial nitrile hydratase.

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