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PURIFICATION AND CHARACTERIZATION OF A NICOTINAMIDE DEAMIDASE RELEASED INTO THE GROWTH MEDIUM OF NEUROBLASTOMA IN VITRO

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Summary

Nicotinamide deamidase (nicotinamide amidohydrolase, EC 3.5.1.19) has been demonstrated in the conditioned growth medium of the M1 clonal cell line of mouse C1300 neuroblastoma. The enzyme has been purified 1200—1500-fold by Sephadex G25, hydroxyapatite, DEAE-cellulose, Sephadex G200 and NAD-Sepharose column chromatographies. The purified protein was characterized by polyacrylamide gel electrophoresis under non-denaturing and denaturing conditions. The apparent molecular weight has been estimated to be 230 000, and the subunits had respective molecular weights of 65 000 and 50 000. Histidine was the only NH_2-terminal amino acid found. The enzyme is a glycoprotein; mannose and *N*-acetyl-glucosamine have been identified. The effects of various ions on its activity have been investigated. The enzyme has a K_m for nicotinamide in the order of 10^{-6} M, a pH optimum of 7.2 and a pH_i of 5.4. It is inhibited by heating and by sulfhydryl reagents. The existence of a nicotinamide deamidase with a high affinity for nicotinamide favors the operation of the Preiss-Handler pathway in M1 cells cultured in vitro. We found an induction of nicotinamide deamidase and a cellular increase of NAD with a higher nicotinamide supply and a repression of the released enzyme with supplying NAD in the nutrition medium of M1 cell cultures.

Introduction

Synthesis of NAD from nicotinamide occurs in the de novo pathway and the Dietrich pathway [1,2]. It has also been shown that nicotinic acid rather than nicotinamide can also be the immediate precursor for diphosphopyridine nucleotide biosynthesis in mammalian cells [3,4]. This latter mechanism (Preiss-

Handler pathway) involves deamidation of nicotinamide into nicotinic acid by a nicotinamide deamidase (nicotinamide amidohydrolase, EC 3.5.1.19). Petrack et al. [5,6] and Greengard et al. [7,8] have suggested that in mammals the deamidation of nicotinamide is the rate limiting step in the biosynthesis of NAD according to the Preiss-Handler pathway. The occurrence of nicotinamide deamidase in Ehrlich ascites [9] and in neuroblastoma [10] also suggests an applicability of the scheme of NAD synthesis to malignant mammalian tissues. The present paper describes the purification and properties of nicotinamide deamidase isolated from the culture medium of M1 mouse neuroblastoma cells and provides some data about the biological role of this enzyme in cultured cells.

Materials and Methods

Cell culture. Clone line M1 derived from C1300 mouse neuroblastoma has been used in these experiments, the conditions for culturing have been previously described [11].

Preparation of the conditioned medium of M1 neuroblastoma cell cultures. All experiments were carried out with medium from M1 cells that were harvested two days after a change of growth medium. The medium was centrifuged for 20 min at $1000 \times g$ and 4°C, and the supernatant thus obtained was used for isolation and purification of nicotinamide deamidase.

Determination of nicotinamide deamidase activity. Nicotinamide deamidase activity was measured by following the labelled nicotinic acid formed from carboxamide[^{14}C]nicotinamide. Aliquots of samples to be analysed (up to 100 µl) were incubated in 0.5 ml of 50 µM sodium phosphate buffer (pH 7.2) in the presence of carboxamide[^{14}C]nicotinamide (40 nCi per assay) and a final concentration of 10 µM nicotinamide for 15 min to several hours (depending upon the purification step of the enzymatic preparation) in a water bath at 37°C. After incubation, the samples were immediately submitted to column chromatography on Dowex 1 X8 (formic form). Beds of anion exchange resin of approx. 0.6 ml were used in Pasteur pipettes for each assay. After adsorption of the sample and washing with 6 ml of distilled water, the column was washed with 6 ml of 0.05 M formic acid to achieve complete removal of unreacted substrate. Nicotinic acid was eluted with 6 ml of 0.5 N formic acid. Under these conditions, no labelled nicotinamide was present in the eluate. The radioactivity of the eluate was counted in 10 ml of Instagel (Packard) using an SL 40 Intertechnique Spectrometer. Nicotinamide deamidase activity is expressed as nicotinic acid (pmol or cpm) produced under standard conditions per h and per mg of protein.

Protein determination. Protein contents of samples were determined by the method of Lowry et al. [12] or by the method of Su et al. [13], absorbance being measured at 215 and 225 nm. Bovine serum albumin (Boehringer, F.R.G.) was used as standard.

Isoelectric focusing. An analytical electrofocusing method on polyacrylamide ampholine gels was employed to determine the isoelectric point of the enzyme. LKB Ampholine polyacrylamide gel plates with a pH range of 3.5 to 9.5 were used. The anode solution was 1 M H_3PO_4 and the cathode solution

was 1 M NaOH. 2 h runs were made at 30 W and 1400 V. After each run, the slab was cut into slices and each slice was eluted with 1 ml of bidistilled water for measurements of pH and enzymatic activity under standard conditions.

Periodic acid-Schiff staining of glycoprotein. After electrophoresis the gel was fixed, treated with periodic acid, and stained with Schiff reagent (Segrest and Jackson, Ref. 14).

Gas-liquid chromatography of monosaccharides. The method of Zanetta et al. [15] was used to determined monosaccharides of the enzymic protein. This method involves the conversion of covalently bound monosaccharides into their *O*-methyl-glycosides and the subsequent analysis of the glycosides as their trifluoroacetate derivatives.

Polyacrylamide gel electrophoresis. Electrophoresis was carried out on 7.5% polyacrylamide disc gels (diameter 6 mm, height, 10 cm) according to Loening [16]. The gels were stained with Coomassie blue R (0.25%) in methanol/water/acetic acid (50 : 40 : 10, v/v/v) for 1 h at 60°C and destained in methanol/water/acetic acid solution. For nicotinamide deamidase activity determination the gels were cut into slices and placed into a dialysis bag containing 1 ml of incubation buffer and attached to a disc polyacrylamide gel (5%). Electrophoresis was run for 2 h to allow elution of enzyme into the incubation buffer; its activity was then measured by the standard assay.

NH_2-terminal amino acid identification. The technique of Gros and Labouesse [17] as modified by Zanetta et al. [18], was used for dansylation and hydrolysis of the enzymic protein. Two-dimensional chromatography on polyamide plates or silica gel plates was carried out using dansylamino acids as markers. The solvents of Zanetta et al. [18] were used on silicate plates, and those of Hartley [19] and Bertrand et al. [20] were used for polyamide plates. The fluorescent spot (examined under a ultraviolet lamp 366 nm) was cut out and subjected to mass spectrography for comparison with spectra of dansyl-amino acids.

NAD determinations. The method of Passonneau and Lowry [21], based on enzymatic cycling, was used for NAD determination in M1 cells. NAD is cycled with lactate dehydrogenase and glutamate dehydrogenase. Pyruvate and glutamate are produced in yields up to 6000-fold per h. Pyruvate was measured by reversing the reaction; i.e., by adding NADH and lactate dehydrogenase. The disappearance of NADH was measured spectrophotometrically at 340 nm. To remove contaminating NAD from lactate dehydrogenase and glutamate dehydrogenase preparations, they were treated with charcoal as described by Lowry and Passonneau [22].

Reagents. Carboxamide[^{14}C]nicotinamide was obtained from the Radiochemical Centre (Amersham U.K., code CFA 199). Sephadex G25, Sephadex G200, Sephacryl S200, Sepharose 4B, concanavalin A covalently bound to Sepharose 4B and the standard proteins for molecular weight determinations in polyacrylamide gel electrophoresis were purchased from Pharmacia Fine Chemicals. DEAE-cellulose was a Whatman (U.K.) product. Hydroxyapatite was prepared as previously described [23]. NAD-Sepharose 4B was prepared according to Barry and O'Carra [24] and Cuatrecasas [25]. Ampholine polyacrylamide gel plates were purchased from LKB. Lactate dehydrogenase from rabbit muscle and glutamate dehydrogenase from beef liver were purchased

from Boehringer (F.R.G.). Other chemicals and biochemicals were of highest purity and from Sigma (USA) or Merck (F.R.G.) when not otherwise specified. TLC Ready Plastic Sheets F 1700 Micropolyamide No. 356 000 (Schleicher and Schull, F.R.G.) and TLC Plastic Sheets Silicagel 60 No. 5748 were purchased from Merck (F.R.G.). Pure dansylamino acids, used as markers, were either from Sigma (U.S.A.) or were prepared according to Zanetta et al. [18]. Liquid and stationary phases for gas chromatography were purchased from Applied Science Laboratories (State College, USA).

Purification procedure of the enzyme. All experiments were carried out at 4°C. Portions (200 ml) of centrifuged used medium of M_1 neuroblastoma cells were treated simultaneously. Two to six preparations were pooled for the last purification step in order to operate on enough material. The purification procedure consisted of the following steps:

Step 1. The used growth medium of M_1 cells was adsorbed on a Sephadex G-25 column (40 × 5 cm) previously equilibrated with 5 mM sodium phosphate buffer (pH 7.2). With Sephadex G-25 column chromatography, the small molecular weight components of growth medium are retarded, especially the phenol red which would interfere with subsequent purification steps. The ultraviolet absorbing material possessing nicotinamide deamidase activity eluted in the exclusion volume was immediately placed onto a hydroxyapatite column (10 × 4.7 cm) equilibrated with 5 mM sodium phosphate buffer (pH 7.2). Most of the nicotinamide deamidase activity was found in the effluent of the hydroxyapatite column and was subsequently purified, whereas most of the protein was retained on the column. The nicotinamide deamidase which remained on the column was not further investigated.

Step 2. The effluent from hydroxyapatite column chromatography was concentrated on an Amicon Diaflo cell membrane (XM 50) to a final volume suitable for adsorption on a DEAE-cellulose column (40 × 1.5 cm) (Fig. 1). The elution was carried out using a gradient of 5 mM to 1500 mM sodium phosphate buffer (pH 7.2; 250 ml of each concentration). Fractions (2.5 ml) were collected, and protein absorbancies were measured at 230 and at 280 nm. Nicotinamide deamidase was assayed as described above. The most active fractions were combined and concentrated on an Amicon Diaflo cell membrane (XM 50) to a volume of 5—8 ml.

Step 3. The concentrated eluate of the DEAE-cellulose column containing the nicotinamide deamidase activity was applied to a Sephadex G200 column (30 × 2 cm) previously equilibrated with 5 mM sodium phosphate buffer (pH 7.2) (Fig. 2). The elution was performed with the same buffer. The fractions containing nicotinamide deamidase were pooled and concentrated as in the foregoing steps. For the more recent enzyme purifications we preferred to use a Sephacryl S-200 instead of a Sephadex G-200 column (same dimensions) since the separation was as good and the elution was more rapid with Sephacryl S-200.

Step 4. Affinity chromatography was performed with freshly prepared NAD-Sepharose 4B (5 × 1 cm). After adsorbing the concentrated enzymic fractions obtained in step 3 on a NAD-Sepharose 4B column, the enzyme was eluted with a linear gradient of sodium phosphate buffer (pH 7.2) of 5—150 mM (2 × 50 ml). The eluted fractions possessing the highest nicotinamide deamidase

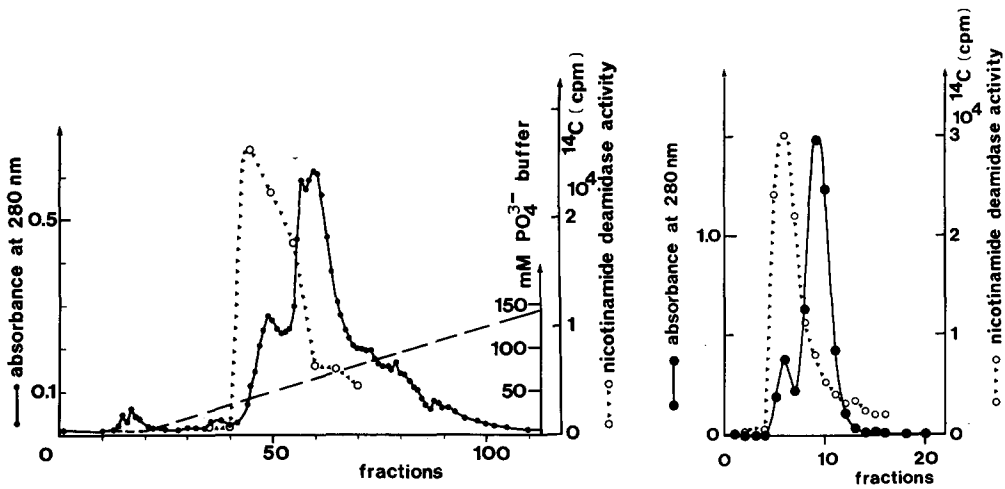

Fig. 1. Elution profile of partially purified enzyme from DEAE-cellulose column chromatography. ●——●, absorbance at 280 nm; ▲▲▲▲▲, nicotinamide deamidase activity measured on an aliquot of the eluted fractions (2 ml) measured by the standard assay (see Methods) by determination of [^{14}C]nicotinic acid formed from carboxamide[^{14}C]nicotinamide used as substrate. The broken line corresponds to the elution gradient of sodium phosphate buffer used.

Fig. 2. Chromatography of nicotinamide deamidase on a Sephadex G200 column. For details see Results. ●——● absorbance at 280 nm. ▲▲▲▲▲ enzymic activity determined on aliquots of the eluted fractions (2 ml) by the standard assay for nicotinamide deamidase (see Methods) based upon the [^{14}C]nicotinic acid formed from labelled nicotinamide.

activity were pooled and concentrated either as described above or in a dialysis bag plunged into sucrose crystals (BDH, U.K., analytical grade).

Results

A typical result of the purification procedure and yields for each step are given in Table I. A 1200—1500-fold purification was obtained over the starting material with a 0.05—0.08% overall yield in terms of protein.

Purity of the enzyme preparations. Electrophoresis of the purified enzyme preparation on polyacrylamide gel at pH 8.3 provided only one major band

TABLE I

PURIFICATION OF NICOTINAMIDE DEAMIDASE FROM THE CULTURE MEDIUM OF M1 NEUROBLASTOMA CELLS

Step	Total enzymic activity (pmol)	Total protein (mg)	Specific activity (pmol/h per mg)	Purification factor	Yield (%)
Growth medium	9040	800	11.3	1	100
Sephadex G-25 hydroxyapatite	8303	115	72.2	6	92
DEAE-cellulose	8085	16.5	490	43	89
Sephadex G-200	1063	0.26	4090	362	12
NAD-Sepharose 4B	902	0.06	15034	1330	10

under non-denaturing conditions. When enzymic activity was tested on the eluates of gel slices it corresponded to the stained major band. The molecular weight was estimated to be 230 000 (average of three separate experiments). Under denaturing conditions we found two protein bands in the region of 65 000 and 50 000. Taken together, these data suggest that the parent enzyme molecule may have a tetrameric structure consisting of two subunits of 65 000 and two subunits of 50 000 daltons.

NH_2-terminal amino acid identification. A bi-dimensional separation of dansylamino-acids from dansylated and then hydrolyzed enzyme was performed. Besides dansyl-OH and dansyl-lysine, a spot was identified as dansyl-histidine corresponding to an NH_2-terminal amino acid.

Evidence for protein-bound carbohydrate. A net positive Periodic acid-Schiff band found after electrophoresis of the enzyme preparation on polyacrylamide gel shows evidence to indicate a glycoprotein. On the other hand, the purified enzyme was totally retained on a concanavalin A-Sepharose 4B column. High molarity (up to 0.05 M) of phosphate buffer (pH 7.2) did not elute the enzyme. 50—200 mM of *O*-methyl-D-glycopyranoside or methyl-mannoside were necessary to liberate the enzyme from concanavalin A-bound to Sepharose 4B. This high retention of the purified enzyme on concanavalin A is attributed to the presence of mannose or glucose residues in the enzyme. Gasliquid chromatography has been performed after methanolysis and derivatization of the enzyme. The major monosaccharides found were mannose and *N*-acetyl-glucosamine, 1.7% (w/w) and 1.5% (w/w) respectively. The amounts of glucose and galactose found were not significant. All these data favor a glycoproteic nature of the nicotinamide deamidase studied.

Properties of nicotinamide deamidase. The properties of nicotinamide deamidase were examined with the enzyme preparations obtained after step 3 or 4 of purification (see above).

Time course of deamidase and effect of varying enzyme concentration. The amounts of nicotinic acid formed were linear with incubation time for at least 2 h for the step 4 enzyme preparation and for over 24 h for initial used growth medium of M1 cells. Nicotinic acid produced was directly proportional to the amounts of enzyme added.

Effect of temperature, and enzymic stability. The optimum incubation temperature for the purified nicotinamide deamidase was 37°C. The enzymic activity was markedly decreased at temperatures higher than 45°C. The Q_{10} value for nicotinamide deamidase over the range of 10 to 37°C was 2.5 ± 0.5. The half-life of the purified enzyme was 2.5 min and 1 min at 55 and 65°C respectively. However, the enzymic activity was stable for months in used medium kept sterile at 4°C. Purified enzyme retained almost all of its activity for at least a month when kept in 50% glycerol at −20°C. Freezing and defreezing of a nicotinamide deamidase preparation inactivated its activity.

Effect of pH on nicotinamide deamidase activity. The optimal range of pH for nicotinamide deamidase activity appeared to lie between pH 6.8 and pH 7.5. Between pH 6 and pH 7, the enzymic activity increased progressively, whereas it decreased sharply beyond pH 7.6; pH 7.2 was used throughout the work herein reported.

Isoelectric point. The purified enzyme preparation had a pH_i of 5.4. Under

the same experimental conditions serum albumin displayed a pH_i of 4.9 which corresponded well with that described in biochemical handbooks.

Effects of ions on enzymic activity. An increase of phosphate buffer concentration up to 50 mM resulted in stimulation of enzymic activity which was followed by a slight decrease of this enzymic activity at higher ionic strengths. Therefore, 50 mM sodium phosphate buffer was used routinely in nicotinamide deamidase assays.

The effects of monovalent and divalent ions on enzymic activity are shown in Fig. 3. Among the divalent ions, the most marked activation was produced by Mg^{2+}, Mn^{2+} and Ca^{2+}, whereas Cu^{2+}, Zn^{2+} and Fe^{2+} significantly inhibited nicotinamide deamidase to the same extent at 5 to 20 μM. Monovalent ions such as Na^+ and K^+ were without effect on enzymic activity, whereas Ag^+ and Hg^+ produced total inhibition.

Effects of various reagents on nicotinamide deamidase activity. The presence of the metal chelating agent EDTA at 20 μM (final concentration) inhibited enzymic activity by 46 ± 4%. The catalytic activity of the enzyme was strongly inhibited by β-mercaptoethanol: at 50 μM it decreased enzymic activity by approx. 50%. Cysteine and dithiothreitol also inhibited enzymic activity by approx. 67 ± 3% and 54 ± 2%, respectively, when present at the same molar

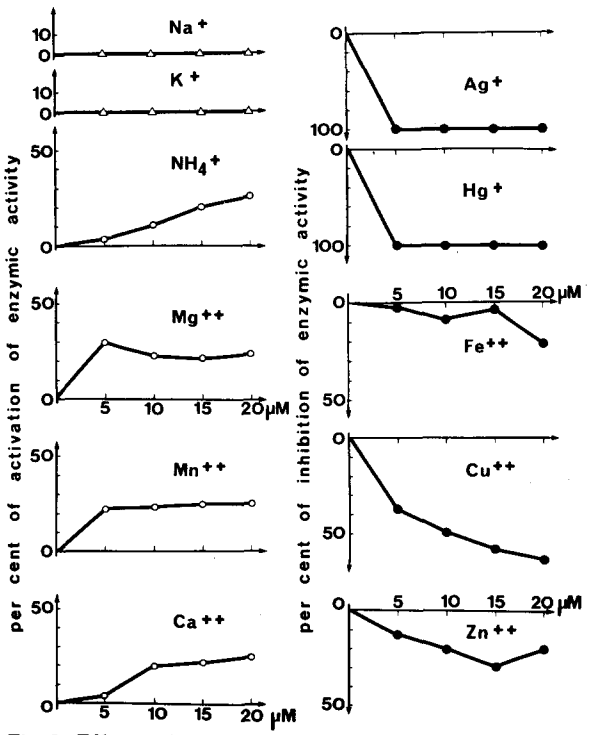

Fig. 3. Effects of ions on nicotinamide deamidase activity. 30 μg of enzymic preparation obtained by Sephadex G-200 chromatography were incubated in 50 mM sodium phosphate buffer under standard conditions (see Methods) with varying concentrations of metal ions as indicated. Results are expressed as percentages of activation or inhibition relative to the nicotinamide deamidase activity found in the absence of added metal ion.

concentration as sulfhydryl reagents. Triton X-100 (0.1%) and urea (1 M) inhibited nicotinamide deamidase activity by 24 ± 2% and 20 ± 1%, respectively. Serum albumin (0.02%, final concentration) activated the nicotinamide deamidase activity slightly but significantly by 9 ± 2%, under standard conditions.

K_m determination. The enzyme activity followed Michaelis-Menten kinetics within experimental error. The apparent Michaelis constant (K_m) for nicotinamide was estimated under standard conditions from a double reciprocal plot of the reaction velocity versus the concentration of nicotinamide. The K_m was $6 \cdot 10^{-6}$ M.

Effects of nicotinic acid and NAD on kinetic characteristics of nicotinamide deamidase activity. Nicotinic acid, at 10 and 25 μM concentration, did not alter the K_m or the V of nicotinamide deamidase. In similar experiments with 80 and 160 μM NAD a competitive inhibition occurred with respect to nicotinamide.

Role of nicotinamide deamidase in M1 cultures. In order to gain insight regarding the role of nicotinamide deamidase released to the growth medium, we investigated the influence of nicotinamide added to the growth medium on the nicotinamide deamidase activity of neuroblastoma M1 cells and in their growth medium, as well as on the cellular level of NAD. As shown in Fig. 4., M1 cells cultured with 4, 10, 20 and 40 μg/ml of the vitamin showed significant increases in their nicotinamide deamidase activity and NAD as compared with control cultures (4 μg of nicotinamide per ml of growth medium). These increases in nicotinamide deamidase in M1 cells were paralleled by an increase in nicotinamide deamidase activity released to the medium. Peaks of enzymic activity and NAD were found for the 10 μg/ml concentration, For higher concentrations the respective increases tended to approach values of cultures

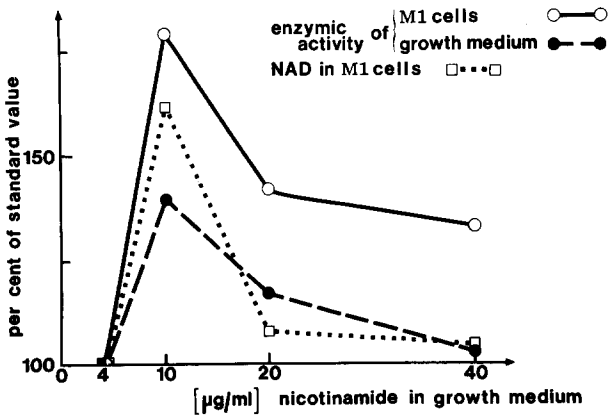

Fig. 4. Nicotinamide deamidase activity of M1 cells and their growth medium, and NAD variations with various amounts of nicotinamide in the growth medium. M1 neuroblastoma cells in logarithmic growth phase were cultivated under standard conditions (4 μg nicotinamide per ml of growth medium) and in the presence of 2.5, 5 and 10 times the usual concentrations of the vitamin. After a 2 day treatment, the cells were analyzed for NAD and nicotinamide deamidase activity, and the corresponding growth media was analyzed for enzymic activity. Results are expressed as percentages of the values found for growth medium and cells cultivated under standard conditions.

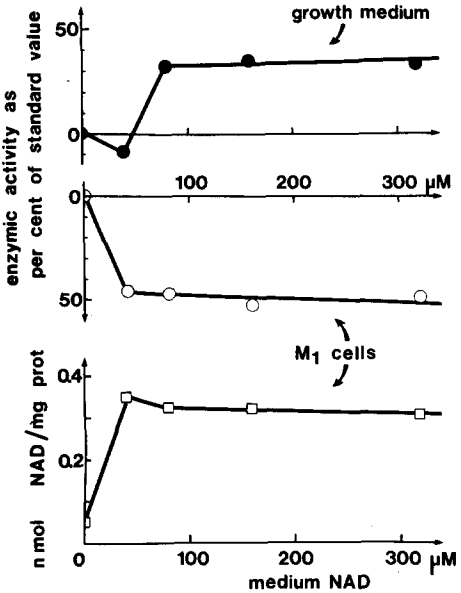

Fig. 5. Nicotinamide deamidase activity of M1 cells and their growth medium, and cellular NAD variations with respect to various amounts of NAD in the growth medium. M1 cells were cultivated under standard conditions (without NAD addition) and in the presence of NAD (up to 300 μM final concentration in the growth medium). After a 2 day treatment, the cells and the corresponding growth media were analyzed. The results were expressed as percentages of the values found for cells and media of cultures grown under standard conditions.

maintained under standard conditions. This latter point may be connected with the previous finding that NAD acts as a competitive inhibitor of nicotinamide deamidase. Thus nicotinamide promotes NAD synthesis in M1 cells and the level of NAD appears to regulate nicotinamide deamidase activity. In one other series of experiments NAD and nicotinamide deamidase in M1 cells, as well as the enzymic activity in the culture medium were studied when NAD was supplied in the growth medium. Results summarized in Fig. 5 show the significant increase of NAD in M1 cells that occurred concomitantly with a decrease of enzymic activity in the same cells. Moreover, the enzymic activity varied inversely with the NAD in the growth medium. Taken together, these findings illustrate the relationships between the nicotinamide deamidase of the growth medium and that of the M1 cells and between this latter parameter and the intracellular NAD level. Induction and repression of nicotinamide deamidase would seem to be linked with NAD biosynthesis.

Discussion

Nicotinamide deamidase is primarily a microsomal enzyme in animal cells [6,13,26] while the enzyme we purified is released in a soluble form to the growth medium of M1 neuroblastoma cells cultivated in vitro. The K_m of our enzyme is similar to that of the microsomal nicotinamide deamidase of Ehrlich's ascites tumor [9] but much lower than that of liver [6,26]. The optimal pH (7.2) differs from that of microsomal nicotinamide deamidase (pH

8.9) of normal [6,26] or malignant (pH 6) tissues [9]. Activation of the nicotinamide deamidase by Mn^{2+} was in agreement with results reported by Tanigawa et al. [28] on *Flavobacterium perigrinum* of rat stomach. The isolated enzyme appears to contain at least one essential SH group, since the inhibitory effect of certain heavy metal ions on enzymic activity was noted. The molecular weight of our nicotinamide deamidase (230 000 daltons) is similar to that determined by Gillam et al. [29] and Su et al. [30] for the rabbit liver microsomal enzyme. This molecular weight is much higher than that estimated for nicotinamide deamidases of microorganisms [27,28,31]. The glycoprotein structure of an animal nicotinamide deamidase (e.g., the presence of mannose) has also been reported by Su and Chaykin [30]. In addition, the relatively high stability of the purified enzyme is a characteristic shared with nicotinamide deamidase activities from other sources [9,10,27,30].

Nicotinic acid which is the end product of the reaction showed no interaction with nicotinamide deamidase activity, whereas NAD produced significant competitive inhibition of the enzyme released by neuroblastoma cells. This finding favors a control of pyridine nucleotide synthesis by feedback inhibition of deamidase. Fig. 6 summarizes the biosynthesis of NAD in the animal cells by its different pathways. Different capacities to use the one or the other pathway of pyridine nucleotide metabolism in various mammalian cells in culture have been reported [32]. In the pathway of Preiss-Handler [3,4] nicotinamide deamidase activity may contribute to a salvage pathway with reutilization of the nicotinamide from pyridine nucleotides by the action of NAD^+ glycohydrolase (EC 3.2.2.5) concomitantly with the use of nicotinamide obtained by nutrition [10,33]. In our experimental system nicotinamide supplied in the growth medium induced intracellular NAD biosynthesis and nicotinamide deamidase in parallel with an increased release of the same enzyme to the growth medium. A release of the enzyme from cells into the growth medium may be due to a lack of physiological regulation. In vivo the hypophysis is known to control the biosynthesis of the free fatty acids which are endogenous inhibitors of nicotinamide deamidase [34], and to contribute to

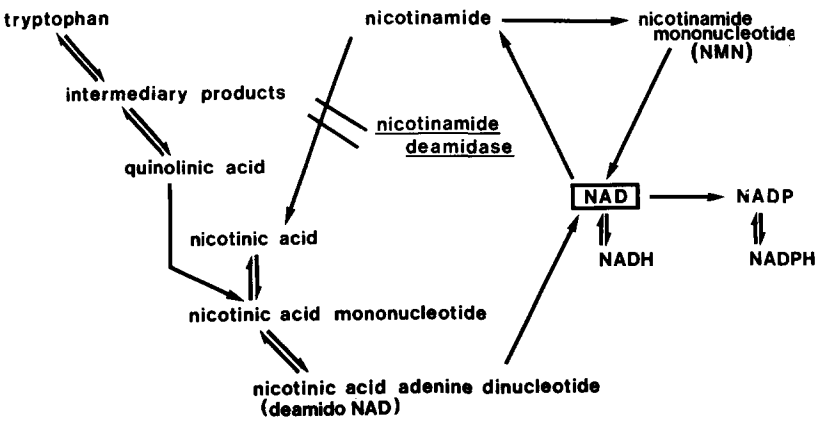

Fig. 6. Scheme of the biosynthesis of NAD. Nicotinamide deamidase plays a primary role in NAD biosynthesis via the Preiss and Handler pathway [3,4].

the mechanism of hormonal regulation of NAD synthesis from nicotinamide [35,36] together with thyroid, adrenals and gonads [29,37].

Previous experiments [10] have shown that the studied enzyme appeared to be absolutely specific for nicotinamide (it had no effect on other amides). Moreover, we have found nicotinamide deamidase activity in growth media of all tissue cultures examined [10]: N1 15 neuroblastoma cells, C6 and NN glial cells, astroblasts and neurones of primary cultures, HeLa cells and fibroblasts. The nicotinamide deamidase isolated and purified from growth medium of M1 cells seems to represent a common feature of cellular metabolic regulation of NAD in various tissue cultures in vitro.

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