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Metabolic Fate of Extracellular NAD in Human Skin Fibroblasts

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Abstract Extracellular NAD is degraded to pyridine and purine metabolites by different types of surface-located enzymes which are expressed differently on the plasmamembrane of various human cells and tissues. In a previous report, we demonstrated that NAD-glycohydrolase, nucleotide pyrophosphatase and 5′-nucleotidase are located on the outer surface of human skin fibroblasts. Nucleotide pyrophosphatase cleaves NAD to nicotinamide mononucleotide and AMP, and 5′-nucleotidase hydrolyses AMP to adenosine. Cells incubated with NAD, produce nicotinamide, nicotinamide mononucleotide, hypoxanthine and adenine. The absence of ADPribose and adenosine in the extracellular compartment could be due to further catabolism and/or uptake of these products. To clarify the fate of the purine moiety of exogenous NAD, we investigated uptake of the products of NAD hydrolysis using U-[^{14}C]-adenine-NAD. ATP was found to be the main labeled intracellular product of exogenous NAD catabolism; ADP, AMP, inosine and adenosine were also detected but in small quantities. Addition of ADPribose or adenosine to the incubation medium decreased uptake of radioactive purine, which, on the contrary, was unaffected by addition of inosine. ADPribose strongly inhibited the activity of ecto-NAD-hydrolyzing enzymes, whereas adenosine did not. Radioactive uptake by purine drastically dropped in fibroblasts incubated with ^{14}C-NAD and dipyridamole, an inhibitor of adenosine transport. Partial inhibition of [^{14}C]-NAD uptake observed in fibroblasts depleted of ATP showed that the transport system requires ATP to some extent. All these findings suggest that adenosine is the purine form taken up by cells, and this hypothesis was confirmed incubating cultured fibroblasts with ^{14}C-adenosine and analyzing nucleoside uptake and intracellular metabolism under different experimental conditions. Fibroblasts incubated with [^{14}C]-adenosine yield the same radioactive products as with [^{14}C]-NAD; the absence of inhibition of [^{14}C]-adenosine uptake by ADPribose in the presence of α-β methyleneADP, an inhibitor of 5′ nucleotidase, demonstrates that ADPribose coming from NAD via NAD-glycohydrolase is finally catabolised to adenosine. These results confirm that adenosine is the NAD hydrolysis product incorporated by cells and further metabolized to ATP, and that adenosine transport is partially ATP dependent. J. Cell. Biochem. 80:360–366, 2001.

Key words: NAD; NAD-glycohydrolase; ectoenzymes; adenosine; uptake; purine; human skin fibroblasts

In all living cells, nicotinamide adenine dinucleotide (NAD) is a cofactor for redox-reactions and substrate for enzymes such as mono and poly-ADP-ribosyl transferase [Ueda and Hayaishi, 1985], ADP-ribosyl cyclase [Lee and Aarhus, 1991] and NAD-glycohydrolase [Travo et al., 1979]. NAD has also been identified as a synaptic modulator in the central nervous system [Snell et al., 1984] and as an inhibitor of excitatory synaptic transmission in the rat hippocampus, acting through activation of specific receptors [Gallareta et al., 1993]. This evidence associated with the presence of NAD in biological fluids [Kim et al., 1993a; Majamaa et al., 1996; De Flora et al., 1996] has revealed much about the physiological functions of this nucleotide.

Extracellular NAD is degraded to pyridine and purine metabolites by different types of surface-located enzymes, which are expressed differently on the plasmamembrane of various human cells and tissues [Zimmermann, 1996; Han et al., 1995]. The main enzyme family is

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the NAD-glycohydrolases. It is NAD specific and hydrolyses NAD to nicotinamide and ADPribose [Bock et al., 1971; Muller et al., 1983; Honma and Mandel, 1986]. In blood cells NAD-glycohydrolase activity is expressed by the transmembrane glycoprotein CD38 [Malavasi et al., 1994], a bifunctional ectoenzyme converting NAD to nicotinamide and cyclic ADPribose (ADP-ribosyl cyclase activity) and cyclic ADPribose to ADPribose (cADPribose hydrolase activity) [Zocchi et al., 1993; Kim et al., 1993b]. Cyclic ADPribose is a newly identified messenger which mobilizes Ca^{2+} from an inositol 1,4,5-trisphosphate insensitive intracellular store [Berridge, 1993; Lee, 1994; De Flora et al., 1998]. NAD therefore acts as extracellular signaling substance or is a precursor of signaling nucleotides [Kato et al., 1995; Okamoto et al, 1995]. A number of enzymes metabolizing extracellular purine nucleotides and nucleosides may also act on exogenous NAD, influencing its physiological role and the fate of the hydrolysis products [Zimmermann, 1996].

In a previous report [Aleo et al., 1996], we demonstrated that NAD-glycohydrolase, nucleotide pyrophosphatase and 5′-nucleotidase are located on the outer surface of human skin fibroblasts. Nucleotide pyrophosphatase cleaves NAD to nicotinamide mononucleotide and AMP, and 5′-nucleotidase hydrolyses AMP to adenosine. Cells incubated in the presence of NAD, produced nicotinamide, nicotinamide mononucleotide (NMN), hypoxanthine and adenine but not ADPribose and adenosine (probably due to uptake and further catabolism of these products).

To clarify the fate of the purine moiety of exogenous NAD and its role in the intracellular metabolism of purines, we investigated the uptake and reutilization of NAD-hydrolysis products, using U-[^{14}C]-adenine-NAD.

METHODS

Materials

Minimum essential medium with Earle's salts (MEM), glutamine, trypsin, and foetal calf serum were from Life Technologies Italia S.r.l. (Gibco BRL). NAD, adenine, adenosine, ADPribose, AMP, ADP, ATP, IMP, inosine, hypoxanthine, dipyridamole, nitrobenzylthioinosine, 2-deoxy-D-glucose, ouabaine, 1,N6-etheno-NAD and methyleneadenosine 5′-diphosphate were from Sigma-Aldrich S.r.l. (Milan, Italy). NaN_3 was from Merck (Bracco S.p.A, Milan, Italy). Methanol and tetrabutylammonium dihydrogen phosphate (TBA) were from Carlo-Erba (Milan, Italy).

Cell Culture

Human skin fibroblasts were grown in 35 mm dishes containing MEM medium with 10% foetal calf serum and 2 mM L-glutamine in a 5% CO_2 atmosphere at 37°C.

Enzyme Assays

Ecto-NAD-hydrolyzing activities (NAD-glycohydrolase and nucleotide pyrophosphatase) were measured by a fluorimetric method described by Muller et al. [1983]: confluent fibroblasts were incubated 10 min at 37°C in the presence of 0.2 mM 1,N6-etheno-NAD in 500 µl phosphate buffered saline (PBS); 400 µl of incubation medium was diluted with the same volume of PBS and fluorescence measured. In some experiments, 1 mM ADPribose or adenosine or 5 mM NaN_3 and 50 mM 2-deoxyglucose (NaN_3/dxg) were added to the incubation medium. Fluorescence was measured using a Jasco FP-770 Spectrofluorimeter exciting at 290 nm and reading at 415 nm.

Determination of ATP in Controls and ATP-Depleted Cells

Depletion of ATP intracellular levels was achieved incubating fibroblasts at 37°C with 10 mM Hepes in Hank's buffer pH 7.4 (H/H buffer) without glucose but containing NaN_3/dxg. After 15, 30, 75 and 90 min, cells were harvested by scraping in 0.1 N $HClO_4$, the cellular extracts centrifuged for 15 min at 12,000 *g* in an Eppendorf 5414 centrifuge and the supernatants neutralized with 1.33 M K_2CO_3. The ATP, ADP and AMP contents of the final clear supernatant were analyzed by HPLC. Protein determination was performed by Bradford assay [Bradford, 1976] on the first pellet resuspended in 1 M NaOH. Control cells were incubated for the same times with H/H buffer.

HPLC analysis was performed as previously described [Aleo et al. 1996] using 0.1 M K-phosphate buffer containing 6 mM TBA at pH 5.5 and methanol as eluants.

^{14}C-NAD and ^{14}C-Adenosine Supply

Confluent fibroblasts were incubated for 60 min at 37°C with 1 µM U-[^{14}C]-adenine-

NAD (252 mCi/mmol, Amersham International plc, England) or 1 μM 8-[^{14}C] adenosine (58 mCi/mmol NEN Life Science Products Italy S.r.l.) in H/H buffer. The reaction was stopped by chilling cells at 0°C. After washing, cells were harvested by scraping with 70% (v/v) ethanol and centrifuged for 10 min at 14,000 *g* in a Hermle Z 230 MA centrifuge. Pellets were resuspended with 1 volume of 18% trichloroacetic acid and dissolved with 2 volume of 1 M NaOH, while supernatants were evaporated to 80 μl in a speed-vac centrifuge (Howe, Gyrovap L) and then used for thin layer chromatography analysis. Aliquots of supernatant or pellet were measured in a Wallac Win Spectral TM 1414 Liquid Scintillation Counter.

ADPribose or adenosine or inosine or dipyridamole (DPR) or nitrobenzylthioinosine (NBT) (100 μM) were added to the incubation medium where indicated.

Protein content of NaOH dissolved pellets was determined according to Bradford [Bradford, 1976] using bovine serum albumin as standard.

TLC Analysis and Autoradiography

The labeled products were estimated by TLC using iso-butyric acid/H_2O/NH_3 (66:33:1.7 v/v/v) as solvent system. Suitable unlabeled internal standards were used and visualised by UV fluorescence. Autoradiography of plates was detected with Hyperfilm-MP X-ray (Amersham) and the radioactive spots were scraped and quantified by liquid scintillation count.

RESULTS

When human skin fibroblasts were incubated 60 min at 37°C with H/H buffer containing 1 μM U-[^{14}C]-adenine-NAD (150 nCi/dish), $25 \pm 3.0\%$ of the total radioactivity, or 1.27 ± 0.1 nmol/mg protein, was incorporated by the cells ($n = 12$). TLC analysis of cell extracts revealed that the labeled species were mainly ATP (79.97%) and ADP plus IMP (11.86%); trace amounts of NAD (1.45%), AMP (1.67%) and other purines such as adenosine, inosine and hypoxanthine (2.17, 2.23, and 0.64%, respectively) were also present.

The main purine products of extracellular NAD hydrolysis in fibroblasts [Aleo et al., 1996], namely ADPribose and adenosine were then added to the incubation medium containing U-[^{14}C]-adenine-NAD. Total radioactive cell uptake drastically decreased to $3.6 \pm 0.9\%$ in the presence of ADPribose and $5.0 \pm 0.76\%$ in the presence of adenosine ($n = 5$), while the relative proportion of intracellular labeled compounds did not vary with respect to control cells (Fig. 1). Thus, extracellular ADPribose and adenosine interfere in the uptake of radioactivity, but do not modify ^{14}C-purine recycling.

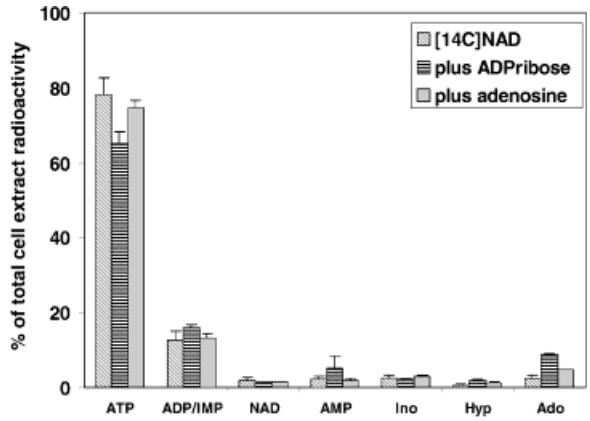

Fig. 1. Distribution of intracellular radioactive adenine compounds after incubation of fibroblasts for 1 h with 1 μM U-[^{14}C]-adenine-NAD in the presence or absence of 100 μM ADPribose or adenosine. Values are given as percentage of total cell extract radioactivity.

ADPribose is a known inhibitor of NAD glycohydrolase [Honma and Mandel, 1986] and it competes with NAD as a substrate of nucleotide pyrophosphatase. When added to the incubation medium of the NAD-hydrolytic ectoenzyme assay, ADPribose strongly inhibited their activity, but adenosine did not (Table I). This suggests that ADPribose and adenosine affect [^{14}C]NAD uptake differently: ADPribose competes with [^{14}C]NAD in the production of the radioactive purine moiety, which is then only partially taken up, whereas adenosine competes directly with the latter compound for membrane passage, without affecting the ectoenzymes.

Uptake of the ^{14}C-purine moiety was also measured in the presence of the purine nucleoside inosine, or DPR, an inhibitor of adenosine transport. Uptake was reduced to $2.1 \pm 0.6\%$ per 100 μM DPR, and $19.5 \pm 2.4\%$ per 100 μM inosine ($n = 4$). All these results suggest that adenosine is the molecule involved in purine moiety uptake by NAD.

TABLE I. Assay of Membrane NAD-Hydrolase Activities (NAD-Glycohydrolase and Nucleotide Pyrophosphatase) in Human Skin Fibroblast[a]

Control	+ADPribose (1 mM)	+Adenosine (1 mM)	+NaN_3 (5 mM) +dxg (50 mM)*
184,30 ± 36,60 (n = 6)	00,00 ± 0,00 (n = 6)	146,70 ± 15,00 (n = 4)	166,30 ± 10,30 (n = 5)

[a]Cells were incubated for 10 min at 37°C in the presence of 0.2 mM 1,N6-etheno-NAD with or without ADPribose or Adenosine or NaN_3/dxg (in the indicated amounts). Activity is expressed as fluorescence units (ΔF/min) per mg of protein.
*Cells were preincubated for 15 min at 37°C in 10 mM Hanks'/Hepes buffer pH 7.4 without glucose and in the presence of NaN_3/dxg before addition of 1,N6-etheno-NAD.

Effect of ATP Depletion on U-[^{14}C]-Adenine-NAD Uptake

The effect of ATP depletion on radioactivity uptake was also examined. When fibroblasts were incubated with H/H buffer without glucose, with 5 mM NaN_3 and 50 mM deossyglucose, 73% of the energy charge of the adenylate pool, as defined by Atkinson formula [Atkinson, 1977], was lost after 15 min and reached a minimum (9%) after 75 min. Figure 2 shows the time course of cell content of AMP, ADP and ATP assayed by HPLC. After 15 min of NaN_3/dxg treatment, 1 μM [^{14}C]NAD was added and 60 min later the radioactivity of cell extracts was measured. These ATP-depletion conditions reduced the uptake of total ^{14}C-purine to 13.0 ± 1.5%, which is 54.7 ± 5.3% (n = 6) of the value found in untreated controls, and modified intracellular radioactivity distribution (Fig. 3). The recycling of labeled purine matches the behavior of adenine nucleotides observed in time-course experiments: ATP production was inhibited, and AMP accumulated. The activities of NAD hydrolyzing ectoenzymes were not affected by incubation with NaN_3/dxg (Table I). These results suggest that transport of U-[^{14}C]-adenine-NAD requires ATP to some extent.

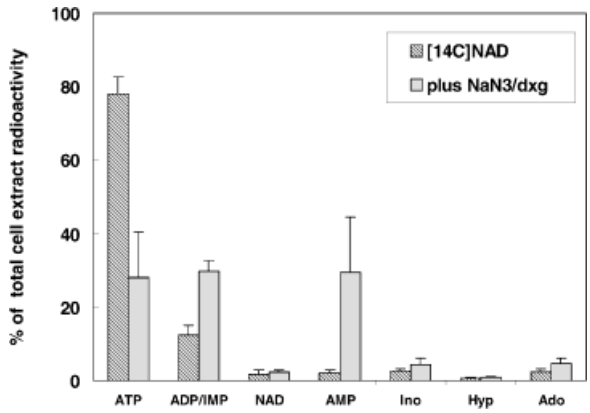

Fig. 3. Distribution of radioactive adenine compounds in cell extract of ATP-depleted fibroblasts. Cells were incubated for 15 min in H/H buffer without glucose containing NaN_3/dxg, then 1 μM U-[^{14}C]-adenine-NAD was added and after 1 h the radioactivity of the different adenine compounds was measured. Control cells were incubated in H/H without NaN_3/dxg. Values are given as percentage of total cell extract radioactivity.

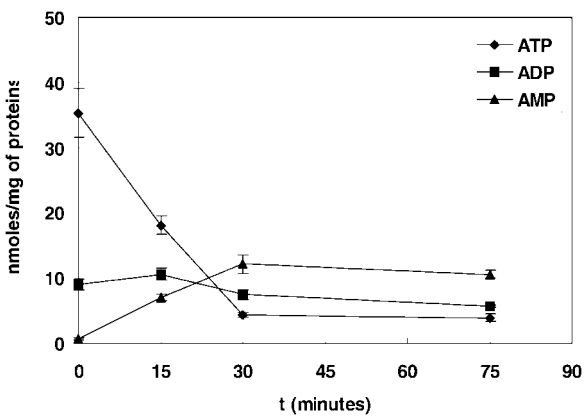

Fig. 2. Time course of ATP, ADP and AMP content in cell extract (nmol/mg of protein), after incubation with 5 mM NaN_3 and 50 mM dxg in H/H buffer without glucose. Data of a representative experiment, made in duplicate, are shown.

[^{14}C]-Adenosine Uptake

To confirm that adenosine is the NAD moiety responsible for [^{14}C]-purine uptake, fibroblasts were incubated in the presence of 1 μM 8-[^{14}C]-adenosine (35 nCi/dish). Uptake of [^{14}C]-adenosine was linear with time for at least 30 min,

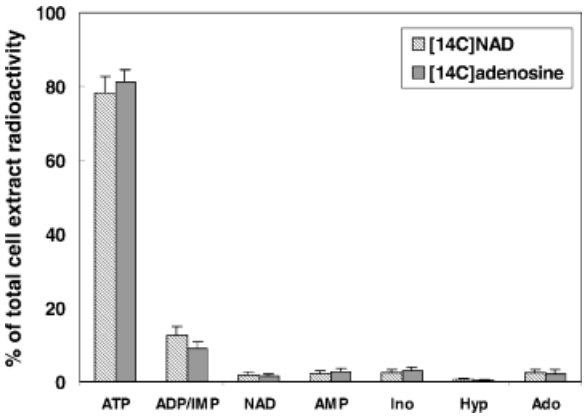

Fig. 4. Distribution of intracellular radioactive adenine compounds after incubation of human fibroblasts for 1 h with 1 μM U-[^{14}C]-adenosine. Values are given as percentage of total cell extract radioactivity.

reaching 1.46 ± 0.26 nmol/mg protein at 60 min ($33.7 \pm 5.6\%$ of total radioactivity, $n = 10$), when cell extract radioactivity showed a same pattern of purine compounds identical to that detected after incubation with [^{14}C]NAD (Fig. 4). [^{14}C]-adenosine uptake was completely depressed by two inhibitors of equilibrative nucleoside transport, namely 10 μM DPR or 10 μM NBT; being 1.64 ± 0.8 and $1.2 \pm 0.1\%$ of total radioactivity, respectively ($n = 6$). To determine whether the uptake was affected by cell content of ATP, the effect of NaN_3/dxg treatment on [^{14}C]-adenosine uptake was assessed. A decrease cell radioactivity to $17 \pm 2.0\%$ ($n = 6$) was found. To verify adenosine active transport in human fibroblasts, the uptake assays were carried out substituting extracellular Na^+ with equimolar choline, which eliminates concentrative Na^+-dependent adenosine permeation, or inhibiting Na^+, K^+-ATPase by 2 mM ouabain, that dissipated the electrochemical gradient of Na^+. The effects were less than those obtained with NaN_3/dxg treatment, leading to [^{14}C]-adenosine uptakes of 25.27 ± 1.0 and $26.9 \pm 1.0\%$, respectively ($n = 4$). Finally, the effect of exogenous ADPribose on [^{14}C]-adenosine uptake was tested. [^{14}C]nucleoside transport was reduced to $19.71 \pm 3.84\%$ of total radioactivity in the presence of 100 μM ADPribose ($n = 6$). This inhibition was removed when the incubation medium was supplemented with 1 mM α-β methyleneADP, an inhibitor of 5′-nucleotidase. α-β methyleneADP was ineffective when incubated with [^{14}C]adenosine alone, but when added to U-[^{14}C]-adenine-NAD uptake assay mixture, strongly depressed uptake ($2.7 \pm 0.4\%$, $n = 2$) thus demonstrating that adenosine is the purine moiety of NAD which permeates the cell membrane.

DISCUSSION

In a previous study, we demonstrated that NAD-glycohydrolase (cleaving NAD into nicotinamide and ADPR), nucleotide pyrophosphatase (cleaving NAD into NMN and AMP), and 5′-nucleotidase are the enzymes involved in extracellular NAD catabolism in human skin fibroblasts. We also showed that the final products of the extracellular NAD metabolism are nicotinamide, NMN, hypoxanthine and adenine [Aleo et al., 1996]. In the present study, we investigated the metabolic fate of the extracellular NAD purine moiety, showing that adenosine is the form which permeates the cell membrane.

This hypothesis is supported by the competition observed between ADPribose or adenosine and [^{14}C]-NAD for uptake of the purine moiety into cells and by the drastic reduction in uptake of labeled purine with an inhibitor of adenosine transport such as DPR in the extracellular medium. Moreover, fibroblasts incubated with [^{14}C]-adenosine yield the same radioactive products as with [^{14}C]-NAD. The fact that ADPribose does not inhibit [^{14}C]-adenosine uptake in the presence of α-β methyleneADP, an inhibitor of 5′-nucleotidase, demonstrates that ADPribose produced from NAD via NAD-glycohydrolase is finally catabolised to adenosine. The product of NAD degradation via nucleotide pyrophosphatase, AMP, has the same fate, being converted by 5′ nucleotidase into adenosine, which enters the cell. Most of the adenosine incorporated into fibroblasts is then phosphorylated and metabolized via AMP to ADP and ATP (90%). The extracellular fates of NAD suggested by our results are summarized in Figure 5.

The partial inhibition of [^{14}C]-NAD uptake observed in fibroblasts depleted of ATP shows that the adenosine transport system requires ATP to some extent. The main transport of nucleosides into the cells is known to be equilibrative (by facilitated diffusion) or concentrative (Na^+ linked) [Dagnino et al., 1991; Gu et al., 1996]. Adenosine kinase, which causes phosphorylation of adenosine, plays a role in

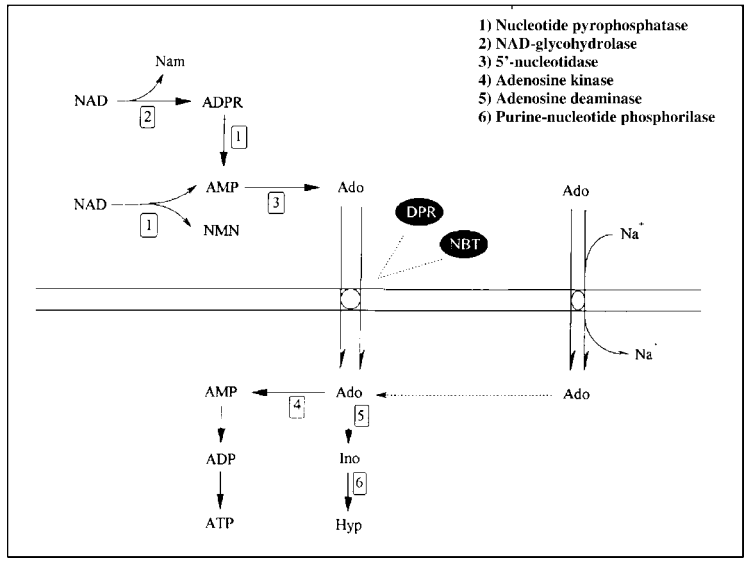

Fig. 5. Extracellular fates of NAD in human fibroblasts.

nucleotide uptake. In our experiments with human fibroblasts, uptake of [^{14}C]-adenosine was completely inhibited by DPR as well as by NBT, known inhibitors of equilibrative nucleoside transport, and was partially inhibited in ATP depleted cells, to the same extent as previously observed for [^{14}C]-NAD. Choline and ouabaine, which depress Na^+-dependent adenosine permeation and the electrochemical gradient respectively, slightly inhibited uptake of [^{14}C]-adenosine, showing that some adenosine (about 20–25% of total uptake) may enter fibroblasts by active transport.

All these findings indicate that adenosine is the main transport form of the purine moiety of NAD, when NAD is metabolized by extracellular enzymes. Passive transport of NAD was recently demonstrated [Zocchi et al., 1999]; the flux across the membranes depended only on the concentration gradient and did not seem to require any energy source. The cells used for the above experiments (HeLa and 3T3 cell lines) were previously shown to be devoid of any NAD hydrolyzing activity. We cannot exclude the possibility that in human fibroblasts, in which NAD hydrolyzing activities do exist, a small amount of NAD permeates the membranes by a similar transport mechanism. The novel finding demonstrated in this study with human fibroblasts is that NAD is cleaved by extracellular enzymes, and the purine form which is taken up is adenosine.

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