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Fungal Degradation of Aromatic Nitriles

ENZYMOLOGY OF C–N CLEAVAGE BY *FUSARIUM SOLANI*

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1. A strain of the fungus *Fusarium solani* able to use benzonitrile as sole source of carbon and nitrogen was isolated by elective culture. 2. Respiration studies indicate that the nitrile, after degradation to benzoate, is catabolized via catechol or alternatively via *p*-hydroxybenzoate and 3,4-dihydroxybenzoate. 3. Cell-free extracts of benzonitrile-grown cells contain an enzyme mediating the conversion of benzonitrile into benzoate and ammonia. 4. The nitrilase enzyme was purified by DEAE-cellulose chromatography, $(NH_4)_2SO_4$ precipitation and gel filtration on Sephadex G-200. The homogeneity of the purified enzyme preparation was confirmed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and isoelectric focusing on polyacrylamide gel. 5. The enzyme showed a broad pH optimum between pH 7.8 and 9.1 and a K_m with benzonitrile as substrate of 0.039 mM. The activation energy of the reaction deduced from an Arrhenius plot was 48.4 kJ/mol. 6. The enzyme was susceptible to inhibition by thiol-specific reagents and certain heavy metal ions. 7. Gel filtration gave a value of 620000 for the molecular weight of the intact enzyme. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis demonstrated that the enzyme was composed of eight subunits of mol.wt. 76000. 8. Rates of enzymic attack on various substrates indicated that the nitrilase has a fairly broad specificity and that the fungus probably plays an important role in the biodegradation of certain nitrilic herbicides in the environment.

Previous work has demonstrated the existence of an enzyme in bacteria capable of degrading nitriles with the formation of either the corresponding acid, as in the hydrolysis of benzonitrile (Harper, 1974, 1976, 1977), or the amide, as in the hydrolysis of acetonitrile (Mimura *et al.*, 1969; Firmin & Gray, 1976). In addition an enzyme (EC 3.5.5.2), albeit of limited substrate specificity, has been isolated and purified from a *Pseudomonas* sp. capable of growth on the naturally occurring cyanopyridine, ricinine (Robinson & Hook, 1964; Hook & Robinson, 1964). In this instance the nitrile is cleaved to the corresponding acid with the formation of a small proportion of amide, though the latter is not a substrate for the nitrilase.

Harper (1977) has described the purification and characterization of a substrate-activated multisubunit enzyme showing unusual kinetic behaviour from a *Nocardia* sp. This enzyme will mediate the conversion of benzonitrile and a number of substituted aromatic nitriles directly into the corresponding acid without formation of the amide as an intermediate or a product. In some higher plants a nitrilase (EC 3.5.5.1), thought to have indol-3-ylacetonitrile as its natural substrate *in vivo*, though of fairly wide substrate specificity, has been identified by Thimann & Mahadevan (1964) and isolated and purified from barley leaves (Thimann & Mahadevan, 1964; Mahadevan & Thimann 1964). Again quantitative conversion of substrate into the acid was observed with this enzyme.

Several workers have isolated fungal species which appear to degrade nitriles. Thus Thimann & Mahadevan (1964) showed that many *Fusarium* species, *Aspergillus niger* and *Penicillium chrysogenum* will convert indol-3-ylacetonitrile into indol-3-ylacetic acid. Recent investigations into the degradation of the herbicides Bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) and Ioxynil (3,5-di-iodo-4-hydroxybenzonitrile) also suggest that *Fusarium solani* may be a major species in the breakdown of such compounds in the environment (Hsu & Camper, 1976). Moreover, Japanese workers have demonstrated that the presence of cyanide will induce a cyanide-degrading enzyme system in strains of this species (Shimizu & Taguchi, 1969). However, no reports have appeared of the enzymology of nitrile cleavage by *Fusarium* or other fungal genera.

The present paper describes the purification and properties of the enzyme responsible for C–N cleavage from a fungal species isolated from Bromoxynil-treated soil, identified as *F. solani*, and found capable of growth on benzonitrile as sole carbon and nitrogen source. The differences between

this enzyme and the bacterial and plant enzymes previously investigated is discussed, and its significance in the breakdown of herbicides in the environment is considered.

Materials and Methods

Isolation and culture of fungus

A fungus with the ability to use benzonitrile as sole carbon, nitrogen and energy source was isolated from the soil of a Bromoxynil-treated field near Downpatrick, County Down, N. Ireland, by elective culture on 0.1% benzonitrile. The white isolate was identified by Dr. C. Booth of the Commonwealth Mycological Institute, Ferry Lane, Kew, Surrey, U.K., as *Fusarium solani* (Mart.) Sacc (I.M.I. 196840). The organism was routinely 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, autoclaved at 120°C for 20min, sterile benzonitrile was added to 0.1% (w/v) and the cultures were incubated at 25°C on an orbital shaker, with an eccentricity of 2.5cm, at 200rev./min. The growth of the mycelium was complete after about 5 days under these conditions.

Preparation of washed mycelial suspensions

The mycelia were harvested after about 4 days, shortly before maximum growth was achieved, with a Sharples Super continuous-action centrifuge at a flow rate of 250ml/min. Mycelia were washed with 100mM-sodium phosphate buffer, pH7.5, and harvested by centrifugation (15000*g*, 30min, 10°C), then resuspended in the wash buffer and again harvested by centrifugation under similar conditions.

Measurement of O_2 uptake by mycelial suspensions

O_2 consumption by mycelial suspensions was recorded at 25°C with a 4ml-capacity Clark-type oxygen electrode (Rank Bros., Bottisham, Cambridge, U.K.). Incubation mixtures contained 100mM-sodium phosphate buffer, pH7.5, mycelial suspension (2.5mg dry wt. of mycelia) and 1mM-substrate in a total volume of 4ml.

Preparation of cell-free extracts

Washed mycelium (20g wet wt.) harvested from 15 litres of culture medium was suspended in 100mM-sodium phosphate buffer, pH8.0 (80ml), containing 2mM-EDTA and 2mM-dithioerythritol. The suspension in volumes of 15ml was then disrupted in a Braun disintegrator with glass beads (0.25–0.30mm diam.; 30g) for a period of 5min, the temperature being kept below 10°C by cooling with liquid CO_2. The pooled suspension from this treatment was centrifuged (60000*g*, 30min, 10°C) and the clear cell-free supernatant decanted. The remaining cell debris and glass beads were washed with further buffer solution (40ml), centrifuged as previously, and the supernatant was pooled with the original extract.

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 fungal enzyme was assayed by measuring the production of ammonia during the hydrolysis of benzonitrile to benzoic acid by using the method of Fawcett & Scott (1960). The standard assay was performed in triplicate at 30°C in tubes containing, unless otherwise stated, 0.9ml of 30mM-benzonitrile in 100mM-sodium phosphate buffer, pH8.0, to which was added 0.1ml of extract. Mixtures were incubated for periods from 10 to 60min depending on the experiment, after which time 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 with a Perkin–Elmer Coleman-model 55 UV-VIS digital spectrophotometer with auto sampler. At ammonia concentrations up to 0.9mM the A_{640} was directly proportional to ammonia concentration. Neither benzonitrile nor residual protein in the assay mixture interfered with the colour reaction at the enzyme and substrate 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, 1,2-dicyanobenzene, 1,3-dicyanobenzene 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). Indol-3-ylacetonitrile was obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. 3,5-Dibromo-4-hydroxybenzonitrile was prepared as described by Carpenter *et al.* (1964). Proteins used for calibration of Sephadex G-200 columns and also used as standards in SDS*/polyacrylamide-gel electrophoresis were purchased from Sigma Chemical Co. as was DEAE-cellulose (coarse grade) used in column chromatography and the acrylamide used in electrophoresis. SDS (Sigma) was recrystallized from ethanol before use. Ampholines of various pH ranges used in isoelectric focusing were purchased from LKB Instruments, South Croydon, Surrey, U.K.

Thin-layer chromatography

The products of enzyme attack on benzonitrile were identified by t.l.c. Nitrilase (10ml) from stage 4 of the purification procedure was incubated for 30min with 100mM-sodium phosphate buffer, pH8.0, containing 30mM-benzonitrile (500ml). 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 samples were applied to t.l.c. plates precoated with silica gel (Eastman–Kodak, Kirby, Liverpool, U.K.). Chromatography was performed with the solvent systems and spray reagents as described by Harper (1977).

Electrophoresis

Isoelectric focusing on polyacrylamide gel. Analytical thin-layer electrofocusing in polyacrylamide gel was performed in an LKB 2117 Multiphor instrument by the method described by Karlsson *et al.* (1973) for isoelectric focusing in the pH range 2.5–6.0, with 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. To prevent dissociation of the nitrilase on electrophoresis, and thus obtain sharper resolution, it was found beneficial to dilute the sample solution with an equal volume of 30mM-benzonitrile immediately before application to the paper. The pH gradient in the gel after electrofocusing at 2°C was determined by means of an Activion surface electrode (Activion Glass Ltd., Halstead, Surrey, U.K.). The staining technique of Vesterberg (1972) was used for locating protein bands with Coomassie Brilliant Blue R-250.

SDS/polyacrylamide-gel electrophoresis. For determination of the homogeneity of the nitrilase and the molecular weight of its constituent subunits, electrophoresis on polyacrylamide gel was performed in the presence of SDS by a thin-layer technique with an LKB 2117 Multiphor apparatus. Details of the preparation of gels, application of samples and the conditions for electrophoresis are given elsewhere (Harper, 1977).

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. The following proteins were used for calibration (mol.wt. of subunit in parentheses): phosphorylase *a* (100000), bovine serum albumin (68000), L-amino acid oxidase (63000), catalase (58000), γ-globulin (H chain 50000, L chain 23500), ovalbumin (43000), alcohol dehydrogenase (41000), glyceraldehyde 3-phosphate dehydrogenase (36000), aldolase (41000), lactate dehydrogenase (36000), carbonic anhydrase (29000) and trypsin (23300).

Results and Discussion

Respiration studies on washed mycelial suspensions

Mycelial suspensions prepared as described in the Materials and Methods section from fungal cultures actively growing on benzonitrile were tested for their ability to oxidize possible intermediates in the degradation of benzonitrile and the results are presented in Table 1. In addition to oxidizing benzonitrile such cells rapidly oxidize benzoate and catechol, but not benzamide, suggesting that degradation proceeds directly to benzoic acid, which is catabolized via catechol by either the *ortho-* or the *meta*-cleavage pathway in a similar manner to the bacterial degradation previously described (Harper, 1977). However, *p*-hydroxybenzoate and 3,4-dihydroxybenzoate also show relatively high O_2 uptakes, indicating that at least a proportion of the benzoate formed may be degraded by ring fission of 3,4-dihydroxybenzoate as in *Aspergillus* (Jamaluddin *et al.*, 1970), though it is possible that O_2 uptake on these substrates represents non-specific induction of enzymes associated with the catabolism of the compounds. Whichever of the latter ring-fission routes is used, the low O_2 uptake with 3,4-dihydroxybenzonitrile as substrate implies that the initial step in degradation of benzonitrile is conversion into benzoic acid.

Purification of nitrilase

All stages of enzyme purification were performed at a temperature between 0 and 3°C. Stages in

* Abbreviation: SDS, sodium dodecyl sulphate.

Table 1. *Oxidation of possible intermediates in benzonitrile metabolism by mycelial suspensions of F. solani*
Suspensions of the organisms were prepared, and the initial rate of O_2 uptake on 1 mM solutions of different substrates was determined as described in the Materials and Methods section. Values for O_2 uptake are initial values and are corrected for an endogenous respiration rate of 14.1 μl of O_2/h per mg dry wt.

Substrate	O_2 uptake (μl/h per mg dry wt.)	Substrate	O_2 uptake (μl/h per mg dry wt.)
Benzonitrile	46.2	*o*-Hydroxybenzoate	1.9
Benzoate	58.0	*m*-Hydroxybenzoate	17.9
Catechol	44.8	*p*-Hydroxybenzoate	26.6
Benzamide	4.1	*o*-Hydroxybenzonitrile	5.3
3,4-Dihydroxybenzoate	37.3	*m*-Hydroxybenzonitrile	11.9
2,5-Dihydroxybenzoate	3.9	*p*-Hydroxybenzonitrile	10.2
3,4-Dihydroxybenzonitrile	10.2		

Table 2. *Purification of nitrilase from F. solani*
The results shown are of a typical enzyme-purification procedure as described in the text. All stages were performed at a temperature between 0 and 3°C.

Stage of preparation	Volume (ml)	Total 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	114	58.7	100	5.88	0.088
2. DEAE-cellulose column eluate	165	38.1	68.5	0.97	0.237
3. $(NH_4)_2SO_4$ precipitate redissolved and dialysed	21	22.4	38.2	2.62	0.407
4. Eluate after gel filtration on Sephadex G-200	38	14.2	24.2	0.225	1.66

purification of the cell-free extract (1) are described below and the results are summarized in Table 2.

(2) *DEAE-cellulose chromatography.* DEAE-cellulose suspended in 100mM-sodium 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 faintly brown cell-free extract (114ml) was applied to the column, which was then eluted with the equilibrating buffer until no further protein was removed. The nitrilase, which had been retained by the column, was then eluted with 200mM-sodium phosphate buffer, pH7.5, containing 1mM-EDTA and 0.25mM-dithioerythritol. Fractions (10ml) were collected, assayed and those possessing high nitrilase activity pooled to give 165ml of partially purified extract.

(3) *Precipitation with* $(NH_4)_2SO_4$. To the enzyme solution (165ml) from stage 2 solid $(NH_4)_2SO_4$ (57.75g) was added with stirring over a period of 1h to give a 0.55%-satd. solution. After a further hour the solution was centrifuged (20000*g*, 30min, 1°C), the supernatant discarded, the white precipitate dissolved in 100mM-phosphate buffer, pH8.0, containing 1mM-EDTA and 0.25mM-dithioerythritol (17ml) and dialysed overnight against 5 litres of the same buffer.

(4) *Gel filtration on Sephadex G-200.* The extract was concentrated by ultrafiltration to 10ml in an Amicon Diaflo cell by using a PM 10 membrane and applied to a Sephadex G-200 column (2.5cm×60cm) equilibrated with buffer of the same composition as that in which the stage-3 precipitate was dissolved. On elution with this buffer, fractions (3.8ml) of eluate were collected and only those containing the highest nitrilase activity were pooled.

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 pI4.19 (see Fig. 1). SDS/polyacrylamide-gel electrophoresis also confirmed the homogeneity of the preparation (see below under 'Determination of molecular weight'). Purification by the above procedure was 19-fold and the overall yield 14.2%.

Properties of nitrilase

In the purified form the enzyme exhibited a relatively short half-life of 48h in 100mM-phosphate

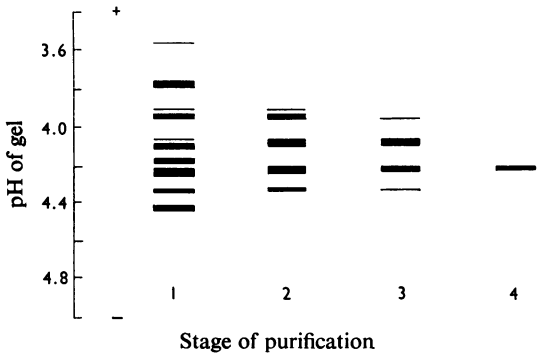

Fig. 1. *Isoelectric focusing of protein fractions obtained in purification of nitrilase*

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, redissolved $(NH_4)_2SO_4$ precipitate; 4, fraction from gel filtration.

buffer, pH8.0, containing 1mM-EDTA and 0.25mM-dithioerythritol at 1°C. Therefore more stable partially purified preparations usually those of stage 3 (with a half-life of 7 days under the above conditions) were, in general, used for enzyme characterization. However, stage-4 preparations were used for determination of molecular weight and the products of enzyme reaction. Enzyme preparations were normally stored in dilute solution in the buffer described above at 1°C, as freezing destroyed up to 50% of activity. In experiments involving the effect of enzyme concentration, incubation during assay was performed in the presence of 0.05% (w/v) bovine serum albumin to lessen enzyme denaturation.

Products of enzyme reaction

The products of nitrilase attack were investigated by t.l.c. Benzoic acid and ammonia were shown to be the sole compounds produced on hydrolysis of benzonitrile. No benzamide was detected, nor did this compound act as a substrate for the enzyme, suggesting that the amide was not an intermediate in benzonitrile degradation. Thus the fungal enzyme displays similarities to the nitrilases isolated from barley by Thimann & Mahadevan (1964) and from *Nocardia* by Harper (1977) rather than to the amide-producing nitrilases observed in *Corynebacterium* and *Pseudomonas* sp. (Mimura *et al.*, 1969; Firmin & Gray, 1976; Robinson & Hook, 1964).

Influence of enzyme concentration

Experiments on the effect of varying enzyme concentration on the initial velocity of the nitrilase reaction demonstrated that the rate of ammonia release was directly proportional to enzyme concentration at enzyme concentrations ranging from 2.5 to 50μg/ml. This observation is in marked contrast with the situation reported with the nitrilase isolated from *Nocardia* (Harper, 1977), where the initial velocity was dependent on enzyme concentration as a result of slow substrate-activated association of enzyme subunits.

Influence of pH

The effect of pH on the activity of the nitrilase was measured under standard assay conditions at pH values obtained by using 100mM solutions of sodium acetate, sodium phosphate, sodium borate and sodium bicarbonate buffers. The enzyme exhibits a fairly broad plateau of activity between pH7.8 and 9.1, quite unlike the sharp pH optimum shown by the bacterial enzyme from *Nocardia* (Harper, 1977), but displaying marked similarities to the maxima given by the barley nitrilase (Thimann & Mahadevan, 1964). Activity decreases sharply at pH values above 10, but falls more slowly at pH values on the acidic side of the maxima, slight activity being manifested at pH5.0.

Influence of temperature

The velocity of nitrile hydrolysis was determined at temperatures between 10 and 55°C under standard assay conditions by using an incubation time of 1h. An Arrhenius plot of the results gave a slope from which the activation energy of the nitrilase reaction was calculated to be 48.4kJ/mol (11580cal/mol) and independent of temperature between 10 and 50°C. Above 50°C the slope decreases, indicating inactivation of the enzyme at higher temperatures. The value for the activation energy is similar to that determined for the reaction when catalysed by the bacterial enzyme, although the latter enzyme is considerably more heat-labile.

Effect of substrate concentration

The initial velocity of nitrile hydrolysis was measured at concentrations of benzonitrile between 0.035 and 27mM under standard assay conditions, and the results are given in Fig. 2 in the form of a Lineweaver–Burk plot (Lineweaver & Burk, 1934). Over the lower part of the substrate concentration range tested, the enzyme displayed Michaelis–Menten kinetics, although at substrate concentrations above 0.3mM a slight deviation from linearity was evident. The Michaelis constant K_m of 39μM obtained from the plot was two orders of magnitude less than that noted for the bacterial enzyme (Harper, 1977) and significantly less than that observed with the nitrilase

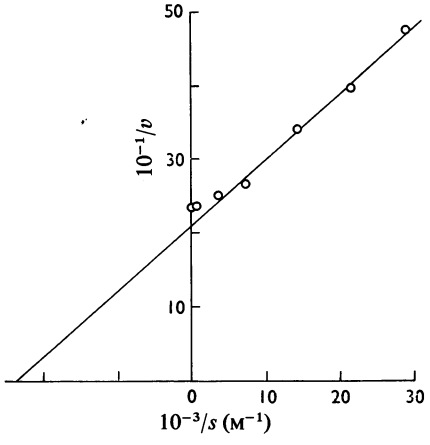

Fig. 2. *Lineweaver–Burk plot for nitrilase*
The initial velocity of nitrile hydrolysis was measured under standard assay conditions by using concentrations of benzonitrile between 0.035 and 27mM.

isolated from barley (Thimann & Mahadevan, 1964). This high affinity of the fungal enzyme for substrate may indicate that the natural nitrilic substrate of the nitrilase in the soil or on the plant host is normally present at rather low concentrations, although other explanations, such as an inherently low catalytic activity or a relatively low rate of uptake of substrate by the cell, are also possible.

Determination of molecular weight

The molecular weight of the enzyme was determined by gel filtration of the pure nitrilase on a Sephadex G-200 column previously calibrated with the following reference proteins (mol.wt. in parentheses); thyroglobulin (680000), catalase (240000), ovalbumin (43000) and myoglobin (17300). Purified enzyme (100μg) was applied to the top of a column (2.5cm × 70cm) of Sephadex G-200 equilibrated with 100mM-sodium phosphate buffer, pH8.0, containing 1mM-EDTA and 0.25mM-dithioerythritol, and the column was eluted with the same buffer. Fractions (3ml) were collected and assayed for nitrilase activity. The eluted enzyme protein emerged as a single discrete peak of activity at an elution volume relative to the void volume of 1.038 corresponding to a mol. wt. of 620000. The presence of 5mM-benzonitrile in the eluting buffer did not alter the elution volume of the protein. The molecular weight of the constituent subunits of the enzyme was measured by SDS/polyacrylamide-gel electrophoresis as described in the Materials and Methods section. This technique indicated a subunit mol.wt. of 76000. Thus it appears that the intact enzyme, mol.wt. approx. 620000, consists of eight subunits each of mol.wt. 76000. This finding is in contrast with the situation observed with the bacterial nitrilase (Harper, 1977), in which the active enzyme had a mol.wt. of 560000 and was formed only in the presence of substrate from 12 subunits each of mol.wt. 45000.

Table 3. *Effect of inhibitors and metal ions on nitrilase*
The enzyme was preincubated in the presence of inhibitor for 10min before the addition of substrate and assayed under standard conditions. The following ions at 100μM showed no inhibition of nitrilase activity: Pb^{+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, Co^{2+}, Fe^{2+}, Fe^{3+}, Ca^{2+}, Mg^{2+}.

Inhibitor or metal ion	Final concentration (μM)	Inhibition (%)
p-Hydroxymercuribenzoate	50	52
Phenylmercuric acetate	50	94
Phenylmercuric acetate+ 1mM-reduced glutathione	50	84
N-Ethylmaleimide	50	11
Iodoacetamide	50	20
KCN	500	0
NaN_3	500	0
Ag^{+}	10	100
Hg^{+}	10	100

Effect of inhibitors and metal ions

The sensitivity of the enzyme to thiol reagents, metal ions and chelating agents was investigated. After dialysis against 100mM-phosphate buffer, pH8.0, the enzyme was preincubated in the presence of the possible inhibitor for 10min before assay, which was performed under standard conditions. The percentage inhibition of hydrolytic activity shown by various compounds compared with the control is given in Table 3.

The susceptibility of the enzyme to inhibition by thiol-specific reagents such as *p*-hydroxymercuribenzoate, phenylmercuric acetate and *N*-ethylmaleimide demonstrates that thiol groups are probably involved at the active site of the enzyme, particularly as inhibition by phenylmercuric acetate can be partially reversed by reduced glutathione. Prolonged dialysis of the enzyme against 1mM-EDTA and incubation with chelating agents such as cyanide and azide had no demonstrable effect on activity, indicating that the enzyme lacked a metal-ion requirement. Of the metal ions tested, only those of the heavy metals Ag^{+} and Hg^{+} caused loss of activity, confirming the importance of thiol groups at the active site. As might be expected with a high-molecular-weight multi-subunit enzyme of this type, incubation with 6M-urea caused inactivation, and only about 13% of the original activity was recovered on dilution.

From the foregoing it is therefore evident that the fungal enzyme displays a broadly similar pattern of inhibition by thiol reagents and heavy metal ions to that shown by the bacterial enzyme (Harper, 1977), although it is considerably less sensitive to such agents than the latter enzyme.

Substrate specificity and environmental significance of nitrilase

Relative rates of hydrolysis of various substituted aromatic and aliphatic nitriles were measured at five different concentrations at 30°C. $V_{max.}$ for each substrate was determined by the Lineweaver–Burk method (Lineweaver & Burk, 1934) and expressed as a percentage of that with benzonitrile as substrate in Table 4.

Clearly both *meta*- and *para*-substitution of benzonitrile by the halogens, methyl, nitro, hydroxyl and nitrile groups is compatible with enzymic attack; *ortho*-substituted nitriles are, however, in general, poor substrates for the enzyme. Steric hindrance of the enzyme is probably responsible for this effect, which was also noted with the barley nitrilase by Mahadevan & Thimann (1964) and the bacterial enzyme by Harper (1977). Although the cyanopyridines were fairly rapidly hydrolysed, aliphatic nitriles, such as acetonitrile and indol-3-ylacetonitrile, acted as rather poor substrates for the enzyme.

Mahadevan & Thimann (1964) in their study of the barley nitrilase demonstrated a strong correlation between the electron-withdrawing power of the substituent as measured by the Hammett σ-value and the velocity of hydrolysis of a monosubstituted benzonitrile. The bacterial enzyme from *Nocardia* exhibited no such relationship (Harper, 1977), and Table 4 demonstrates that the Hammett σ-values quoted for the aromatic substrates tested likewise show no correlation with their rate of hydrolysis by the fungal enzyme, even when the anomalous *ortho*-substituted compounds are ignored. Although this observation could indicate a different mechanism of hydrolysis, the overall similarities between the plant and fungal enzymes, together with the finding that benzamide did not act as a substrate, tend to suggest a somewhat similar mechanism.

Although the di-*ortho*-substituted herbicide Dichlobenil (2,6-dichlorobenzonitrile) was not cleaved by the fungal enzyme, both Bromoxynil and Ioxynil (3,5-dibromo- and 3,5-di-iodo-4-hydroxybenzonitrile respectively) were hydrolysed at significant rates, indicating that unlike the bacterial enzyme, the fungal nitrilase probably plays an important role in the degradation of these compounds in the environment, particularly in view of the widespread occurrence of *F. solani* in the soil. Support for this conclusion is provided by investigations in the U.S.A. by Hsu & Camper (1976), who have found that, of a number of isolates of micro-organisms able to degrade Ioxynil when provided with exogenous nutrients, only two, the fungus *F. solani* and the bacterium *Klebsiella ozaenae*, could release $^{14}CO_2$ from *ring*-^{14}C-labelled Ioxynil. Of these organisms *F. solani* was a more effective degrader of the herbicide than was the bacterium. It seems likely that hydrolysis of the nitrile group is the limiting factor in the degradation of nitrilic herbicides and that the comparative persistence of Dichlobenil in the environment (Verloop, 1972) compared with the rapid disappearance of Bromoxynil and Ioxynil (Carpenter *et al.*, 1964; Smith, 1971) simply reflects the invulnerability of *ortho*-substituted nitriles to attack by fungal, plant and bacterial nitrilases compared with the ready hydrolysis of most *meta*- and *para*-substituted compounds by both the fungal and plant enzymes.

Table 4. *Relative rates of hydrolysis of different nitriles*
Rates of hydrolysis of various nitriles by enzyme at 30°C in 100mM-sodium phosphate buffer, pH8.0, were measured at five different substrate concentrations. $V_{max.}$ was determined by the Lineweaver–Burk method and is expressed as a percentage of that with benzonitrile. For most of the substituted benzonitriles the Hammett σ-value (Harper, 1977) is also quoted in the Table.

Compound	$V_{max.}$ (% of $V_{max.}$ with benzonitrile)	Hammett σ-value
o-Fluorobenzonitrile	13.8	+0.93
m-Fluorobenzonitrile	74.3	+0.33
p-Fluorobenzonitrile	150.2	+0.06
o-Chlorobenzonitrile	0	+1.26
m-Chlorobenzonitrile	40.4	+0.37
p-Chlorobenzonitrile	85.6	+0.21
o-Bromobenzonitrile	0	+1.35
m-Bromobenzonitrile	34.4	+0.39
p-Bromobenzonitrile	37.6	+0.20
o-Nitrobenzonitrile	2.1	+2.03
m-Nitrobenzonitrile	7.0	+0.75
p-Nitrobenzonitrile	27.6	+0.76
o-Hydroxybenzonitrile	7.4	+1.22
m-Hydroxybenzonitrile	21.0	+0.12
p-Hydroxybenzonitrile	9.0	−0.38
1,2-Benzodinitrile	2.7	—
1,3-Benzodinitrile	8.5	+0.60
1,4-Benzodinitrile	213.3	+0.65
o-Tolunitrile	6.4	+0.29
m-Tolunitrile	17.9	−0.04
p-Tolunitrile	9.9	−0.14
Bromoxynil	2.2	—
Ioxynil	3.9	—
Dichlobenil	0	—
Acetonitrile	6.6	—
Indol-3-ylacetonitrile	1.3	—
Benzyl cyanide	0	—
o-Cyanopyridine	7.2	—
m-Cyanopyridine	31.0	—
p-Cyanopyridine	124.7	—

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