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Microbial Metabolism of Aromatic Nitriles

ENZYMOLOGY OF C–N CLEAVAGE BY *NOCARDIA* SP. (*RHODOCHROUS* GROUP) N.C.I.B. 11216

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1. An organism utilizing benzonitrile as sole carbon and nitrogen source was isolated by the enrichment-culture technique and identified as a *Nocardia* sp. of the *rhodochrous* group. 2. Respiration studies indicate that nitrile degradation proceeds through benzoic acid and catechol. 3. Cell-free extracts of benzonitrile-grown cells contain an enzyme that catalyses the conversion of benzonitrile directly into benzoic acid without intermediate formation of benzamide. 4. This nitrilase enzyme was purified by DEAE-cellulose chromatography and gel filtration on Sephadex G-100 in the presence and absence of substrate. The purity of the enzyme was confirmed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and isoelectric focusing on polyacrylamide gel. 5. The enzyme shows a time-dependent substrate-activation process in which the substrate catalyses the association of inactive subunits of mol.wt. 45000 to form the polymeric 12-unit active enzyme of mol.wt. 560000. The time required for complete association is highly dependent on the concentration of the enzyme, temperature and pH. 6. The associated enzyme has a pH optimum of 8.0 and K_m with benzonitrile as substrate of 4mM. The activation energy of the reaction as deduced from the Arrhenius plot is 51.8kJ/mol. 7. Enzyme activity is inhibited by thiol-specific reagents and several metal ions. 8. Studies with different substrates indicate that the nitrilase is specific for nitrile groups directly attached to the benzene ring. Various substituents in the ring are compatible with activity, though *ortho*-substitution, except by fluorine, renders the nitrile invulnerable to attack. 9. The environmental implications of these findings and the possible significance of the enzyme in the regulation of metabolism are discussed.

Despite the widespread use of herbicides containing the nitrile group such as Dichlobenil (2,6-dichlorobenzonitrile) and Bromoxynil (3,5-dibromo-4-hydroxybenzonitrile), comparatively little is known of the microbial metabolism of nitriles and in particular the mechanism of cleavage of the C–N bond by microorganisms. Robinson & Hook (1964) reported the isolation and Hook & Robinson (1964) the subsequent purification of a nitrilase enzyme (EC 3.5.5.2) of rather restricted substrate specificity from a *Pseudomonas* species that would grow on the naturally occurring cyanopyridine, ricinine, as sole carbon source. The main product of the action of this enzyme on ricinine is the corresponding carboxylic acid, although a small but constant proportion of the amide is also formed in spite of the fact that the amide is not a substrate for the enzyme. Mimura *et al.* (1969) have described a *Corynebacterium* sp., *C. nitrilophilus*, that can use aliphatic nitriles such as acetonitrile as sole carbon source. In this case metabolism appears to proceed via the corresponding amide, although no enzyme isolation was attempted. Similarly Firmin & Gray (1976) have, on the basis of ^{14}C-labelling experiments, concluded that acetonitrile is degraded via acetamide by a *Pseudomonas* sp. that can use acetonitrile a sole carbon source.

Mahadevan & Thimann (1964) have examined a nitrilase (EC 3.5.5.1) purified from barley leaves which would attack indol-3-ylacetonitrile and a number of other aliphatic and aromatic nitriles. The enzyme mediated the direct conversion of the nitrile into the corresponding acid, with no detectable formation of amide (Thimann & Mahadevan, 1964).

Harper (1974) showed that a number of microorganisms isolated from soil were capable of utilizing benzonitrile or 4-hydroxybenzonitrile as sole carbon

and nitrogen source. In the present paper the metabolism of benzonitrile by one such organism, *Nocardia* sp. (*rhodochrous* group) N.C.I.B. 11216, is described, and the isolation, purification and properties of the nitrilase enzyme involved are reported. The environmental implications of these findings for the degradation of herbicides and the significance of the allosteric nature of the enzyme in the metabolism of the organism are also considered. A preliminary report of part of this work has appeared (Harper, 1976).

Materials and Methods

Isolation and culture of micro-organism

A micro-organism capable of using benzonitrile as sole carbon, nitrogen and energy source was isolated by elective culture from mud obtained from the bed of the River Lagan in Belfast. The pink isolate, initially tentatively classified as a *Corynebacterium* sp. (Harper, 1974), has now been positively identified as a *Nocardia* sp. (*rhodochrous* group) N.C.I.B. 11216. The organism was grown in 2-litre conical flasks plugged with cottonwool and containing 1 litre of mineral-salt medium of the following composition per litre: KH_2PO_4 (1.5g); K_2HPO_4 (3.5g); $MgSO_4,7H_2O$ (0.1g); yeast extract (50mg); trace elements as described by Barnett & Ingraham (1955). The medium was adjusted to pH7.5, 0.25% benzonitrile added and the cultures were incubated at 25°C on an orbital shaker. The growth of the organism was monitored by measuring the A_{690} of the culture medium.

Preparation of washed cell suspensions

The organism was harvested when the A_{690} of the culture attained 0.6, i.e. in the early exponential phase of growth, by using a Sharples Super centrifuge and a flow rate of 250ml/min. Cells were washed twice with 100mM-sodium phosphate buffer, pH7.5, and again harvested by centrifugation (15000*g*, 30min, 10°C).

Measurement of oxygen uptake by cell suspensions

O_2 consumption by whole-cell suspensions was recorded at 25°C with a 4ml-capacity Clark-type oxygen electrode (Rank Bros., Bottisham, Cambridge, U.K.). Incubation mixtures contained 100mM-phosphate buffer, pH7.5, cell suspension (approx. 30mg wet wt. of cells equivalent to 5mg dry wt.) and 1mM substrate in a total volume of 4ml.

Preparation of cell-free extracts

Cells (15g) harvested from 20 litres of culture medium as described above were suspended in 100mM phosphate buffer, pH7.5 (45ml), containing 2mM-EDTA and 2.5mM-dithioerythritol and disrupted by sonication for a total duration of 15min by using an MSE 150W ultrasonic disintegrator at maximum amplitude. Cooling in an ice/NaCl bath ensured that the temperature did not rise above 15°C. The resulting suspension was centrifuged (60000*g*, 45min, 2°C) and the pellet containing cell debris discarded.

Determination of benzonitrile, benzoic acid and ammonia in growing cultures

Changes in the concentration of benzonitrile, benzoic acid and NH_3 were monitored in 50ml samples of culture medium extracted at intervals during growth. Centrifugation (25000*g*, 20min, 10°C) of such samples gave a clear supernatant, which was made alkaline with NaOH and extracted with 3×25ml of diethyl ether. The ether extracts were pooled, dried with anhydrous Na_2SO_4 and diluted with ether to 250ml in a volumetric flask. Benzonitrile concentrations were determined by measuring A_{277}, by using the experimentally observed absorption coefficient of 1.60×10^3 litre·mol^{-1}.cm^{-1}. The aqueous solution remaining after ether extraction was acidified to pH2.0 with HCl and extracted with ether as previously described. The resulting ethereal solution adjusted to a volume of 250ml with ether was assayed for benzoic acid at 279.5nm, at which wavelength the absorption coefficient of the latter compound was 1.62×10^3 litre·mol^{-1}·cm^{-1}. NH_3 concentrations in the supernatant were determined by the phenol/hypochlorite method of Fawcett & Scott (1960).

Protein and enzyme assays

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Nitrilase activity of the microbial enzyme was assayed by measuring the production of NH_3 during the hydrolysis of benzonitrile to benzoic acid by the method of Fawcett & Scott (1960). The standard assay was performed in duplicate at 30°C in tubes containing, unless otherwise stated, 0.9ml of 30mM-benzonitrile in 100mM-phosphate buffer, pH8.0, to which was added 0.1ml of extract. Mixtures were incubated for periods of 4, 8, 12, 16, 20 and 24min (longer in certain instances), after which times the reaction was terminated by the addition of 330mM-sodium phenoxide (1ml), followed by 0.01% sodium nitroprusside (1ml) and 20mM-sodium hypochlorite (1ml). The assay mixture was thoroughly shaken, heated for 10min at 100°C in a boiling-water bath to allow colour development, then diluted with water (6ml), and the A_{640} measured in a Perkin–Elmer Coleman model 55 UV-VIS digital spectrophotometer with auto sampler. At NH_3 concentrations up to 1.5mg/100ml of solution, the A_{640} was directly proportional to NH_3 concentration. Residual protein in the assay mixture did not interfere with the colour reaction at the enzyme concentrations used.

Chemicals

Benzonitrile was obtained from BDH Chemicals, Poole, Dorset, U.K., redistilled and the fraction boiling between 190 and 192°C at normal atmospheric pressure collected. *o*- and *m*-Fluorobenzonitrile, *m*- and *p*-bromobenzonitrile and *m*- and *p*-tolunitrile, phthalodinitrile, isophthalodinitrile and 2-, 3- and 4-cyanopyridine were purchased from Koch–Light Laboratories, Colnbrook, Bucks., U.K. *m*- and *p*-Chlorobenzonitrile, *m*-nitrobenzonitrile, *p*-fluorobenzonitrile, *o*-bromobenzonitrile and 1,4-dicyanobenzene were obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A. 3,5-Di-iodo-4-hydroxybenzonitrile, also purchased from the latter company, was recrystallized from ethanol. *o*-, *m*- and *p*-Hydroxybenzonitrile, *o*- and *p*-nitrobenzonitrile, *o*-tolunitrile and 2,6-dichlorobenzonitrile were acquired from Ralph N. Emanuel, Wembley, Middx., U.K. *o*-Chlorobenzonitrile, also from this supplier, was recrystallized from light petroleum (b.p. 60–80°C). 3,5-Dibromo-4-hydroxybenzonitrile was prepared as described by Carpenter *et al.* (1964) and *cis,cis*-muconic acid as described by Elvidge *et al.* (1950).

Proteins used for calibration of Sephadex G-75 and G-200 columns and also used as standards in SDS*/polyacrylamide-gel electrophoresis were purchased from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K., as was DEAE-cellulose (coarse grade) used in column chromatography and the acrylamide used in electrophoresis. SDS (Sigma) was recrystallized from ethanol before use. Ampholine of various pH ranges used in isoelectric focusing was purchased from LKB Instruments Ltd., South Croydon, Surrey, U.K.

T.l.c.

Products of enzyme action on benzonitrile were identified by t.l.c. Nitrilase (5ml) from stage 4 of the purification procedure was incubated for 30min with 30mM-benzonitrile (500ml) in 100mM-phosphate buffer, pH7.5. The solution was then extracted with ether (3×100ml), acidified with HCl to pH2.0 and again extracted with ether (3×100ml). After drying over anhydrous $MgSO_4$ the two extracts were pooled and ether was removed under reduced pressure. The residue was taken up in 1ml of ether and portions were applied to t.l.c. plates precoated with silica gel (Eastman–Kodak, Kirkby, Liverpool, U.K.). Chromatograms were developed in solvent system (1) [hexane/ether/formic acid (85:15:3, by vol.)] or (2) [toluene/ethyl acetate/formic acid (10:4:1, by vol.)]. Benzoic acid and benzamide were detected on chromatograms by using either 0.04% (w/v) Bromocresol Green in ethanol or 1% (w/v) 5,7-dichlorofluorescein in ethanol followed by examination under u.v. light. Benzoic acid and benzamide were well resolved by each solvent system, showing R_F 0.91 and 0.13 respectively in system (1) and R_F 0.84 and 0.64 respectively in system (2).

Reference proteins used in gel filtration

To calibrate the Sephadex G-200 and G-75 columns used in molecular-weight determination the following reference proteins were used (mol.wt. in parentheses): thyroglobulin (680000), catalase (240000), aldolase (145000), bovine serum albumin (67000), ovalbumin (43000), carbonic anhydrase (29000), myoglobin (17300) and cytochrome *c* (11200).

Electrophoresis

Isoelectric focusing on polyacrylamide gel. Analytical thin-layer electrofocusing in polyacrylamide gel was performed with an LKB 2117 Multiphor apparatus by the method described by Karlsson *et al.* (1973) for isoelectric focusing in the pH range 2.5–6.0, by using riboflavin as the catalyst for polymerization of the acrylamide. Before application to the gel, the protein fraction for focusing was dialysed against 10mM-sodium phosphate buffer, pH7.0, containing 0.5mM-dithioerythritol. Volumes of sample solution containing 10–50μg of protein were applied to the surface of the gel absorbed on 5mm×10mm pieces of Whatman 3MM chromatography paper. The pH gradient in the gel after electrofocusing at 2°C was determined by means of an Activion surface electrode. The staining technique of Vesterberg (1972) was used for localizing protein bands with Coomassie Brilliant Blue R-250

SDS/polyacrylamide-gel electrophoresis. Molecular weight and purity determinations on the nitrilase were performed by electrophoresis on polyacrylamide gel in the presence of SDS by a thin-layer technique that enabled up to 18 samples to be analysed simultaneously on the same gel slab by using an LKB 2117 Multiphor apparatus. The method used was based on that described by Weber *et al.* (1972) for disc gel electrophoresis. Thin-layer gels of 10% acrylamide containing 0.2% (w/v) SDS dissolved in 100mM-sodium phosphate buffer, pH7.2, were suitable, and were polymerized by using ammonium persulphate as catalyst and *NNN'N'*-tetramethylenediamine as accelerator in the usual manner. Samples of standard proteins and of nitrilase were prepared for application to the gel as described by Weber *et al.* (1972), in 10mM-sodium phosphate buffer, pH7.0, containing 1% (w/v) SDS and 1% (v/v) mercaptoethanol at 100°C. Pieces (1.5mm×6mm) of Whatman 3MM chromatography paper were soaked in the sample solution, dried to remove superficial moisture and placed vertically in slits along the length of the gel

* Abbreviation: SDS, sodium dodecyl sulphate.

(25cm×12.5cm) on the side adjacent to the cathode. By this method up to 18 individual samples could be subjected to electrophoresis simultaneously on the same gel slab. After introduction of the sample, each slit was filled to the surface of the gel with 10mM-sodium phosphate buffer, pH7.2, containing 0.1% (w/v) SDS and 0.1% (v/v) mercaptoethanol, with a micro-pipette. Bromophenol Blue was used as the tracking dye. The gels were subjected to transverse electrophoresis on the LKB 2117 Multiphor instrument, the reservoir buffer in the anode and cathode compartments being 50mM-sodium phosphate buffer, pH7.2, containing 0.1% (w/v) SDS. With a voltage of 80–100V and a current of 40–50mA at a temperature of 17.5°C, a running time of about 16h was required to complete electrophoresis, i.e. for the tracking dye to approach the anodic side of the gel. The position of the dye was then marked with waterproof black ink and the gel stained with Coomassie Brilliant Blue as described by Vesterberg (1972). The distance migrated by the stained protein bands and the dye was measured and the relative mobilities of the sample proteins with respect to the tracking dye were calculated. From a plot of the molecular weights of the polypeptide chains of standard proteins against their electrophoretic mobility the molecular weight of the subunits of the isolated nitrilase enzyme was determined from their relative mobility. In addition to certain of the standard proteins used in gel-filtration experiments, the following proteins were also used for calibration (mol.wt. of subunit in parentheses): L-amino acid oxidase (63000), γ-globulin (H-chain 50000, L-chain 23500), alcohol dehydrogenase (41000), glyceraldehyde 3-phosphate dehydrogenase (36000), lactate dehydrogenase (36000).

Results and Discussion

Growth of Nocardia N.C.I.B. 11216 on benzonitrile and benzamide

The course of growth of the micro-organism on benzonitrile is illustrated in Fig. 1, which shows that in the intital stages after inoculation benzonitrile is degraded, with concomitant formation of benzoic acid and NH_3, which accumulate in the culture medium. Little growth occurs until benzonitrile is exhausted, at which point the exponential phase of growth ensues on the benzoic acid present in the culture medium. Growth of benzonitrile-adapted cells of the organism on 0.1% benzamide only ensued after a period of several months, and no nitrilase activity could be detected in cell-free extracts of such cells.

Respiration studies on whole cells

The ability of cell suspensions from cultures in the early exponential phase (i.e. after 40h incubation) to oxidize possible intermediates in the degradation of benzonitrile was measured (Table 1). In addition to oxidizing benzonitrile, such cells rapidly utilize both catechol and benzoate. However, the rates of oxidation of *o*- and *p*-hydroxybenzoate, the corresponding

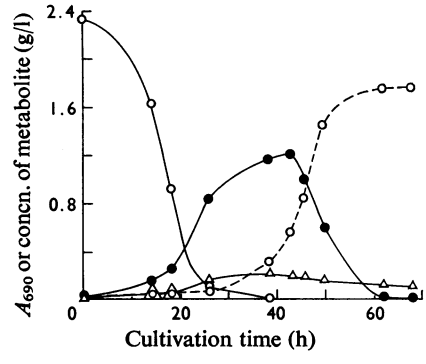


Fig. 1. *Growth of Nocardia sp. on benzonitrile showing changes in concentrations of metabolites in the culture medium*

The organism was grown, intermediates were assayed and cell density was assessed as described in the Materials and Methods section. Growth (--○--); benzonitrile concn. (—○—); benzoic acid concn. (—●—); ammonia concn. (—△—).

Table 1. *Oxidation of possible intermediates in benzonitrile metabolism by cell suspensions of Nocardia N.C.I.B. 11216*

Suspensions of the organism were prepared and the initial rates of O_2 uptake on different substrates determined as described in the Materials and Methods section. Values are corrected for an endogenous respiration rate of 0.37μl/min per 30mg wet wt.

Substrate	O_2 uptake (μl/min per 30mg wet wt.)	Substrate	O_2 uptake (μl/min per 30mg wet wt.)
Benzoate	1.12	*o*-Hydroxybenzoate	0.12
Catechol	1.84	*m*-Hydroxybenzoate	0.18
Benzonitrile	1.01	*p*-Hydroxybenzoate	0.14
Benzamide	0.02	*o*-Hydroxybenzonitrile	0.05
3,4-Dihydroxybenzoate	0.12	*m*-Hydroxybenzonitrile	0.23
2,5-Dihydroxybenzoate	0.27	*p*-Hydroxybenzonitrile	0.14
cis,cis-Muconic acid	0.03		

nitriles and benzamide is slow and seems to indicate that benzonitrile is degraded directly to benzoic acid and catabolized via catechol by either the *ortho*- or *meta*-cleavage pathway. The presence of benzamide did not appear to alter significantly the rate of oxidation of either benzonitrile or benzoate. Though *cis,cis*-muconic acid is not oxidized at an appreciable rate, the *ortho*-cleavage pathway is not excluded, since cell permeability may be an important factor with this substrate.

Purification of nitrilase

All stages of enzyme purification were performed at a temperature of between 0 and 3°C. Stages in purification of the cell-free extract (1) are described below and the results are summarized in Table 2. The duration of the entire purification procedure was approx. 4 days.

(2) *DEAE-cellulose chromatography.* DEAE-cellulose suspended in 100mM-phosphate buffer, pH7.5, was packed into a column (2.5cm×45cm), washed with 0.5M-NaOH and equilibrated with 100mM-phosphate buffer, pH7.5, containing 1mM-EDTA and 0.25mM-dithioerythritol. The orange–pink cell-free extract (46ml) was applied to the column and elution performed with the equilibrating buffer. The orange pigment and much of the protein of the extract remained bound to the column, but the nitrilase was not appreciably retarded in passage through the column. Fractions (10ml) were collected and those possessing high nitrilase activity were pooled.

(3) *Gel filtration in absence of substrate.* The enzyme solution from stage (2) was concentrated by ultrafiltration in an Amicon Diaflo cell by using a PM10 membrane, and applied to a column (2.5cm×60cm) of Sephadex G-200 equilibrated with 100mM-phosphate buffer, pH7.5, containing 1mM-EDTA and 0.25mM-dithioerythritol. On elution with this buffer fractions (3.8ml) of eluate were collected, and those fractions containing most of the nitrilase activity, at a relative elution volume between 1.9 and 2.1 V_0, were combined.

(4) *Gel filtration in presence of substrate.* The stage-(3) preparation was concentrated to 2.5ml by ultrafiltration and then added to 2.5ml of 30mM-benzonitrile in 100mM-phosphate buffer, pH8.0, and incubated at 30°C for 3min. After rapid cooling to 0°C, the solution was applied to a column (2.5cm×80cm) of Sephadex G-200 equilibrated with 100mM-phosphate buffer, pH8.0, containing 1mM-EDTA, 0.5mM-dithioerythritol and 30mM-benzonitrile. Fractions (3.8ml) of eluate were collected, and portions (0.1ml) diluted with 30mM-benzonitrile and assayed in the usual manner. The low activity of the enzyme at 0°C prevented appreciable hydrolysis of substrate in the eluate before assay. Any products of hydrolysis in the original preparation applied to the column were retarded to relative elution volumes in excess of 2.5 V_0 and so did not interfere with enzyme assay. Fractions eluted at relative elution volumes between 1.0 and 1.2 V_0 contained nitrilase activity, and were combined, concentrated by ultrafiltration, and dialysed against 10mM-phosphate buffer, pH8.0, containing 1mM-EDTA and 0.5mM-dithioerythritol.

Examination of samples of the fractions from various stages of purification by isoelectric focusing on polyacrylamide gel revealed the presence of only one protein band in the stage-4 fraction, with pI 4.22 (see Fig. 2). SDS/polyacrylamide-gel electrophoresis also confirmed the homogeneity of the preparation (see below, under 'Determination of molecular weight'). Purification by the procedure described was 37-fold and the yield 0.7%.

Properties of nitrilase

The enzyme was exceedingly labile in the purified form, with a half-life of 10h in 100mM-phosphate buffer, pH8.0, containing 1mM-EDTA and 0.25mM-dithioerythritol at 0°C, and undergoing 90% loss of activity on freezing. The stability could be improved by the addition of 5mM-benzonitrile. Such treatment increased the half-life to about 30h, but necessitated dialysis before use. However, the partially purified preparations from stages (2) and (3) had a half-life of 4 days at 0°C in the buffer described above without addition of substrate, and in general such preparations were used for enzyme characterization. Pure enzyme was used for the determination of the products of enzyme reaction and the molecular weight of enzyme

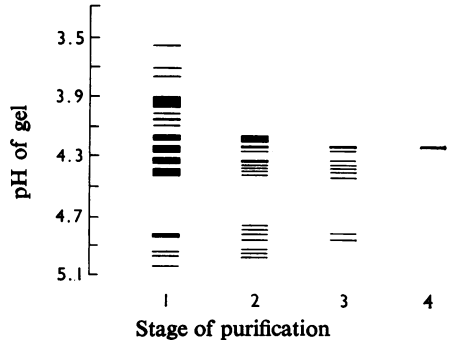


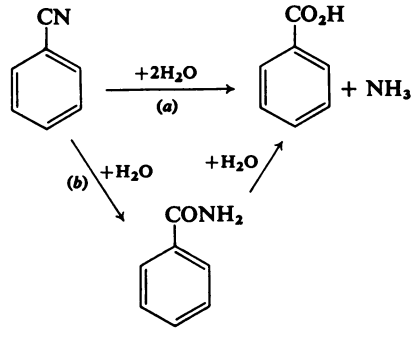
Fig. 2. *Isoelectric focusing of protein fractions obtained in purification of nitrilase*

Isoelectric focusing of samples containing 20μg of protein was performed on polyacrylamide gel as described in the Materials and Methods section. Fractions focused correspond to the four stages of purification of the enzyme: 1, cell-free extract; 2, DEAE-cellulose eluate; 3, fraction obtained after first gel filtration in the absence of benzonitrile; 4, fraction obtained after second gel filtration in the presence of benzonitrile.

Table 2. *Purification of nitrilase from Nocardia N.C.I.B.* 11216

The results shown are of typical enzyme purification procedure as described in the text. All stages were performed at a temperature of between 0 and 3°C.

Preparation	Volume (ml)	Total units of enzyme activity (μmol of NH_3 formed/min)	Yield (%)	Protein (mg/ml)	Specific activity (μmol of NH_3 formed/min per mg of protein)
(1) Cell-free extract	46	204.9	100	9.9	0.45
(2) DEAE-cellulose column eluate	138	78.6	38.4	0.32	1.78
(3) Eluate after ultrafiltration and passage through Sephadex G-200 in absence of benzonitrile	45	20.7	10.1	0.091	5.06
(4) Eluate after passage through Sephadex G-200 in presence of benzonitrile, ultrafiltration and dialysis	5	1.41	0.7	0.018	15.7



Scheme 1. *Alternative routes for hydrolysis of benzonitrile*

subunits by using SDS/polyacrylamide-gel electrophoresis. In experiments on the effect of enzyme concentration, assay was performed in the presence of 0.05% (w/v) bovine serum albumin to lessen enzyme denaturation.

Products of enzyme reaction

Investigation by t.l.c. as described in the Materials and Methods section of the products of the action of nitrilase demonstrated that benzoic acid and NH_3 were the sole compounds produced on hydrolysis of benzonitrile by the enzyme and were formed at identical rates. No benzamide was detected, suggesting that the amide was not an intermediate in the degradation of benzonitrile. This was confirmed by the observation that benzamide did not act as a substrate for the pure enzyme, even when assay was performed with enzyme preparations 5-fold more concentrated than normal. Thus the nitrilase from *Nocardia* N.C.I.B. 11216 appears to be similar to that isolated from barley by Thimann & Mahadevan (1964) in using route (*a*) in Scheme 1 rather than the amide route (*b*), which is used in the cleavage of acetonitrile by *Corynebacterium nitrilophilus* (Mimura *et al.*, 1969) and *Pseudomonas* sp. (Firmin & Gray, 1976).

Influence of enzyme concentration

The effect of varying the enzyme concentration on the time-course of the nitrilase reaction has been described (Harper, 1976). Clearly the reaction does not follow first-order kinetics, especially at the lower enzyme concentrations, where linearity of velocity with respect to time was not achieved for 20–30min. However, provided that velocity measurements were made on the linear portion of the time-course plot, the rate of benzonitrile hydrolysis as measured by NH_3 release was directly proportional to enzyme concentration. The variable lag period implies some form of activation process. A quantitative assessment of this time-period was obtained by measurement of the intercept of the linear portion of the time-course plot on the time axis. Further mention of lag period or association time will refer to this parameter.

Neither the products of the nitrilase reaction, NH_3 and benzoic acid, nor benzamide had any effect on the length of this period, but a plot of lag period against enzyme concentration (see Fig. 3) shows an inverse relationship between these functions, indicating some form of substrate activation which, when considered in conjunction with the changes in molecular weight in the presence of substrate described below, probably involves the aggregation of protein subunits.

Determination of molecular weight

The molecular weight of the nitrilase was determined by gel filtration on Sephadex G-200 in the presence and absence of substrate as described by Harper (1976). In the absence of substrate the nitrilase had an elution volume corresponding to a mol.wt. of 47000, whereas in the presence of substrate the apparent mol.wt. increased to 560000. A more accurate determination of the mol.wt. of the enzyme in the absence of substrate by using Sephadex G-75 gave a value of 44500. This estimate of subunit size was confirmed by SDS/polyacrylamide-gel electrophoresis of the pure enzyme as

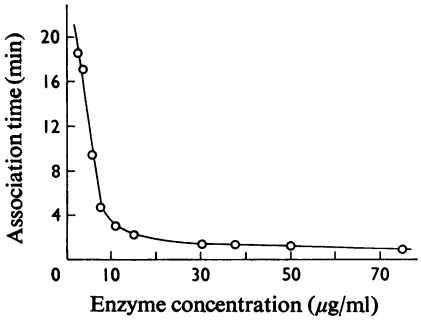


Fig. 3. *Association time of the enzyme as a function of enzyme concentration*

Enzyme was preincubated at 30°C for 10min in 100mM-phosphate buffer, pH8.0, contining 1mM-EDTA and 0.25mM-dithioerythritol. Final concentrations in the assay mixture incubated at 30°C were 27mM-benzonitrile, 0.1mM-EDTA and 0.025mM-dithioerythritol, 100mM-phosphate buffer, pH8.0, and enzyme concentrations as shown. Association time was defined as the intercept of the linear portion of the time-course graph on the time axis.

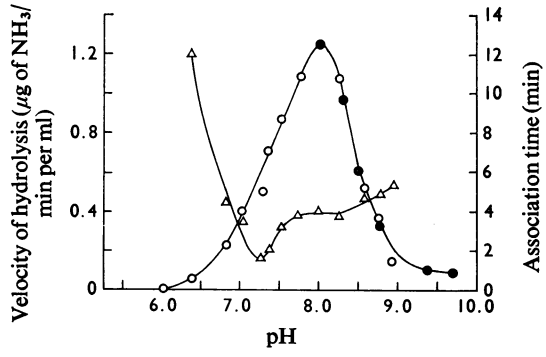


Fig. 4. *Effect of pH on velocity of benzonitrile hydrolysis by nitrilase and on association time*

Enzyme was incubated at the appropriate pH in either 100mM-sodium phosphate buffer (○) or 100mM-sodium borate buffer (●) at 30°C in the presence of 27mM-benzonitrile. Enzyme activities in borate buffer have been adjusted as described in the text. Velocity of hydrolysis (—○—); association time measured only in phosphate buffer (—△—).

described in the Materials and Methods section. A mol.wt. of 45000 was indicated by this technique. In the presence of benzonitrile the nitrilase therefore underwent an association process involving probably 12 identical subunits of mol.wt. about 45000 to form an active enzyme of mol.wt. approx. 560000.

Influence of pH

The velocity of the reaction and the association time were measured as previously described at various pH values between 6.0 and 9.7 in both phosphate and borate buffers. Enzyme activity in 100mM-sodium borate buffer at pH8.0 was 44% of that of 100mM-sodium phosphate buffer at the same pH. In Fig. 4, which shows the effect of pH on nitrilase activity and association time, enzyme activities in borate buffer have accordingly been adjusted to make them comparable with results obtained in phosphate buffer. Activity was maximal at pH8.0, falling sharply at both higher and lower pH values. However, the association time is at a minimum at pH7.3 and rises to a plateau between pH7.75 and 8.50, indicating that the pH optimum for the substrate-initiated association process differs from that for the substrate–enzyme reaction at the active centre involved in nitrile hydrolysis.

Effect of temperature

The influence of temperature on the velocity of nitrile hydrolysis and on the assocation time of the enzyme was determined under standard assay conditions. The activation energy of the nitrilase reaction between 20 and 40°C, as deduced from the slope of

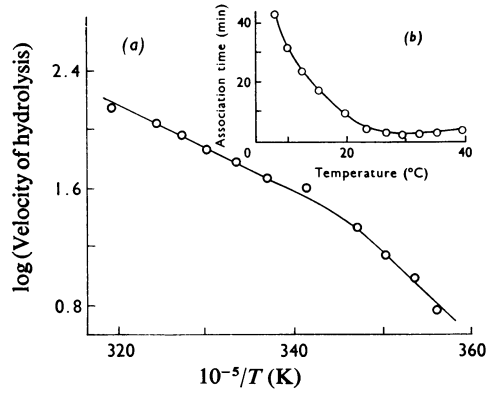


Fig. 5. *Influence of temperature on the velocity of benzonitrile hydrolysis by nitrilase (a) and on association time (b)*

Enzyme was incubated with 100mM-phosphate buffer, pH8.0, in the presence of 27mM-benzonitrile at the appropriate temperature. Velocity of nitrile hydrolysis was measured when it became linear with respect to time and is represented as an Arrhenius plot (*a*). The effect of temperature on association time is shown in the inset (*b*).

the Arrhenius plot (Fig. 5*a*), was 51.8kJ/mol (12400cal/mol). The slope of the plot is discontinuous below 20°C, and between 15 and 8°C an apparent activation energy of 119.3kJ/mol (28550cal/mol) is observed. One possible explanation of this behaviour may be that the enzyme is predominantly in the inactive monomeric form even at equilibrium with the substrate (i.e. when linearity of the velocity of

nitrile hydrolysis is attained) at temperatures below 20°C. The rapid increase in association time at temperatures below 20°C (see Fig. 5*b*) tends to support this hypothesis.

Effect of inhibitors and metal ions

Possible inhibition of enzyme activity by a variety of compounds and metal ions was investigated. After dialysis against 100mM-phosphate buffer, pH8.0, the enzyme was preincubated in the presence of inhibitor for 10min before the addition of substrate. The velocity of nitrile cleavage was measured when linearity with respect to time was achieved. Table 3 demonstrates the extreme sensitivity of the enzyme to certain thiol reagents. Thus *p*-hydroxymercuribenzoate and phenylmercuric acetate show complete inhibition at concentrations as low as 5nM, although this could be at least partially reversed by the addition of reduced glutathione. *N*-Ethylmaleimide and iodoacetamide also gave strong inhibition of the enzyme at relatively low concentrations. Such results indicate that thiol groups play an important role in the activity of the enzyme, but whether at the active site or in the association process is not known.

Metal-chelating agents such as azide and cyanide do not cause inhibition at 0.1mM, which, together with the finding that prolonged dialysis against 1mM-EDTA did not affect activity, demonstrates that the nitrilase lacks a metal-ion requirement. The involvement of thiol groups in enzyme activity explains the complete inhibition of the enzyme by 10μM solutions of Zn^{2+}, Cu^{2+}, Ag^{+} and Hg^{+} ions. The lower valency states of iron, cobalt and manganese also produce large decreases in enzyme activity, probably owing to redox effects on thiol linkages or alternatively to complexing with such groups. As might be expected with such a multimeric enzyme, 6M-urea produces an irreversible inactivation.

Table 3. *Effect of inhibitors and metal ions on nitrilase*
After preincubation of the enzyme in the presence of inhibitor for 10min before the addition of substrate, assay of the enzyme was performed under standard assay conditions.

Inhibitor or metal ion	Final concentration (μM)	Inhibition (%)
p-Hydroxymercuribenzoate	0.5	100
p-Hydroxymercuribenzoate	0.005	36
p-Hydroxymercuribenzoate+0.5mM-glutathione	0.5	42
Phenylmercuric acetate	5	100
N-Ethylmaleimide	5	100
Iodoacetamide	500	100
Iodoacetamide	5	39
Zn^{2+}	10	100
Cu^{2+}	10	100
Ag^{+}	10	100
Hg^{+}	10	100
Pb^{2+}	10	0
Mg^{2+}	100	0
Ca^{2+}	100	0
Fe^{3+}	100	0
Fe^{2+}	10	100
Fe^{2+}	1	37
Co^{2+}	100	97
Co^{2+}	10	36
Mn^{2+}	100	100

Effect of substrate concentration

The velocity of the nitrilase reaction was determined after association of the enzyme was complete at concentrations of benzonitrile between 0.5 and 18mM. The associated enzyme obeyed Michaelis–Menten kinetics throughout the substrate-concentration range tested, and the K_m as determined graphically by the method of Lineweaver & Burk (1934) was 4.0mM. A similar determination performed at pH7.5 close to the pH optimum for association of the enzyme gave a K_m value of 1.1mM. Thus the affinity of the enzyme for substrate is higher at the optimum pH for association of the enzyme rather than at the optimum for hydrolytic activity. This perhaps suggests that *in vivo* the enzyme operates at an environmental pH nearer 7.5 than 8.0, despite the fact that $V_{max.}$ is higher at the latter pH. At the concentrations of benzonitrile examined, no significant effect of substrate concentration on association time was noted, implying that the K_m for substrate-activated association of the enzyme was considerably less than that for nitrilase activity.

Substrate specificity

The relative rates of hydrolysis of the number of substituted aromatic and aliphatic nitriles were measured at five different concentrations. In all cases the velocity of hydrolysis was determined when it became linear with respect to time. $V_{max.}$ was calculated from a Lineweaver–Burk plot when the substrate–enzyme interaction followed Michaelis–Menten kinetics. However, in a number of instances, as might be expected from the dual role of substrate in substrate–enzyme interaction, certain substrates (marked with an asterisk in Table 4) failed to give linear Lineweaver–Burk plots. Instead, upward-curved reciprocal plots were obtained, from which values of $V_{max.}$ were impossible to calculate by the normal method. Such results probably arise in cases where the K_m value for association of the enzyme subunits is high in relation to that for hydrolytic activity, resulting in failure of the substrate to produce complete association of the enzyme at equilibrium.

Fortunately this behaviour can be analysed in terms of substrate acting as an activator (Dixon & Webb, 1971), and the curved Lineweaver–Burk reciprocal plots obtained were found to conform to the quadratic relationships predicted by this treatment, enabling $V_{max.}$ to be determined by extrapolation.

The rates of hydrolysis of different nitriles relative to that of benzonitrile are shown in Table 4, together with the Hammett σ value (Brown *et al.*, 1955) of the substituent in the case of the substituted aromatic nitriles. It is evident that *meta*- or *para*-substitution of the aromatic ring by fluoro, chloro, bromo, methyl or hydroxyl groups is consistent with enzymic attack on the nitrile, in some instances at rates even greater than that of the unsubstituted compound. *m*-Nitrobenzonitrile and 1,3-benzodinitrile are also attacked, although the corresponding *para*-substituted compounds are not substrates for the enzyme.

Mahadevan & Thimann (1964) in their study of the nitrilase isolated from barley leaves reported a strong correlation between the electron-withdrawing capacity of the substituent as measured by the Hammett σ value and the velocity of hydrolysis of a monosubstituted benzonitrile. No such relationship is discernible in the present study with the bacterial enzyme. However, this finding does not necessarily imply a different mechanism of hydrolysis, since for the bacterial enzyme the electronic and steric requirements of the subunit binding site which governs the degree of association and hence the activity of the enzyme must also be considered. It is clear that *ortho*-substitution in the aromatic ring except by the small fluoro group is incompatible with enzyme attack. Steric hindrance of enzymic hydrolysis exerted by *ortho*-substituents was also reported by Mahadevan & Thimann (1964) and has been noted in the alkaline hydrolysis of both nitriles and amides (Verloop, 1972). The latter author reported that, although mono-*ortho*-substitution decreases the rate of alkaline hydrolysis of aromatic nitriles to the acid, di-*ortho*-substitution will completely inhibit this process. Whether in the present case *ortho*-substitution hinders the association process or hydrolysis at the active site or both is not known.

The cyanopyridines acted as rather poor substrates for the enzyme, and aliphatic nitriles such as indol-3-ylacetonitrile and acetonitrile itself were not attacked at all. This is in marked contrast with the situation with the barley enzyme, where cyanopyridines and some aliphatic nitriles are hydrolysed at rates in excess of those for benzonitrile. Neither the di-*ortho*-substituted herbicide Dichlobenil (2,6-dichlorobenzonitrile) nor Bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) were attacked by the bacterial nitrilase even at high concentrations, indicating that the rather rigid substrate requirements imposed by the unusual nature of the enzyme render it of little significance in the breakdown of such nitrilic herbicides in the environment.

Table 4. *Relative rates of hydrolysis of various nitriles*

Rates of hydrolysis of nitriles by enzyme at 30°C in 100mM-sodium phosphate buffer, pH8.0, were measured at five different substrate concentrations. In most instances the enzyme used was of specific activity and dilution of stage-(3) partially purified preparations. However, with substrates giving less than 1% of the rate of hydrolysis of benzonitrile, 5-fold more concentrated enzyme preparations were also used in an attempt to demonstrate enzymic attack. $V_{max.}$ was determined by the Lineweaver–Burk method and is expressed as a percentage of that with benzonitrile. Where substrates did not follow Michaelis–Menten kinetics (substrates marked with an asterisk in the Table) $V_{max.}$ was calculated as described in the text. For the substituted benzonitriles the Hammett σ value (see Brown *et al.*, 1955) is quoted in the Table. Aliphatic nitriles such as acetonitrile, propionitrile, indol-3-ylacetonitrile and chloroacetonitrile do not act as substrates, nor do the herbicides 3,5-dibromo-4-hydroxybenzonitrile (Bromoxynil) and 2,6-dichlorobenzonitrile (Dichlobenil).

Compound	$V_{max.}$ (% of $V_{max.}$ with benzonitrile)	Hammett σ value
o-Fluorobenzonitrile*	29.4	+0.93
m-Fluorobenzonitrile	58.4	+0.33
p-Fluorobenzonitrile*	113.2	+0.06
o-Chlorobenzonitrile	0	+1.26
m-Chlorobenzonitrile	140.5	+0.37
p-Chlorobenzonitrile*	130.8	+0.21
o-Bromobenzonitrile	0	+1.35
m-Bromobenzonitrile	174.8	+0.39
p-Bromobenzonitrile	49.1	+0.20
o-Nitrobenzonitrile	0.5	+2.03
m-Nitrobenzonitrile*	65.2	+0.75
p-Nitrobenzonitrile	0	+0.76
o-Tolunitrile	0	+0.29
m-Tolunitrile*	95.5	−0.04
p-Tolunitrile*	116.6	−0.14
1,2-Benzodinitrile	0	—
1,3-Benzodinitrile*	34.4	+0.60
1,4-Benzodinitrile	0	+0.65
o-Hydroxybenzonitrile	0	+1.22
m-Hydroxybenzonitrile	7.6	+0.12
p-Hydroxybenzonitrile	9.0	−0.38
2-Cyanopyridine	4.9	
3-Cyanopyridine	13.6	
4-Cyanopyridine	26.2	

Action of benzamide and possible mechanism of nitrile hydrolysis

As previously noted, benzamide does not act as a substrate for washed suspensions of cells grown on benzonitrile, nor does it modify O_2 uptake with other substrates, suggesting that benzamide is not an inter-

mediate in nitrile hydrolysis. Although after a lengthy induction period the bacterium would grow on benzamide as carbon and nitrogen source, suspensions of cells grown in this way will not utilize benzonitrile as substrate and cell-free extracts show no nitrilase activity, implying that in this case benzamide is degraded by an amidase rather than a nitrilase.

Studies with the purified enzyme demonstrate that benzamide is not attacked by the nitrilase, nor does it affect the hydrolysis of benzonitrile by the enzyme. Observations indicate that benzamide does not cause association of the enzyme and does not change the association time of the enzyme in the presence of benzonitrile. In addition no trace of benzamide could be detected when homogeneous enzyme was incubated with benzonitrile. These findings are again consistent with a mechanism of nitrile hydrolysis not involving the formation of benzamide as an intermediate. Such a mechanism has been proposed by Mahadevan & Thimann (1964) to account for the formation of carboxylic acid direct from the nitrile by the nitrilase isolated from barley. In this scheme the nitrile carbon atom is subjected to nucleophilic attack by a thiol group at the active site of the enzyme. The resulting enzyme imino thioether is hydrolysed via a hydroxyamino thioether to the corresponding ketone, liberating NH_3. The acyl-enzyme is then hydrolysed, liberating the acid. The activated thiol group at the enzyme active site proposed in this mechanism would necessarily be very reactive and would explain the extreme sensitivity of the bacterial enzyme to thiol reagents if such a mechanism is operative in the present instance.

Hook & Robinson (1964) have concluded that a similar mechanism is responsible for the hydrolysis of ricinine by ricinine nitrilase. However, in this instance, in order to account for the observed formation of a small but constant proportion of amide in the hydrolysis, it is assumed that the enzyme hydroxyamino thioether can also decompose, with the enzyme rather than NH_3 acting as the leaving group, so resulting in the formation of the amide.

As would be predicted from this mechanism both of the above-mentioned groups of workers reported that the amide was not a substrate for the nitrilase studied. It therefore seems probable that an identical mechanism is operative in the hydrolysis of benzonitrile by the enzyme from *Nocardia*, though in this case the enzyme hydroxyamino thioether is cleaved solely via the ammonia-elimination route described by Mahedevan & Thimann (1964) leading only to the formation of acid.

Role of the enzyme in metabolism

Only a few instances of enzymes displaying slow association or dissociation reactions have been described in the literature, e.g. phosphorylase *a* and phosphofructokinase in muscle (see Frieden, 1968), and none of these reports involves slow substrate-induced association of an enzyme from a bacterial source. The occurrence of such an enzyme in *Nocardia* sp. is therefore somewhat unexpected. It has been suggested by Frieden (1968) that the slow association or dissociation phenomena observed in enzymes from muscle may serve a regulatory function *in vivo* by buffering the system against certain metabolic alterations which might be produced under stress, i.e. conditions of high metabolic activity leading to pH changes. As the primary initiator of attack on the carbon and nitrogen source of the organism the bacterial nitrilase may be required to play a somewhat similar role. Unlike most enzyme transformations initiating the bacterial degradation of substrate, the nitrilase reaction is non-aerobic, and hence under conditions of limited O_2 supply might well continue at a rate far in excess of the ability of the cell to metabolize the products. Under such a regime, cleavage of benzonitrile to form benzoic acid and NH_3 could lead to unfavourable and ultimately toxic changes in the pH balance of the bacterial cell or its environment if either of these products accumulates to a significant extent within the cell. The unusual associative behaviour of the nitrilase may prevent this eventuality, since the association reaction is sharply dependent on pH and any change from the optimum at about 7.4 would result in slower or incomplete association and hence an effective decrease in total nitrilase activity. Such a feedback mechanism might thus prevent the excessive or toxic accumulation of either product of the reaction in the cell or its immediate environment. That benzoic acid is formed by the organism at a rate in excess of that at which it is catabolized is illustrated by the rapid increase in its concentration in the culture medium during the first stages of growth on benzonitrile, as shown in Fig. 1. It is perhaps pertinent to note that the organism was isolated from the bottom mud of a polluted section of river, an environment where O_2 supply was obviously restricted for most of the time.

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