

Degradation of NAD(H) by Endogenous Enzymes of Yeasts and *Clostridia*

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Dedicated to Professor Hans Grisebach on the occasion of his 60th birthday

NAD(H) Degradation, *Clostridia*, Yeasts, Bioconversion, Enzymatic Reductions

The time courses of degradation of exogenous NAD and NADH (2.5 mM) catalyzed by endogenous enzymes present in *Saccharomyces cerevisiae*, *Candida utilis*, *Clostridium spec. La 1*, *Clostridium kluyveri*, and *Clostridium sporogenes* have been determined. The half lives of the pyridine nucleotides depend extremely on the organism and, for the same organism, on the growth conditions. *C. spec. La 1* as well as *C. kluyveri* possess only negligible enzyme activities for NAD degradation. However, *C. sporogenes* shows activities leading to half lives of less than 2 h for NAD and 5 h for NADH. At 25 °C half lives in the order of 5–17 h have been observed for *Candida utilis* under different conditions. The half lives of NAD are roughly 5 times higher in the presence of *Saccharomyces cerevisiae*.

Introduction

For many decades microorganisms have been used for the stereospecific reduction of suitable substituted unsaturated compounds in order to obtain chiral products or synthons [1, 2]. The cells, usually yeasts, are used either as growing cultures or in a resting state. The latter technique has some advantages [1], *i.e.* resting cells become often permeable or are rendered permeable for effective in- and efflux of substrates and products. Most bioreductions are catalyzed by pyridine nucleotide-dependent reductases. Not much is known yet about the limiting factors of the often not very effective bioreductions [3]. Pyridine nucleotide-degrading enzymes are present in yeasts [4] as well as in clostridia [5]. Their action could lead to a retardation or inactivation of the systems. Takei *et al.* [6] report that about 75% of endogenous NAD is decomposed within 1 h by the autolysate of *Saccharomyces oviformis*, and Kasarov and Moat [7] describe that about 20% of exogenous NAD is degraded after 1 h by the cell-free extract of *Clostridium butylicum*. Up to now no further studies

have been presented about the time course and the specific activity of the NAD(H) decomposition by crude extracts and/or freeze-thawed cells of yeasts and clostridia. Degradation studies carried out with *Saccharomyces cerevisiae*, *Candida utilis*, *Clostridium kluyveri*, *C. spec. La 1*, and *C. sporogenes* are presented here. The comparison of yeasts and the aforementioned clostridia was of interest since we use clostridia for the biohydrogenation of many unsaturated compounds and yeasts for electromicrobial reductions [3].

Material and Methods

Chemicals

The pyridine nucleotides, adenine nucleotides, luciferin of *Photinus pyralis*, tetracycline hydrochloride, and the enzymes were supplied by Boehringer Mannheim (D-6800 Mannheim), Amp and chlorhexidine diacetate were purchased from Serva (D-6900 Heidelberg), and methylviologen from Sigma (D-8000 München). Other chemicals were products of Merck (D-6100 Darmstadt).

Organisms

C. spec. La 1 DSM 1460, and *C. kluyveri* DSM 555 were grown according to Bader and Simon [8]. *C. sporogenes* was grown as described by Giesel and Simon [9]. Before use, the cells were washed twice with a 10-fold volume of nitrogen saturated 0.2 M Tris-acetate, pH 7, or 0.2 M Amp-acetate, pH 9, depending on the individual experiments.

Abbreviations: Amp, 2-amino-2methyl-1,3-propanediol; MV^{•+}, methylviologen cation radical; NMNH, reduced nicotinamide mononucleotide; deamido-NAD, nicotinic acid adenine dinucleotide. Tris, tris(hydroxymethyl)aminomethane; rpm, revolutions per minute; specific activities are expressed in mU/mg, *i.e.* the disappearance of nmol pyridine nucleotide per minute and mg protein.

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For the growth of yeasts the following media were used (weight per liter deionized water):

Medium I: Glucose (10 g), glycerol (6 g), peptone (Oxoid) (15 g), yeast extract (Difco) (3 g), NaCl (5 g), pH adjusted to 6.0.

Medium II: Glycerol (20 g), peptone (Merck) (15 g), yeast extract (Difco) (3 g), NaCl (5 g), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (40 mg), riboflavin (4 mg), pH adjusted to 6.0.

S. cerevisiae was grown in medium I on a rotatory shaker at 250 rpm at 35 °C and harvested at the stationary phase (24 h), or in a 16 l Kiel fermenter at 30 °C with an aeration rate of 2 l air/min, stirring 200 rpm and harvested from midexponential phase (12 h). *Candida utilis* DSM 70167 was cultivated in medium II at 30 °C in a 16 l Kiel fermenter under the conditions mentioned above, for 11.5 h (late exponential phase). A further fermentation of *C. utilis* was performed under the same conditions but using medium II with a glycerol content of 10 g/l for 63 h (late exponential phase). The harvested yeast cells were washed twice with a 10-fold volume of 1% NaCl and stored at -20 °C.

Extracts of yeasts were obtained by sonifying cell suspensions (0.15–0.2 g dry weight per ml of the appropriate buffer) at 5–10 °C for 30 min with a Branson Sonifier B 12, equipped with the micro-tip applying 20–30 Watts (crude extract), or, followed by centrifugation at 4 °C and $27\,000 \times g$ for 10 min (supernatant).

Conditions for degradation

The reaction mixture for the determination of NAD cleavage activity contained in a total volume of 2.5 ml: 0.1 M Tris-buffer, pH 7, or phosphate buffers of various concentrations, pH 7, 0.05 mM tetracycline hydrochloride, 2.5 mM NAD, different additions depending on the individual experiments, and 5 mg/ml protein either in form of supernatant or crude extract or freeze-thawed cells. The reaction mixture for determining NADH cleavage activity consisted of 0.1 M Tris-acetate, pH 7, 0.05 mM chlorhexidine diacetate, 1.5–2 mM MV^{++} , 2.5 mM NADH and 5 mg/ml cell protein, as described above, in a total volume of 2.5 ml. In some cases experiments were performed in 0.1 M Amp-acetate, pH 9, 0.05 mM tetracycline hydrochloride without MV^{++} .

The incubations were performed in heat sterilized brown 5 ml flasks equipped with a septum under

shaking in a thermostated water-bath at 25 °C or 37 °C or in some cases under magnetical stirring in an anaerobic chamber at an average temperature of 27 ± 2 °C. Buffers, stock solutions, the suspensions of yeasts and yeast extracts were freed from oxygen by repeated degassing and flushing with nitrogen for 5 min and were then added to the flasks in an anaerobic chamber (Coy, Ann Arbor) under an atmosphere of 95% N_2 and 5% H_2 . A 5 mM stock solution of MV^{++} in 0.1 M Tris-acetate, pH 7, was obtained by reduction in an electrochemical cell as described [10] and was also handled in the anaerobic chamber.

The reactions were started by the addition of the pyridine nucleotide. At zero time and at appropriate time intervals aliquots of 0.12–0.16 ml were taken, using a nitrogen flushed syringe, if not performed in the anaerobic chamber. The samples were immediately frozen in liquid nitrogen and stored at -20 °C. After thawing and centrifugation at 17000 rpm (Haemofuge, Heraeus) for 1 min suitable amounts of the clear supernatant were immediately added to the test mixture in order to determine the enzymatically active pyridine nucleotides. The time needed for these manipulations was kept approximately constant for each measurement.

The experiments for determination of NAD decomposition by *C. utilis* in the presence of adenine nucleotides were performed as described above but under aerobic conditions.

Analytical procedures

Protein was determined by the Biuret method as described by Herbert *et al.* [11] using bovine serum albumin as the standard. The ATP degradation was estimated by bioluminescence according to the recommendations of Boehringer Mannheim. The NAD determination was carried out by the common method using ethanol and alcohol dehydrogenase (EC 1.1.1.1.) [12a]. For NADH the following procedure was used: The dinucleotide was first oxidized by sodium pyruvate in the presence of lactate dehydrogenase (EC 1.1.1.27) [12b]. After subtraction of a background absorption of the cell extracts the remaining extinction value at 340 nm gave the concentration of NMNH. In an independent test NAD was determined by the alcohol dehydrogenase method. The concentration of compounds possessing the nicotinamide-ribose linkage was determined from

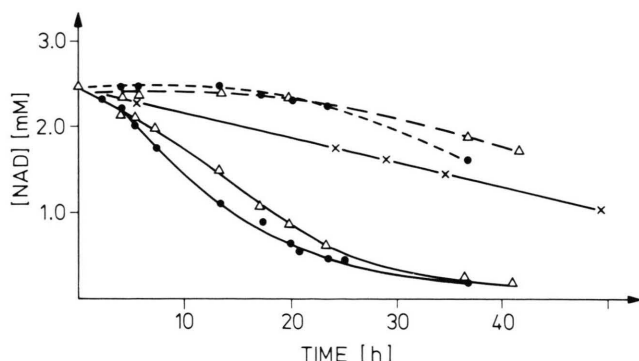


Fig. 1. Time course of the decomposition of NAD by supernatant (●) and 5 times freeze-thawed cells of *S. cerevisiae* at 37 °C (△) and 25 °C (×). The incubations were carried out with 5 mg protein/ml in 0.1 M Tris-chloride, pH 7, 5 mM MgCl₂, 0.05 mM tetracycline. Progress curves for NAD decomposition are given by continuous lines and the hydrolysis of the glycosidic linkage of nicotinamide-ribose by the interrupted lines. The nonenzymatic degradation of NAD at 37 °C is shown in Fig. 2.

the increased absorbance at 328 nm caused by the addition of cyanide to the nicotine-ribose according to l.c. [13].

Results

Freeze-thawed cells and cell extracts of *S. cerevisiae*, *C. utilis*, and *C. sporogenes* catalyze the decomposition of NAD and NADH. Fig. 1 demonstrates the time course of NAD degradation by *S. cerevisiae*. The initial rates of the supernatant and of the freeze-thawed cells are in the same range; maximum rates of the NAD degradation amount to 0.4 mU/mg for the extract and to 0.3 mU/mg for the cells, respectively. At 37 °C the half life of NAD is about 12 h for the extract and 16 h for the cells. In both experiments the splitting of the nicotinamide-ribose linkage as determined by the cyanide method starts when more than 50% of the initial NAD has been degraded, suggesting a reaction that does not affect NAD but the product nicotinamide-ribose. Incubations of freeze-thawed cells at 25 °C result in an apparently linear course. The degradation rate (0.09 mU/mg) is only 30% of the maximum rate observed at 37 °C leading to a half life of 43 h. The nonenzymatic degradation of NAD in 0.1 M Tris-acetate, pH 7, at 37 °C is slow when compared to the enzymatic degradation (Fig. 2). At 25 °C NAD shows no decrease up to 60 h (not shown). During the studies controls were repeated and always showed the same results. At 25 °C the NAD decomposition by freeze-thawed cells of *C. utilis* occurs with initial rates about 3–7 times faster than that observed for *S. cerevisiae* under the same conditions (Fig. 2). An incubation with freeze-thawed

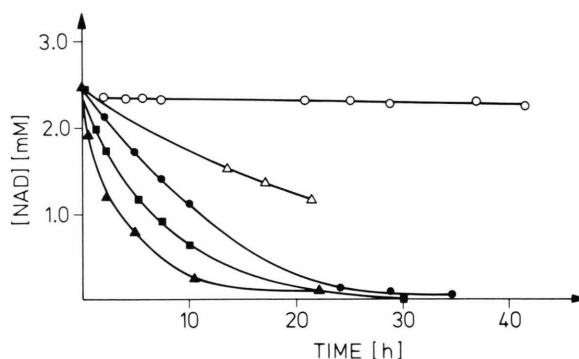


Fig. 2. Time course of the decomposition of NAD by *C. utilis*. The incubations were performed at 25 °C and contained 5 mg protein/ml in 0.1 M Tris-acetate, pH 7, 0.05 mM tetracycline. Filled symbols: *C. utilis* grown on 20 g glycerol/l; ●, 5 times freeze-thawed cells; ▲, as ● with additional 25 mM Na₂P₂O₇; ■, crude extract; △, 5 times freeze-thawed cells of *C. utilis* grown on 10 g glycerol/l; ○, control for nonenzymatic degradation of NAD in 0.1 M Tris-chloride, pH 7, at 37 °C.

cells grown on 10 g glycerol per l results in an initial NAD splitting rate (0.3 mU/mg) only 50% of that observed for cells grown on 20 g glycerol per l. Crude extract of *C. utilis* obtained from the glycerol richer medium shows an initial reaction rate (0.8 mU/mg) about 30% higher than that of freeze-thawed cells. The splitting of the nicotinamide-ribose linkage by crude extract starts with a velocity of 0.25 mU/mg after a lag phase of about 1 h (not shown). The influence of the growth conditions, the pre-treatment of the cells, the incubation buffers, admixtures of salts and adenine nucleotides on the NAD degradation was examined for *C. utilis* as listed in the Table. The presence of 5 mM nicotinamide

Table. NAD decomposition catalyzed with 5 times freeze-thawed cells (section A) or crude extracts (section B) of *C. utilis* grown on 20 g glycerol/l expressed by specific activities (initial rates) and apparent half lives. The experiments were performed at 25 °C with 5 mg protein/ml in various buffers, 0.05 mM tetracycline and different admixtures.

0.1 M ^a Buffer	pH	mM admixture	Specific activity [mU/mg]	Half life [h]
Section A:				
Tris-chloride	7	—	0.4	10
Tris-chloride	7	5 MgCl ₂	0.6	9
Tris-acetate	7	—	0.6	9
Tris-acetate	7	0.1 EDTA	0.5	9.5
Tris-acetate	7	10 NaF	0.5	9.8
Tris-acetate	7	25 Na ₄ P ₂ O ₇	4	2
Section B:				
Tris-acetate	6.5	—	0.8 0.25 ^c	6
Tris-acetate	6.5	5 nicotinamide	0.8 0.25 ^c	6.4
Tris-acetate	7.0 ^b	—	1.0	5.2
0.05 M K-phosphate	6.6 ^b	—	0.6	7
0.05 M K-phosphate	7.0 ^b	2.5 ATP	0.25	13.4
0.05 M Na/K-phosphate	6.9 ^b	5 ATP	0.13	13.7
0.05 M Na/K-phosphate	7.0 ^b	2.5 AMP	0.43	7.2
0.05 M Na/K-phosphate	7.0 ^b	2.5 AMP, 2.5 ATP	0.18	12.1
Na/K-phosphate	6.9 ^b	—	1.0	5.2
Na/K-phosphate	6.9 ^b	2.5 ATP	0.27	11.5
Na/K-phosphate	7 ^b	5 ATP	0.18	16.8

^a Unless otherwise indicated.
^b Assays under aerobic conditions in the presence of 5 mM Mg-acetate.
^c Splitting of the nicotinamide-ribose linkage.

does neither influence the NAD degradation nor the nicotinamide-ribose splitting. Admixtures of EDTA or NaF to freeze-thawed cells diminish the initial rates of NAD decomposition to about 80%. However, the addition of 25 mM Na₄P₂O₇ increases the degradation rate to about 560%. The initial rates of degradation by crude extract are the same in 0.1 M

Tris-acetate and 0.1 M phosphate buffer, pH 6.9–7.0. In 0.1 M Tris-acetate and 0.05 M phosphate buffer, pH 6.5–6.6, the specific activity of 1.0 mU/mg decreases about 20% and 40%, respectively.

ATP is a substrate for both the NAD degrading enzyme pyrophosphatase as well as the NAD synthesizing enzyme pyrophosphorylase [4, 6] and might

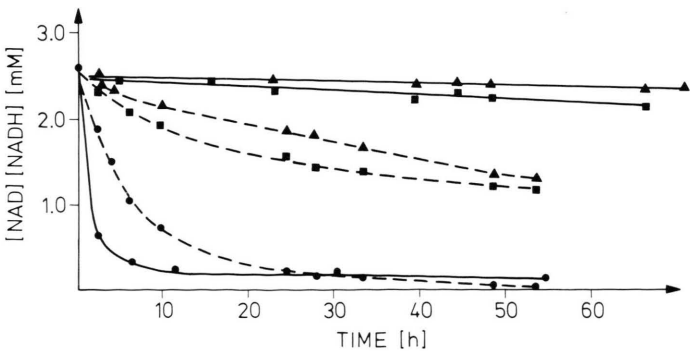


Fig. 3. Time course of the decomposition of NAD (continuous lines) and NADH (interrupted lines) by 5 times freeze-thawed cells of clostridia. The incubations were carried out as mentioned in Fig. 2. However, the NADH degradation was observed at 27 °C in the presence of 0.05 mM chlorhexidine instead of tetracycline and 1.5 mM MV⁺⁺. ▲, *C. spec. La 1*; ■, *C. kluyveri*; ●, *C. sporogenes*. For the control experiment without protein see Fig. 4.

influence the NAD decomposition. Compared to an assay in 0.1 M Tris-acetate without any admixture, an ATP concentration of 2.5 mM clearly decreases the initial rate of the NAD degradation by crude extract from a specific activity of 1.0 mU/mg to 0.25, and 5 mM ATP causes a decrease to about 15%, regardless of a 0.05 or 0.1 M phosphate buffer. ATP (2.5 mM) in 0.05 M phosphate buffer, pH 6.7, was degraded by a crude extract at 25 °C with an initial rate of 0.25 mU/mg and a half life of about 15 h (not shown).

Fig. 3 represents the NAD and NADH decomposition by freeze-thawed cells of different clostridia at 25 °C. Compared to the yeasts previously mentioned *C. spec. La 1* and *C. kluyveri* possess very low specific activities of NAD degradation. Values of 0.005 and 0.015 mU/mg, respectively, and half lives much higher than 100 h have been observed. In contrast, the NAD degradation by *C. sporogenes* occurs with the specific activity of 2.5 mU/mg; the splitting of the nicotinamide-ribose linkage starts with the same activity and yields an almost congruent progress curve (not shown). When the NAD incubation with *C. sporogenes* is performed in the presence of 5 mM nicotinamide, a slight decrease (12%) of both activities is observed.

The studies on the NADH degradation had to differ from the studies on NAD degradation in two aspects. Firstly NADH is oxidized nonenzymatically in the presence of 0.05 mM tetracycline at pH 7.0, therefore at this pH the antiseptic chlorhexidine was used. This compound does not influence the NADH. Secondly yeast extract reoxidizes NADH very fast even under strict anaerobic conditions. In order to regenerate NADH MV^{++} was added. Yeasts contain enzymes which reduce NAD at the expense of MV^{++} [3, 14].

Fig. 4 represents some experiments with crude extracts from yeasts in the presence of MV^{++} . The rate of NADH degradation is influenced by the yeast species, the growth conditions and the pH-value of the assay. *C. utilis* grown in the glycerol richer medium yields a higher specific activity (2.8 mU/mg) than *C. utilis* grown in the glycerol poorer medium (0.65 mU/mg); *S. cerevisiae* harvested in the stationary phase (not shown) or in the exponential phase show similar activities (0.63 and 0.5 mU/mg, respectively). *C. utilis* degrades NADH faster than both breedings of *S. cerevisiae*. Incubations of freeze-thawed cells at pH 9 in the presence of 5 mM $MgCl_2$ enhance the specific activity of degrading enzymes in *S. cerevisiae* (1.2 mU/mg) about twofold and that of *C. utilis* (7 mU/mg) to about 1.5-fold of the value obtained at pH 7 (not shown). The nonenzymatic decay of NADH at pH 7 and 27 °C is shown in Fig. 4; in the observed time span NADH is not influenced by the addition of MV^{++} .

In contrast to the rather fast NADH decomposition by yeasts the NADH degradation by freeze-thawed cells of *C. spec. La 1* and *C. kluyveri* at pH 7 and 27 °C yield progress curves (Fig. 3) proceeding almost parallel to the curve for nonenzymatic degradation (Fig. 4). However, *C. sporogenes* degrades NADH with the specific activity of 1.1 mU/mg leading to a half life of only 5 h. Experiments at pH 9 (not shown) demonstrated that freeze-thawed cells of *C. spec. La 1* have no observable NADH degradation activity. At this pH NADH incubated with freeze-thawed cells of *C. kluyveri* or *C. sporogenes* showed half lives of about 70 h and 40 h, respectively.

Fig. 5 demonstrates the formation and the consecutive degradation of NMNH during incubations of NADH with crude extracts of *C. utilis* at pH 7 and

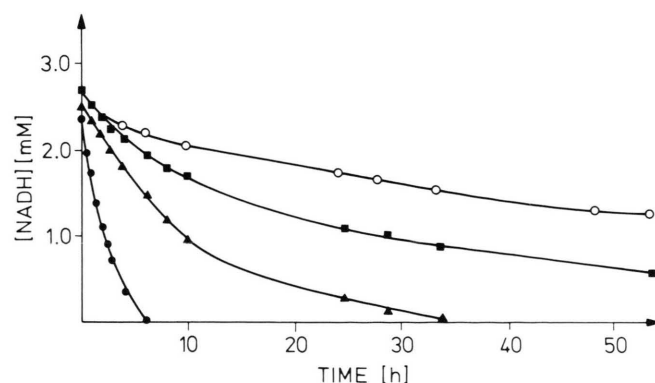


Fig. 4. Time course of the decomposition of NADH by crude extracts of yeasts under conditions described in Fig. 3 in the presence of 2 mM MV^{++} . ●, *C. utilis* grown on 20 g glycerol/l; ▲, *C. utilis* grown on 10 g glycerol/l; ■, *S. cerevisiae* harvested in the exponential phase; ○, control for nonenzymatic degradation of NADH.

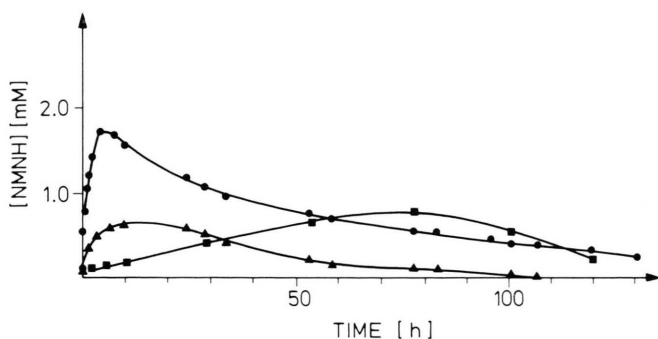


Fig. 5. Time course of the formation and degradation of NMNH by crude extracts of *C. utilis* and 5 times freeze-thawed cells of *C. kluyveri*. The incubations contained 2.5 mM NADH at zero time. The conditions for yeasts are given in Fig. 4. *C. kluyveri* was incubated in 0.1 M Amp-acetate, pH 9.1, 0.05 mM tetracycline at 25 °C. ●, *C. utilis* grown on 20 g glycerol/l; ▲, *C. utilis* grown on 10 g glycerol/l; ■, *C. kluyveri*.

freeze-thawed cells of *C. kluyveri* at pH 9.1. NMNH is produced by *C. utilis* grown in the glycerol richer medium with an initial rate of 2.7 mU/mg, attaining a maximum concentration of 1.7 mM when NADH has totally disappeared. NMNH is decomposed with an initial velocity of 0.08 mU/mg and a half life of 36 h. The crude extract of *C. utilis* harvested from the glycerol poorer medium forms NMNH with an initial activity of only 0.6 mU/mg and yields a maximum concentration of about 0.7 mM when about 75% of the initial NADH has been degraded. Freeze-thawed cells of *C. kluyveri* split NADH to NMNH with an initial rate of 0.04 mU/mg, the maximum is reached at 0.7 mM NMNH after a breakdown of 55% of the initial NADH.

Discussion

If resting cells, especially yeasts, are used for bioreductions, one often faces the problem that the reduction rates decline rather quickly. Therefore often growing cells are used nevertheless that this technique may lead to the following problems: (i) relatively small amounts of material have to be separated from huge volumes of fermentation broth and from large amounts of the biocatalysts. (ii) Growing cells often form unwanted by-products or (iii) the substrate inhibits the growth of the cells. Our results clearly indicate that resting cells capable of bioreductions are very different with respect to their pyridine nucleotide-degrading enzymes. Since the pyridine nucleotides leave resting cells under operational conditions [14] their concentration will be limiting rather soon. Therefore, the decline in reduction activity of yeasts in 5–30 h, a time span often used for bioreductions, is probably due to the degradation of the pyridine nucleotides and to a lesser extent to the instability of enzymes. On the other hand, resting cells of *C. spec. La 1* and *C. kluyveri* which we used

for many bioreductions show longer time stability [3]. This can now be explained by the very low enzymatic degradation of pyridine nucleotides by these organisms (Fig. 3).

The enzymes decomposing the NAD skeleton occur in the various pyridine nucleotide cycles which are salvage pathways for the regeneration of NAD from NMN, nicotinamide or nicotinic acid [15]. Yeasts [4] and some clostridia [5] decompose NAD by hydrolysis of the pyrophosphate bond catalyzed by nucleotide pyrophosphatase (EC 3.6.1.9). A further NAD-degrading enzyme found in brewery yeasts [16] is NAD pyrophosphorylase (EC 2.7.7.1). According to the data presented in Fig. 1 and 2 the NAD decompositions by *S. cerevisiae* and *C. utilis* are mainly progress curves of the enzymatic pyrophosphate bond hydrolysis. While these yeast strains degrade NAD with average specific activities of about 0.1 and 1.0 mU/mg, respectively, the proteolytic *C. sporogenes* degrades exogenous NAD with a rate of 2.5 mU/mg (Fig. 3). Because the splitting of the nicotinamide-ribose linkage by *C. sporogenes* proceeds with the same rate and is slightly inhibited by nicotinamide, NAD might be converted by a very active glycohydrolase (EC 3.2.2.5). So far only *C. butylicum* was checked for this enzyme and it could not be detected [7].

Similar time courses are observed regardless whether supernatants of crude extract or freeze-thawed cells are used. Cells of *C. utilis* which have been frozen and thawed several times have an increased permeability for NAD without releasing enzymes [14]. Freeze-thawed cells of the clostridia applied in these studies become completely desintegrated during incubation in 0.1 M buffer for several hours as was shown by microscopy [17].

Besides pyrophosphate admixtures of various salts and organic compounds to the NAD incubation with

C. utilis exert either a slightly inhibiting or a slightly stimulating effect (Table). The increase of NAD degradation in the presence of inorganic pyrophosphate (Fig. 2) can be explained by the presence of pyrophosphorylase for which inorganic pyrophosphate is the second substrate. One important reason for the net NAD decomposition by resting cells may be explained by the fact that ATP-requiring steps in the pyridine nucleotide cycle do not proceed due to the lack of ATP. As was described for *S. cerevisiae* [7], an admixture of ATP almost completely inhibits the degradation of exogenous deamido-NAD, probably because of reconversion of the degradation products. However, the degradation of deamido-NAD by *C. butylicum* always prevails over synthesis regardless of the ATP content [7]. ATP clearly decreases the NAD conversion by crude extract of *C. utilis* (Table). Because ATP is enzymatically degraded too, an ATP addition influences the initial rate more than the half life of NAD, leading to sigmoidal progress curves (not shown). The presence of ATP may cause an inhibition of the pyrophosphatase as well as the resynthesis of NAD by the pyrophosphorylase.

Even at 37 °C NAD is stable in buffers of pH 7 for 40 h (Fig. 2). However, NADH is chemically transformed in Tris-buffer of pH 7 with a half life of about 50 h (Fig. 4) (*cf.* [18]). Thus, the NADH decomposition by the organisms is superimposed by the non-enzymatic reactions. Since only the total NADH de-

gradation is of practical interest, the nonenzymatic decay has not been subtracted. While *C. kluyveri* and *C. spec. La 1* do not metabolize NADH at pH 7 (Fig. 3), in the presence of *S. cerevisiae* and *C. utilis* the NADH degradation proceeds faster than the corresponding NAD conversion leading to specific activities in the range of 0.5 to 2.8 mU/mg (Fig. 4). Surprisingly, *C. sporogenes* exerts a slower NADH than NAD decomposition with an about treefold longer half life of NADH than NAD (Fig. 3). Nucleotide pyrophosphatases show a broad substrate specificity and frequently split NADH faster than NAD [19]. As has been found for *C. utilis* (Fig. 5) and *S. cerevisiae* (not shown), the faster the NADH decomposition proceeds, the higher is the NMNH enrichment. While *C. spec. La 1* does not metabolize NADH at pH 7 or at pH 9, a slow formation of NMNH at pH 9 by *C. kluyveri* has been observed (Fig. 5). Although *C. sporogenes* clearly degrades NADH both at pH 7 and at pH 9, a formation of NMNH could not be detected photometrically.

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