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A Heat-Stable Nicotinamide–Adenine Dinucleotide Glycohydrolase from *Pseudomonas putida* KB1

PARTIAL PURIFICATION AND SOME PROPERTIES OF THE ENZYME AND AN INHIBITORY PROTEIN

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A thermostable $NAD(P)^+$ glycohydrolase (EC 3.2.2.6) detected in cell-free extracts of *Pseudomonas putida* KB1 was purified to a single component on polyacrylamide-gel electrophoresis. A heat-labile inhibitor of the enzyme was also partially purified. Enzyme free of inhibitor is present in culture supernatants. After an ultrasonic treatment enzyme–inhibitor complex and excess of inhibitor are present in both the cell-debris and soluble fractions. The general properties of the enzyme and inhibitor are described. The molecular weights of enzyme, inhibitor and enzyme–inhibitor complex, determined by gel filtration are about 23500, 15000 and 35000 respectively. The binding of inhibitor and enzyme is inhibited by the presence of substrate.

$NAD(P)^+$ glycohydrolases (EC 3.2.2.6) catalyse the reaction:

$$NAD^+ + H_2O \rightarrow \text{nicotinamide} + \text{ADP-ribose} + H^+ \quad (1)$$

They have been demonstrated in animal and higher-plant tissues and in micro-organisms, and enzymes from mammalian and microbial sources have been purified.

Mammalian and microbial NADases† differ in several respects. Many of the mammalian enzymes are associated with microsomal particles and can only be purified after solubilization by treatment with enzymes (Dickerman *et al.*, 1962; Windmueller & Kaplan, 1962; Green & Bodansky, 1965; Swislocki *et al.*, 1967), whereas the microbial enzymes are soluble (see, e.g., Kern & Natale, 1958) and can even be isolated from the culture supernatant (Carlson *et al.*, 1957). The mammalian enzymes are more sensitive to inhibition by nicotinamide than are the microbial enzymes, and they are able to catalyse an exchange reaction between the nicotinamide moiety of NAD^+ and exogenous nicotinamide:

$$NAD^+ + [^{14}C]\text{nicotinamide} \rightleftharpoons [^{14}C]NAD + \text{nicotinamide} \quad (2)$$

This reaction is not catalysed by the microbial enzymes, although the presence of ergothioneine will impart sensitivity to nicotinamide inhibition to the NADase from *Neurospora crassa* (Grossman & Kaplan, 1958). The microbial enzymes are insensitive to inhibition by isonicotinic acid hydrazide, which is an inhibitor of some animal enzymes, nor do they catalyse either the imidazolysis of NAD^+ or the formation of an acid-insoluble polymer of ADP-ribose, both of which are catalysed by mammalian enzymes (Alivisatos & Wolley, 1955; Römer *et al.*, 1968).

The most striking difference between microbial and mammalian NADases is the stability of some of the microbial enzymes to heat. The NADase of *Mycobacterium tuberculosis* retains 70% of its activity after 1 min at 100°C (Gopinanthan *et al.*, 1964), that of *Mycobacterium butyricum* retains 60% after 20 min at 100°C (Kern & Natale, 1958), and that of *Bacillus subtilis* is described as 'heat-stable' (Everse & Kaplan, 1968). The NADases of *Streptococcus* sp. (Fehrenbach, 1969) and *N. crassa* (Kaplan *et al.*, 1951) are not heat-stable. Each heat-stable enzyme is associated *in vivo* with a heat-labile inhibitor, so that NADase activity is not detectable until preparations have been activated by heating.

We report here the partial purification and some properties of an NADase (comparable in its stability to heat with that of *M. butyricum*) and its inhibitor from *Pseudomonas putida* KB1. A preliminary account of this work has been published (Mather & Knight, 1969).

Materials and Methods

Microbiological methods

Nomenclature of Pseudomonas putida KB1. The organism used in this study was a fluorescent pseudomonad first isolated by Kogut & Podoski (1953). They found that it was intermediate in characteristics

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† Abbreviations: NADase, NAD glycohydrolase; NHD, nicotinamide–hypoxanthine dinucleotide; NHDP, nicotinamide–hypoxanthine dinucleotide phosphate; APAD, acetylpyridine–adenine dinucleotide; thio-NAD, thionicotinamide–adenine dinucleotide.

between *Pseudomonas convexa* and *Pseudomonas ovalis* and referred to it as 'a member of the *Pseudomonas fluorescens* species group'. It has, however, usually been called *P. fluorescens* KB1 (e.g. London & Knight, 1966; Meers & Tempest, 1970). By the criteria of Stanier *et al.* (1966) (ability to grow with creatine, hippurate or glycine as sole carbon source, inability to liquefy gelatin or to use trehalose or *meso*inositol as carbon sources, and negative egg-yolk reaction) this organism should be assigned to *P. putida*, and since it uses nicotinate, but neither tryptophan nor anthranilate, probably to biotype A. In the present paper the organism is referred to as *P. putida* KB1. A culture has been deposited with the National Collection of Industrial Bacteria with the accession number N.C.I.B. 10521.

Maintenance and growth of P. putida KB1. The culture was maintained by sub-culture every 2 months on slopes of Oxoid nutrient agar supplemented with 0.5% sodium succinate.

The organism was grown in liquid culture at 29°C in the minimal medium used by London & Knight (1966) with succinate as the carbon source. Inoculum cultures (300ml) were grown in 1-litre baffled flasks aerated by shaking in a Gallenkamp orbital shaker. Larger cultures (10 or 50 litres) were aerated by passing sterile air into the culture. The 50-litre cultures were grown in a polyethylene carboy, which was sterilized by washing with 80% (v/v) ethanol. In this case, the medium was not sterilized, but was prepared in freshly distilled water. Because of the risk of contamination, these large cultures were started with a 20% (v/v) inoculum.

Cultures were harvested in a Sharples air-turbine continuous-flow centrifuge. The cell paste was stored at −20°C.

Preparation of cell-free extracts. Stored cell-paste was thawed, suspended in about 10vol. of 0.1M-potassium phosphate buffer, pH7.0, centrifuged at 10000*g* for 10min and resuspended in the same buffer at about 20–25mg dry wt./ml. This cell suspension was then broken in 20ml portions by exposure to the full output of an MSE 100W ultrasonic generator, the suspension being cooled by antifreeze at 0°C circulated round the container. Cell debris was removed by centrifuging at 38000*g* for 1h. The extract was stored at −20°C.

Growth of other bacteria. Escherichia coli N.C.T.C. 4071 was grown on Oxoid nutrient broth supplemented with 0.5% (w/v) glucose. *Aeromonas punctata* N.C.M.B. 640, *Pseudomonas aeruginosa* 8602A (obtained from Dr. P. H. Clarke, Department of Biochemistry, University College, London, U.K.), *P. aeruginosa* T1, *P. fluorescens* Millstead (both obtained from Mr. R. J. Watkinson, Shell Research Ltd., Sittingbourne, Kent, U.K.), *Pseudomonas aureofaciens* N.C.I.B. 9030, *Pseudomonas testosteroni* N.C.I.B. 8893, *P. testosteroni* N.C.I.B. 8895 and *Pseudomonas multivorans* N.C.I.B. 9085 were grown on the succinate–salts medium used for *P. putida* KB1. Conditions of growth and harvesting were those used by London & Knight (1966). Cell-free extracts of these suspensions organisms in 0.1M-potassium phosphate buffer, pH7.0 were made with a French pressure cell (Aminco Instrument Co., Silver Spring, Md., U.S.A.), at a working pressure of 110MN/m² (16000lb/m²).

Assays

NADase assays. The unit of enzyme activity referred to is the international unit (1μmol of substrate transformed/min). Values for enzyme activity are relative, as the substrate concentration was not saturating.

NADase was assayed by a modification of the cyanide-addition method of Colowick *et al.* (1951). The assay mixture contained, in a volume of 0.6ml: potassium phosphate buffer, pH7.0, 60μmol; NAD^+, 0.5μmol; and enzyme, 0.06 unit. After incubation for 5min at 25°C, the reaction was stopped with 3ml of 1M-KCN. The residual NAD^+ concentration was calculated from the ϵ_{327} for the NAD–cyanide addition compound (5.9×10^3 litre·mol^{-1}·cm^{-1}; P-L Biochemicals, 1961).

A second assay method was developed based on the measurement of acid produced during the reaction (eqn. 1). The assay was done at 25°C in an automatic-titration assembly (Radiometer, Copenhagen, Denmark). The pH was kept constant by the addition of 2mM-NaOH. Full-scale deflection was equivalent to the addition of 1μmol of NaOH, and so to the hydrolysis of 1μmol of NAD^+. Assay mixtures contained in an initial volume of 1.2ml: potassium phosphate buffer, pH7.0, 0.1μmol; NAD^+, 1.2μmol; and enzyme, 0.2 unit. An assay of NADase based on this principle was described by Zervos *et al.* (1970).

Inhibitor assays. The unit of inhibitor activity is defined as the amount causing a 50% inhibition of 1 unit of NADase under the conditions of the assay.

Inhibitor activity was assayed by measurement of the residual NADase activity (by the cyanide-addition method) after enzyme (0.06 unit, 0.1ml) had been pre-incubated for 30min with inhibitor (0.1ml). Inhibitor combined with excess of enzyme was assayed by measuring enzyme activity before and after heating the mixture at 100°C for 10min.

Protein determination. Protein was determined either by the biuret reaction (Gornall *et al.*, 1949) with bovine serum albumin fraction V as the standard, or by the method of Warburg & Christian (1942). The two methods gave comparable results in the later stages of enzyme purification.

Partial purification of the NADase and its inhibitor

Partial purification of the NADase. For purification of the enzyme, use was made of the observation that

the enzyme–inhibitor complex is precipitated over a different ammonium sulphate saturation range from the enzyme. All operations were done below 5°C.

Stage I: Fractionation with ammonium sulphate. Crude cell-free extract (430ml) was adjusted to 40% saturation with 104g of finely powdered solid ammonium sulphate, and was left to equilibrate for 1h. The precipitate was collected by centrifugation for 15min at 23000*g* and redissolved in 0.1M-potassium phosphate buffer, pH7.0, to a final volume of 180ml. The solution was dialysed for 18h against 10vol. of the same buffer, with two changes.

Stage II: Heat treatment. The dialysed solution from stage I was heated, in two batches, on a boiling-water bath for 10min. After cooling in a crushed ice–salt mixture, the denatured protein was removed by centrifugation at 38000*g* for 1h. The precipitate was washed with 0.1M-potassium phosphate buffer, pH7.0. After a further centrifugation, the washings were added to the supernatant, which was then dialysed overnight against 10vol. of 50mM-potassium phosphate buffer, pH7.0.

Stage III: Fractionation with ammonium sulphate. The protein solution from stage II was fractionated with solid ammonium sulphate. Finely powdered ammonium sulphate was added to the protein solution over a period of 15min. After a further 30min of equilibration the precipitates were collected by centrifugation at 38000*g* for 10min, and were redissolved in 0.1M-potassium phosphate buffer, pH7.1. They were then dialysed against 50vol. of the same buffer, with two changes. Fractions were collected over the ranges 0–25% (14.4g of ammonium sulphate/100ml of solution), 25–35% (6.0g/100ml), 35–55% (12.8g/100ml), 55–57% (13.8g/100ml) and 75–85% (10.9g/100ml) saturation. Over 95% of the activity was recovered in the 35–55% and 55–75% fractions.

Stage IV: Chromatography on DEAE-Sephadex. DEAE-Sephadex was allowed to swell for 24h in a large excess of the initial buffer (0.1M-potassium phosphate, pH7.1). The column (30cm×2.5cm) was packed under gravity and was equilibrated overnight in the cold with the initial buffer circulating through it.

The dialysed protein solution from stage III (24.5ml) was divided into two portions and each portion was chromatographed separately. The column was washed with the initial buffer until no more protein was eluted, and the enzyme was then eluted from the column by use of a gradient of KCl (0–0.5M) in the buffer. The flow rate was about 20ml/h; 6ml fractions were collected. The active fractions were concentrated by precipitation with ammonium sulphate, redissolved in 0.1M-potassium phosphate buffer, pH7.0 (final volume 2.0ml), and dialysed against 20vol. of the same buffer.

The purification procedure is outlined in Table 1. The initial activity was determined by heating a sample of the crude cell extract. If it is assumed that there was no destruction of the NADase during heat treatment, then the observed 52-fold purification over stages III and IV can be extrapolated to a net purification of about 950-fold. Electrophoresis on polyacrylamide gel in a Shandon disc gel electrophoresis apparatus was performed by the methods described by Davis (1964). In a 50mM-tris–glycine, pH9.5, running buffer (Davis, 1964) the preparation was shown to contain three major components (one of them NADase) and five minor ones. When a slice of gel containing the active band was transferred directly

Table 1. *Partial purification of the NADase*

The enzyme was assayed by the cyanide method. Protein was assayed both by the biuret and (values in parentheses) the Warburg & Christian (1942) methods.

Stage	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purity	Yield (%)
Crude extract	430	17200 (22300)	1370	—	—	—
I Ammonium sulphate precipitate (0–40% satn.)	183	4750	—	—	—	—
II Heat treatment	188	950 (1180)	1440	1.5 (1.2)	1.0 (1.0)	100
III Ammonium sulphate precipitate (35–75% satn.)	24.5	563 (441)	1210	2.15 (2.75)	1.4 (2.3)	84
IV DEAE-Sephadex	4.1	(5.6)	346	(62)	(52)	24

to a fresh gel column and re-run either in the same buffer or in diethylbarbiturate buffer, pH8.0, (Williams & Reisfeld, 1964) it was homogeneous.

The position of protein bands was determined by staining the columns with Naphthalene Black in 7% (w/v) acetic acid followed by electrophoretic destaining with 7% (w/v) acetic acid. The position of enzyme activity on the columns was determined by the following method. An unstained column was frozen on a Pel-Cool cold plate [C. W. Brown (Engineers) Ltd., Hertford, Herts., U.K.] and divided in two longitudinally. One half was stained for protein, and the other was sliced into 1mm sections with a razor blade. Each section was broken up with a glass rod in 2ml of 0.1M-potassium phosphate buffer, pH7.0, the gel was allowed to settle and the supernatant was tested for enzyme activity. Sections containing enzyme activity could be correlated with a single protein band on the stained half of the gel.

Partial purification of the inhibitor. Stage I: Fractionation with ammonium sulphate. The fractionation was done as described above (enzyme purification, stage I). Free inhibitor was precipitated between 35% (20.8g of ammonium sulphate/100ml of solution) and 55% (12.8g/100ml) saturation. The precipitate was redissolved in 0.1M-potassium phosphate buffer, pH7.0, and dialysed overnight against 20vol. of the same buffer.

Stage II: Chromatography on DEAE-Sephadex. DEAE-Sephadex (Pharmacia, Uppsala, Sweden) was allowed to swell for 24h in a large excess of 0.1M-potassium phosphate buffer, pH7.0, with four changes. The column (29cm×2.5cm diam.) was packed under gravity, and equilibrated overnight in the cold with the initial buffer circulating through it.

The dialysed extract was applied to the column and the inhibitor was eluted by applying a gradient of KCl concentration (0–0.5M) in 0.1M-potassium phosphate buffer, pH7.0. The flow rate was about 24ml/h; 4.5ml fractions were collected. The inhibitor was eluted at a KCl concentration of 0.30M. Enzyme–inhibitor complex and free enzyme were eluted at 0.38M and 0.45–0.50M respectively.

Fractions that contained inhibitor were concentrated by surrounding a small dialysis bag containing the solution with dry Aquacide I (Calbiochem, Basingstoke, Hants., U.K.). The dialysis bag was then partially immersed in 0.1M-potassium phosphate buffer, pH7.0, while cold air was blown over the exposed surface of the bag to induce evaporation. In this way, the protein solution was evaporated without any increase in the ionic strength of the solvent buffer.

Stage III: Gel filtration on Sephadex G-200. Sephadex G-200 (Pharmacia) was equilibrated with 0.1M-potassium phosphate buffer, pH7.0, on a boiling-water bath. The column (96cm×3.5cm diam.) was packed under gravity; to prevent the gel bed from packing down and decreasing the flow rate the head was not allowed to exceed 15cm of buffer. The column was allowed to equilibrate in the cold with the 0.1M-potassium phosphate buffer, pH7.0, circulating through it.

The material from stage II (10.5ml) was applied to the column. The flow rate was about 12ml/h; 5ml fractions were collected. The combined active fractions were concentrated to 4.6ml with Aquacide I, as described above (stage II). The purified inhibitor was then dialysed overnight against 20vol. of 0.1M-potassium phosphate buffer, pH7.0.

The purification procedure is summarized in Table 2. The overall purification was about 18-fold with a yield of 2–3%. The inhibitor on electrophoresis on polyacrylamide gel as described above contained two major bands (one of them the inhibitor) and five minor ones.

Table 2. *Partial purification of the inhibitor*

The unit of inhibitor assay is defined as the amount of inhibitor causing 50% inhibition of 1 unit of enzyme after 30min preincubation. Enzyme activity was assayed by the cyanide method. Protein was assayed by the biuret method.

Stage	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purity	Yield (%)
Crude extract	170	6370	37800	5.9	1.0	100
I Ammonium sulphate precipitate (35–55% satn.)	47.5	1870	22300	12.1	2.0	59*
II DEAE-Sephadex	10.5	157	4460	28.4	4.8	12
III Sephadex G-200	4.6	9.0	975	108	18.3	3

* The loss is largely due to retention of 0–35% fraction for enzyme purification.

Chemicals

NAD^+ (grade III), $NADP^+$, NADH, NADPH, NHD, NHDP, APAD, thio-NAD, NMN, ADP-ribose and ribonuclease A were obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Biuret reagent, chemicals for polyacrylamide-gel electrophoresis, AnalaR ammonium sulphate (low in heavy metals) and ovalbumin were bought from BDH Chemicals Ltd., Poole, Dorset, U.K. Lysozyme, cytochrome *c*, D-glyceraldehyde 3-phosphate dehydrogenase and aldolase were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. Protamine sulphate (salmon roe) came from Koch-Light Laboratories, Colnbrook, Bucks., U.K.; myoglobin was from Mann Biochemicals, New York, N.Y., U.S.A.; bovine serum albumin was from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.; and t.l.c. adsorbents were from Camlab (Glass) Ltd., Cambridge, U.K.

Results

Preliminary observations with cell-free extracts

When known amounts of NAD^+ were added to a washed-cell suspension of *P. putida* KB1, only minimal amounts of NAD were recovered. NADase activity was demonstrated in washed-cell suspensions, but not in cell-free extracts unless they had first been heated at 100°C. Maximum activity in extracts was obtained after heating for 3min; activity then declined slowly, the activity after 30min being about 70% of the maximum. The protein concentration fell very sharply during the first 3min and then remained constant (Fig. 1); fresh extract inhibited the activity in a boiled extract.

The factor catalysing the reaction was shown to be an enzyme by the following criteria.

(*a*) It was precipitated when ammonium sulphate was added to the solution, and was excluded from Sephadex G-25.

(*b*) When the factor was tested in the cyanide assay system (Colowick *et al.*, 1951) it behaved like an NAD^+ glycohydrolase. Since this assay is dependent on retention of a quaternary nitrogen in the nicotinamide ring, this implies cleavage of the nicotinamide–ribose bond.

(*c*) After deproteinization with 20% (w/v) $ZnSO_4$, $6H_2O$–satd. $Ba(OH)_2$ (1:1, v/v), (mixture added to the protein solution in the ratio 1:1), samples of reaction mixture were chromatographed either on Whatman no. 4 paper in ethanol–0.1M-acetic acid (Zatman *et al.*, 1953), or on cellulose powder (MN300; Macherey Nagel, Düren, Germany) in ethanol–0.1M-ammonium acetate (7:3, v/v), or on silica gel (Kieselgel G; E. Merck A.G., Darmstadt, Germany) in ethanol–0.1M-ammonium acetate (7:3, v/v) or in 5M-ammonium chloride. When the chromatograms were viewed under u.v. light, the reaction products were found, in all four systems, to behave identically with nicotinamide and ADP-ribose.

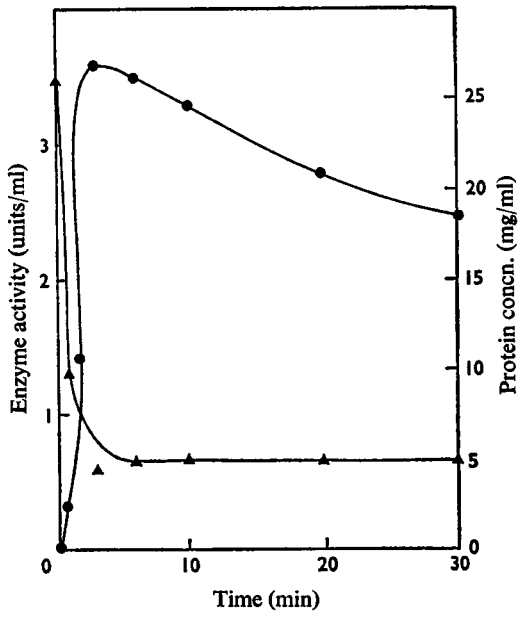

Fig. 1. *Effect of temperature on NADase activity and protein concentration in crude extract*

Samples containing, in a final volume of 2.0ml, crude extract, 51.5mg of protein and 0.1M-potassium phosphate buffer, pH7.0, 200μmol, were heated in a water bath at 100°C for the time indicated and cooled in a crushed ice–salt mixture. Denatured protein was removed by centrifugation. NADase activity (●) was assayed by the cyanide method; protein (▲) was determined by the biuret method.

(*d*) Activity was abolished when the factor was preincubated for 10min with trypsin.

(*e*) The factor resembled the microbial NADases in its resistance to nicotinamide inhibition (only 10% inhibition by 10mM-nicotinamide) and in failing to catalyse the nicotinamide exchange reaction. An attempt was made to demonstrate exchange of added [*carbonyl*-^{14}C]nicotinamide (obtained from The Radiochemical Centre, Amersham, Bucks., U.K.) with NAD^+, in the presence of enzyme. Purified enzyme was incubated at 25°C for 10min in the presence of 20mM-NAD^+ and [*carbonyl*-^{14}C]nicotinamide at two concentrations, 100mM (10μCi/mol) or 25mM (40μCi/mol). The deproteinized reaction mixture was then chromatographed on cellulose t.l.c. plates in ethanol–0.1M-ammonium acetate. No trace of [^{14}C]-NAD^+ was detected when the plates were scanned with a Packard chromatogram scanner.

Table 3. *Concentrations of NADase and inhibitor in culture supernatant and in cells*

Culture supernatant, cell debris and soluble fractions were prepared and assayed for NADase and inhibitor activity as described in the text. Inhibitor concentration in the supernatant was determined indirectly from the difference in enzyme activity before and after heat treatment. The concentration of inhibitor in the soluble fraction refers to both free inhibitor and that combined with enzyme. Values for cell debris and soluble fractions are directly comparable with those for the supernatant. Abbreviation: n.d. = not determined.

Fraction	Enzyme (units/litre of culture) Before heat-treatment	After heat-treatment	Inhibitor (units/litre of culture)
Culture supernatant	65	74	18
Cell debris	0	80	n.d.
Soluble fraction	0	70	334

Location of the enzyme. A sample (200ml) was taken from a 10-litre culture when the E_{610} had reached the equivalent of 500mg dry wt./litre of culture. The supernatant was retained; the cells were washed and broken as described above. The cell-free extract was centrifuged at 38000*g* for 2h. The soluble fraction was retained, and the cell-debris fraction was washed twice with and finally resuspended in 10ml of 0.1M-potassium phosphate buffer, pH7.0. The supernatant and soluble fraction of each sample were assayed for NADase and inhibitor activity before and after heat treatment. The cell-debris fraction was assayed for enzyme activity only.

The results are shown in Table 3. NADase activity could be detected in the culture supernatant before it was heated, although heating caused a stimulation in activity. No enzyme activity was detected in either soluble or cell-debris fractions before they were heated. After heat treatment, enzyme activity was found in both fractions. The amount of free inhibitor present in the soluble fraction varied over a wide range, being approx. 3–15-fold in excess of the amount of enzyme activity. Further experiments have shown that inhibitor is also present in excess over enzyme–inhibitor complex in the cell-debris fraction (B. J. Taylor, personal communication).

The enzyme–inhibitor complex appears to be associated with the cell wall. Cells (45g dry wt.) were harvested and fractionated as described above. The cell-debris fraction, suspended in 200ml of 0.1M-potassium phosphate buffer, pH7.0, was incubated overnight at room temperature with lysozyme (300mg). After the cell debris had been centrifuged down at 38000*g* for 2h, the supernatant was tested for NADase activity before and after heating. Lysozyme treatment released enzyme–inhibitor complex from the cell-debris fraction (Table 4). Thus, the inconsistent results for the content of inhibitor in the soluble fraction, mentioned above, could be due to variable release of inhibitor from the cell debris during ultrasonic treatment.

Table 4. *Solubilization of enzyme–inhibitor complex in cell debris with lysozyme*

The cell-debris fraction was prepared and treated with lysozyme as described in the text. NADase activity in the supernatant was assayed by the cyanide method after heat treatment.

Fraction	NADase activity (units) Before heat treatment	After heat treatment
Soluble fraction	0	646
Cell-debris supernatant (no lysozyme treatment)	0	0
Cell-debris supernatant (after lysozyme treatment)	0	2340

When crude cell-free extracts were chromatographed on a column of Sephadex G-200, prepared as described above (inhibitor purification, stage III), before heat treatment, enzyme activity (measured after heat treatment of the individual fractions) was eluted in the void volume. Inhibitor present in excess over enzyme–inhibitor complex ran as a peak corresponding to a molecular weight of 16500. These results imply a molecular weight of greater than 800000 for the enzyme–inhibitor complex in crude cell-extracts; this probably reflects the particulate origin of some, if not all, of the enzyme–inhibitor complex. A minor peak of activity was also obtained, of mol.wt. 36000. The fraction eluted with the void volume was collected and heated in a water bath at 100°C for 5min. Application of this sample to the same column again gave two peaks of activity. This experiment would tend to indicate that the association of the enzyme–inhibitor complex with particulate material is only partially disrupted by heat. A similar sample of enzyme–inhibitor complex treated with

lysozyme also gave two peaks of activity on gel filtration on Sephadex G-200.

Occurrence of NADase or inhibitor activity in other organisms. NADase could not be detected in cell-free extracts heat-treated at 85°C for 1 min or at 100°C for 10 min, of the following organisms: *A. punctata*, *E. coli*, *P. aeruginosa* 8602A, *P. aeruginosa* T1, *P. aureofaciens*, *P. fluorescens*, *P. multivorans*, *P. testosteroni* N.C.I.B. 8895. NADase activity was detected in unheated extracts of *P. fluorescens* at about 0.02 unit/mg of protein (compared with 0.1–0.2 unit/mg for heated extracts of *P. putida* KB1). Untreated cell-free extracts of *P. aureofaciens* and *P. multivorans* inhibited the NADase activity of heated extracts of *P. putida*. The specific activity was about 0.1 unit/mg, compared with 4–5 units/mg in untreated *P. putida* extracts.

General properties of the NADase

Stability. The enzyme was stable for several weeks when stored at −20°C in 0.1 M-potassium phosphate buffer, pH 7.0. When it was stored overnight at 4°C in 0.1 M-tris–HCl buffer, pH 7.0 there was a 50% loss of activity.

Effect of enzyme concentration on activity. In the acidimetric assay, enzyme activity was proportional to enzyme concentration over the range tested.

Effect of pH on activity and stability of NADase. The variation of NADase activity with pH is shown in Fig. 2. The activity was determined in 1 mM-potassium phosphate buffer by both the cyanide and the acidimetric assays, and in 50 mM-potassium phosphate buffer and 50 mM-sodium citrate buffer by the cyanide method. The enzyme appears to have a wide pH optimum, activity being essentially unchanged between pH 5.0 and 10.0 in citrate buffer and between pH 6.0 and 10.0 in phosphate buffer. Below pH 4.0, it was very difficult to measure the activity, and it sometimes fell to zero: values shown are the maximum obtained. Non-enzymic hydrolysis of NAD^+ prevented the accurate determination of enzyme activity above pH 10.

The activity of the purified enzyme was determined at pH 7.0 by the cyanide method, after preincubation at various pH values for 5 min. The results are shown in Fig. 3. In the presence of 50 mM-citrate the enzyme appears to be stable for 5 min between pH 1.0 and 10.0: between pH 4.0 and 5.0, more than 90% of the original activity can be recovered. In the presence of 1 mM-potassium phosphate there is a range of minimum stability between pH 4.0 and 5.5, where only about 20% of the original activity remains. Below this pH the enzyme is more stable; at pH 2.0, about 60% of the original activity can be recovered. This minimum in the pH-stability curve was less marked in preparations at earlier stages in the purification, an effect that might have been partly due to the protective effect of thiol groups, since the addition of 2 mM-2-mercaptoethanol to the preincubation mixture increased the activity recovered after 5 min of incubation at pH 4.5 by about 20%.

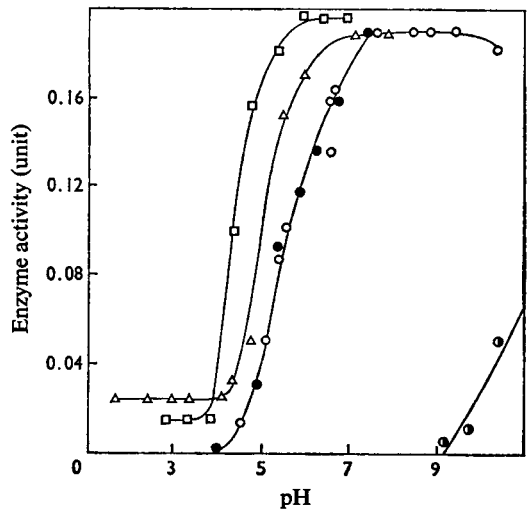

Fig. 2. *Effect of pH on activity of purified enzyme*

For the acidimetric assay (●) the reaction vessel contained: potassium phosphate buffer, 1.2 μmol; NAD^+, 1.2 μmol; enzyme (stage V), 0.2 unit. The reaction was started by the addition of the enzyme in 0.2 ml of 1 mM-potassium phosphate buffer, pH 7.0. For the cyanide assay (○, △, □) the reaction vessel contained in a final volume of 1.2 ml: potassium phosphate buffer, 1.2 μmol (○) or phosphate buffer, 60 μmol (△) or citrate buffer, 60 μmol (□); NAD^+, 1.2 μmol; enzyme (stage IV), 0.2 unit. The reaction was started as described above and a 0.5 ml sample was added to 3 ml of 1 M-KCN after 5 min of incubation. Non-enzymic hydrolysis of NAD (◑) was measured acidimetrically.

Specificity of the enzyme. β-NAD^+ and $NADP^+$ were hydrolysed at equal rates. Thio-NAD^+ was hydrolysed at 35% and $APAD^+$, NHD^+ and $NHDP^+$ at rates below 10% of this rate. NADH, NADPH, α-NAD^+, NMN and thiamin were not hydrolysed.

K_m values and their standard errors (Table 5) were determined for all the active substrates by the method of Wilkinson (1961) by using a BASIC computer program devised by Williams (1969).

Molecular-weight determination. The molecular weights of purified enzyme, purified inhibitor and purified enzyme–inhibitor complex (made by combining purified enzyme and inhibitor) were measured by Sephadex-gel filtration (Andrews, 1964, 1965). A column (100 cm × 1.5 cm diam.) of Sephadex G-100,

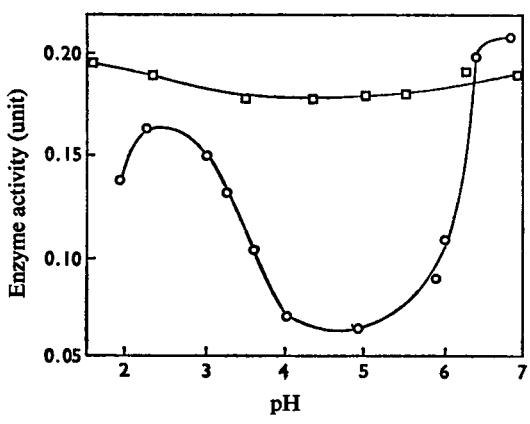

Fig. 3. *Effect of pH on stability of the purified enzyme in 1 mM-potassium phosphate buffer or 50 mM-sodium citrate buffer*

The reaction vessel contained in a final volume of 0.65ml: citric acid, 32.5μmol (□) or KH_2PO_4, 0.65μmol (○); HCl or NaOH to the required pH; enzyme (stage IV), 0.2 unit. After 5min the pH was readjusted to pH7.0 and activity was assayed by the cyanide method as described in Fig. 2.

Table 5. *Substrate specificity of NADase*

NADase activity was assayed acidimetrically in reaction vessels containing in a final volume of 1.0ml: potassium phosphate buffer, pH7.0, 1.0μmol; enzyme (stage IV), 0.27 unit; substrate, 0.2–1.0μmol. Abbreviation: n.d. = not determined.

Compound	Relative $V_{max.}$ ($NAD^+ = 100$)	K_m (mM) ±S.E.M.
NAD^+	100	0.85±0.04
$NADP^+$	100	0.66±0.09
Thio-NAD	35	0.25±0.03
NHD^+	4	1.44±0.30
$NHDP^+$	6	0.51±0.22
APAD	5	n.d.

equilibrated with the eluting buffer (0.1 M-potassium phosphate buffer, pH7.0) was used. The buffer was applied under gravity and the flow rate was kept at 0.15–0.20ml/min. The column was calibrated with proteins of known molecular weight, each one being applied separately to the column at a concentration of 3mg/ml; fractions were assayed for the standard proteins as described by Andrews (1965). The void volume was measured by using Blue Dextran.

Purified enzyme (stage IV) (68μg) and purified inhibitor (stage III) (1.0mg) were each dissolved in 1.0ml of the eluting buffer. Purified enzyme–inhibitor complex was prepared by dissolving 68μg of purified enzyme (stage IV) and 186μg of purified inhibitor (stage III) in 1.0ml of the elution buffer. The three protein preparations were then separately applied to the column. The purified complex was afterwards heated on a boiling-water bath for 5min, concentrated with Aquacide I, dialysed against the eluting buffer and run once more on the column. The following values for V_e/V_0 were obtained: enzyme, 1.79 (corresponding to a mol.wt. of 23500); inhibitor, 2.04 (mol.wt. 15000); enzyme–inhibitor complex, 1.60 (mol.wt. 35500); boiled enzyme–inhibitor complex, 1.72 (mol.wt. 27000).

The enzyme–inhibitor complex appears to involve 1mol of each, since the measured molecular weight is close to the predicted molecular weight of such a complex (38500). Heat treatment of the enzyme–inhibitor complex apparently gives active enzyme with a molecular weight higher than that of the purified enzyme. The elution curve for this material is bimodal, with peaks at mol.wt. 27000 and 35500. This is caused by some denatured inhibitor remaining attached to the enzyme, as demonstrated by treatment with DEAE-cellulose, which yields a relatively homogeneous product with mol.wt. of 24000.

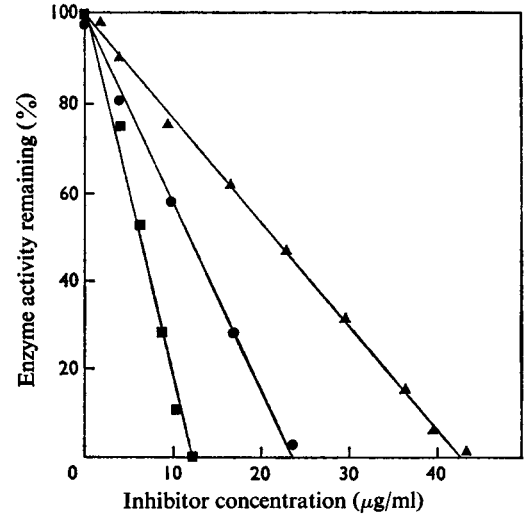

Fig. 4. *Stoicheiometry of enzyme–inhibitor interaction*

Purified enzyme (stage IV), 0.117 unit/ml (■), 0.235 unit/ml (●) or 0.47 unit/ml (▲) in 0.1 M-potassium phosphate buffer, pH7.0, was incubated for 30min with purified inhibitor (stage III) at the concentrations indicated. Residual NADase activity was determined by the cyanide method.

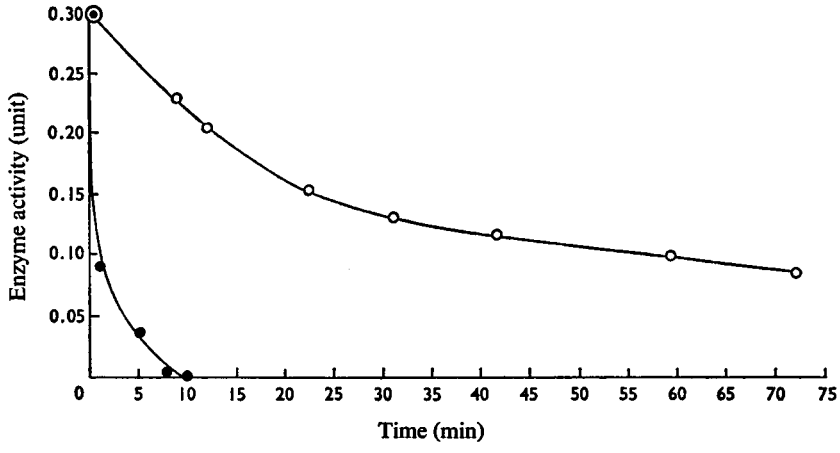

Fig. 5. *Effect of substrate on the binding of enzyme and inhibitor*

NADase activity was assayed acidimetrically. The reaction vessel contained in a final volume of 2.0ml: potassium phosphate buffer, pH 7.0, 0.2μmol; NAD^+, 10μmol; enzyme (stage IV), 0.30 unit; inhibitor (stage III), 0.78 unit. Inhibitor was added either before (●) or after (○) NAD^+. In the latter case, the points are derived from tangents drawn at the times indicated to a continuous trace of acid production.

Kinetics of interaction of enzyme and inhibitor. The results of the titration of enzyme at three different concentrations with inhibitor are shown in Fig. 4. In each case there is a linear relationship between residual activity and inhibitor concentration, and also between the enzyme concentration and the amount of inhibitor needed just to inhibit it completely. The linear relationship between enzyme activity and inhibitor concentration at a fixed enzyme concentration indicates that under the conditions of this experiment the enzyme–inhibitor complex is undissociable (Kern & Natale, 1958).

The rate of combination of enzyme and inhibitor is markedly decreased in the presence of substrate. Fig. 5 summarizes the results of measuring the rate of decrease of enzyme activity in the presence of inhibitor, with or without the prior addition of substrate (5mM). Conditions in the experiment with prior addition of substrate were such that the enzyme remained saturated with substrate throughout the experiment. The decrease in enzyme activity is therefore a direct measure of the rate of combination of enzyme with inhibitor. After 80min, the reaction mixture was dialysed against 0.1mM-potassium phosphate buffer, pH 7.0, for 10h. Recovery of enzyme activity in the dialysed solution was 13% before and 90% after a standard heat treatment (5min at 100°C). Thus the decrease in the rate of combination of inhibitor with enzyme, in the presence of NAD^+, is not due to irreversible denaturation of the inhibitor by substrate.

Thermostability of the enzyme

In crude preparations, NADase activity decreased by about 30% after 30min at 100°C (Fig. 1). The purified enzyme was rather more sensitive, losing about 60% of its activity after 30min and 80% after 1h.

In contrast, the purified inhibitor is inactivated by 50% after heating to 65°C for 10min (Fig. 6). The activation of the enzyme by heating seems to be mainly the result of the deactivation of the inhibitor.

The purified enzyme appears to be stable to hydrogen-bond reagents at 100°C. After incubation in 6M-guanidine–HCl for 20min at 25°C, followed by tenfold dilution with 0.1M-potassium phosphate buffer, pH 7.0, 52% of the original activity (measured by the acidimetric assay) was recovered. When the enzyme was incubated in 6M-guanidine–HCl for 15min at 25°C and then for 5min at 100°C and assayed in the same way, the proportion of the activity recovered (42%) was not considered significantly different.

The variation of specific activity with temperature was studied first with a relatively crude preparation (stage I). The optimum temperature over a 5-min assay period was 40°C; above 60°C the enzyme was inactive. Full activity was recovered on cooling. This effect could be due to conformational changes in either the enzyme or the substrate.

The effect of temperature on $V_{max.}$ and K_m was studied with purified enzyme. Both parameters respond in a similar way to increasing temperature.

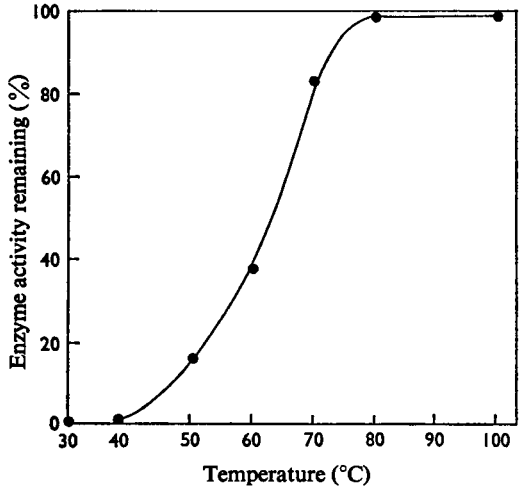

Fig. 6. *Effect of temperature on the inhibitor*

Purified inhibitor (stage III) (2.5 unit) in 2.0ml of 0.1M-potassium phosphate buffer, pH7.0, was incubated at the temperatures indicated for 10min and was then cooled in an ice–salt bath. Inhibitor activity was determined from the enzyme activity remaining after a 0.1ml sample had been incubated for 30min with 0.1ml (0.055 unit) of enzyme.

Both increase with temperature to maximum values at about 47°C; the Arrhenius plot of $V_{max.}$ against $1/T$ is linear over the range 15–38°C. The curve of K_m against temperature appears to be biphasic; below 39°C, K_m increases little with temperature, but above 39°C it changes very rapidly, increasing from 1.4mM at 39°C to 6.5mM at 47°C. Above 47°C, both parameters decrease sharply with temperature, reaching zero at 55–60°C.

To reveal any changes in the conformation of the enzyme with temperature, its circular-dichroism spectrum was determined between 230 and 300nm as a function of temperature. The instrument used was a Dichographe model 1B (Roussel-Jouan, Paris, France). A positive Cotton effect was observed, but it was not pronounced at any temperature. It did, however, decrease as the temperature was raised from 2° to 62°C and was restored immediately on cooling to 10°C.

Discussion

The NADase described here, though apparently very striking in its thermostability, is quite similar in its properties to the NADases of *M. butyricum* and *B. subtilis* (Table 6). The three enzymes have associated with them heat-labile inhibitors, and the three are also similar in thermal stability. The molecular weights of the enzymes and inhibitors seem to be of the same order and the binding of enzymes and inhibitors seems to be on an equimolar basis. The NADase of *M. tuberculosis* is similar in some respects to the above enzymes, but is less heat stable, losing 30% of its activity in 1min at 100°C (Gopinathan *et al.*, 1964). The enzymes from *Streptococcus* sp. and *N. crassa* differ in lacking the inhibitor and in being heat-labile.

The *P. putida* NADase, in common with most microbial and animal NADases, hydrolyses β-NAD^+ and $NADP^+$ at equal rates, but hydrolyses neither NADH, NADPH, NMN nor α-NAD^+. NHD^+ is hydrolysed at less than 5% of the rate for NAD^+, whereas the enzyme from pig spleen hydrolyses it at 50% of the rate with NAD^+.

The marked specificity of these enzymes for NAD^+ and $NADP^+$ might reflect the structure of the substrate. Kaplan & Sarma (1970) and Sarma & Kaplan (1970) propose, mainly on a basis of n.m.r. measurements, that NAD^+ can exist in at least three forms. One is a straight chain, whereas the other two are folded structures, in which some interaction occurs between the purine and nicotinamide rings. The folded structures are helical, owing to a half turn in the pyrophosphate backbone. Kaplan & Sarma (1970) and Sarma & Kaplan (1970) suggest that these helical structures are in equilibrium, and that interconversion proceeds through the straight-chain configuration. However, the interpretation of the n.m.r. results leading to these conclusions has been questioned (Jacobus, 1971).

The inactivity of NADases with analogues lacking either the carboxamide group of the nicotinamide moiety or the amino group of adenine indicates that both moieties are essential for the binding of substrate and enzyme. This is consistent with the idea that the interaction involves both the adenine and the nicotinamide moieties of one helical form. The lack of activity with the reduced forms could be due to the difference in configuration of the oxidized and reduced forms.

The thermostability of this enzyme is comparable with that of some enzymes from thermophilic bacteria. Optical-rotatory-dispersion studies in the presence of hydrogen-bond reagents with the α-amylase of *Bacillus stearothermophilus* (Manning *et al.*, 1961; Campbell & Cleveland, 1961) show that in the native state this protein exists as a random coil with little α-helix. Similar studies with the 3-phosphoglyceraldehyde dehydrogenase of *B. stearothermophilus* (Amelunxen, 1967) indicate a structure stable to hydrogen-bond reagents, but the optical-rotatory-dispersion spectra are not characteristic of an unfolded conformation. By contrast, a protease from *Bacillus thermoproteolyticus* (Ohta *et al.*, 1966; Ohta, 1967) appears to exist as a compact globular protein with a large number of tyrosine and tryptophan

Table 6. *Comparative properties of microbial NADases*

Values in parentheses were obtained from analytical ultracentrifuge measurements. All others were obtained by gel filtration. References: 1, Kern & Natale (1958); 2, Ogasawara *et al.* (1970); 3, Gopinathan *et al.* (1964); 4, Everse & Kaplan (1968); 5, Fehrenbach (1969). Abbreviation: n.d. = not determined.

	Molecular weight			K_m (mM)		
Organism	Enzyme	Inhibitor	Complex	NAD^+	$NADP^+$	Reference
P. putida	23500	15000	35500	0.85	0.66	Present work
M. butyricum	39000	26000	60000	1.88	n.d.	1, 2
M. tuberculosis	n.d.	n.d.	n.d.	0.33	0.31	3
B. subtilis	24000 (26200)	24000 (26200)	44000 (52700)	n.d.	n.d.	4
Streptococcus sp.	56000	—	—	0.48	n.d.	5
N. crassa	31500 (36500)	—	—	0.50	n.d.	4

residues buried within it. In this case, inactivation by 8M-urea was accelerated by heat. In preliminary experiments with *P. putida* NADase, hydrogen-bond reagents seemed not to affect the thermostability.

The presence of large quantities of carbohydrate in the *B. subtilis* NADase (Everse & Kaplan, 1968) might explain the thermostability of the enzyme. Since, however, the heat-labile inhibitor from *B. subtilis* and the heat-labile NADase of *N. crassa* are both also glycoproteins, this explanation seems unlikely. When, after electrophoresis on polyacrylamide gel, the *P. putida* NADase was stained for glycoprotein by the procedure of Zacharius *et al.* (1969) the result was negative.

The apparently reversible thermal denaturation observed with the *P. putida* NADase has been found in two similar enzymes, the NADase of *M. butyricum* (Kern & Natale, 1958) and the nucleotide pyrophosphatase (EC 3.6.1.9) of *Proteus vulgaris* (Swartz *et al.*, 1958). The affinity of the *P. putida* NADase for substrate decreases with increasing temperature to a minimum at 47°C, after which it increases once more. The decrease is consistent with a change in conformation of the active site to a form less favourable for binding of substrate to enzyme. The apparent optimum in the curve of $V_{max.}$ against temperature at 47°C is probably the result of two opposing processes: first, the increase in rate of the enzymic reaction with temperature; and secondly, a decrease in the amount of active enzyme as the change in conformation proceeds.

A positive Cotton effect was seen between 240 and 300nm in the circular-dichroism spectrum of the *P. putida* enzyme; this effect decreased as the temperature was raised and reappeared on cooling. This is probably caused by a reversible change in conformation. Changes in the circular-dichroism spectrum in the near-u.v. are interpreted as being due to aromatic side chains and disulphide bonds (Glazer & Simmons, 1966), and Cotton effects in the far-u.v. are due to changes in the α-helix (Beychok, 1966). The temperature-dependent spectral changes seen with the *P. putida* enzyme seem likely to reflect relative movements of amino acid residues rather than changes in helicity.

The assumption that the apparent temperature optimum is due purely to conformational changes in the protein must be regarded with caution. The conformation of NAD is thought to be temperature-dependent (Kaplan & Sarma, 1970; Sarma & Kaplan, 1970). Assuming that the enzyme preferentially binds one structure rather than the others, the phenomenon of reversible denaturation might be partly due to changes in the conformation of the substrate.

Gholson (1966) proposed a metabolic role for NADase, as part of a 'pyridine nucleotide cycle' in which NAD^+ is hydrolysed to nicotinamide and the nicotinamide moiety is then converted back into NAD^+ via nicotinate and nicotinic acid dinucleotide. If this were its function, then its K_m should be of the same order as the NAD^+ concentration in the cell. Calculation from values reported by London & Knight (1966) indicates that the intracellular NAD^+ concentration in *P. putida* KB1 is 0.7mM; Lundquist & Olivera (1971) report a value of 1.3mM for *E. coli*. These values are close to that reported above for the K_m of the enzyme (0.8mM).

Modulation of the binding of inhibitor to enzyme may therefore have some significance in regulating the turnover of NAD^+ in the 'pyridine nucleotide cycle', and the observation that NAD^+ inhibits the combination of enzyme and inhibitor is consistent with this. High concentrations of NAD^+ should lead to inhibition of enzyme–inhibitor complex-formation, resulting in the presence of active enzyme *in*

vivo. High concentrations of NAD^+ do lead to a partial activation of the enzyme–inhibitor complex (B. J. Taylor, personal communication), a result in agreement with observations on the *B. subtilis* NADase inhibitor system (Kaplan, 1968).

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