Biochemical Pharmacology, Vol. 45, No. 6, pp. 1191–1200, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

SPECIFIC BINDING AND UPTAKE OF EXTRACELLULAR NICOTINAMIDE IN HUMAN LEUKEMIC K-562 CELLS

ANDERS OLSSON,*† TOR OLOFSSON‡ and RONALD W. PERO*†
*Department of Molecular Ecogenetics and ‡Department of Internal Medicine, University of Lund, Lund, Sweden

(*Received* 23 *June* 1992; *accepted* 18 *December* 1992)

Abstract—Extracellular nicotinamide is well recognized as the primary precursor to the cellular synthesis of NAD. NAD is a pivotal molecule in regulating the energy and redox potentials of cells via synthesis of ATP and NAD(P)/NAD(P)H ratios. NAD turnover in cells is very rapid due to NAD catabolism via ADP-ribosylation reactions. These facts suggest that the cellular uptake and transport of nicotinamide may not be a passive process but a highly regulated cellular event. We have utilized radiometric procedures to characterize the uptake of [^{14}C]nicotinamide in human leukemic K-562 cells. At physiologically relevant doses of nicotinamide (<100 μM), the uptake was saturable with a K_m of 2.3 ± 1.0 μM and a V_{max} of about 1.5 ± 0.5 pmol/10^6 cells/min. Kinetic studies revealed that nicotinamide was first taken up intracellularly and then immediately converted to NAD and 1-methyl nicotinamide. All of the nicotinamide taken up into the cell was bound tightly to plasma membranes (25,000 *g* pellet) with K_d values between 3.2 and 12.7 μM and a B_{max} of 1.56 pmol/10^6 cells. The specificity of nicotinamide binding was demonstrated by competitive inhibition experiments using NAD analogs, nicotinamide derivatives, and agonists or antagonists of plasma membrane receptors. We conclude that there is specific binding of nicotinamide, followed by intracellular uptake and immediate synthesis to NAD.

Nicotinamide (NAM§), the amide form of the B_3-vitamin nicotinic acid (niacin), is an important precursor for the synthesis and maintenance of cellular NAD. Data from both *in vivo* and *in vitro* cell model systems have strongly supported that extracellular NAM or "serum nicotinamide" is the main source of NAM for synthesis of NAD via nicotinamide mononucleotide (NMN) [1–4]. The NAD synthesis is performed in two steps [3–5] via the enzymes NAM phosphoribosyltransferase (EC 2.4.2.12) and NMN:ATP adenyltransferase (EC 2.7.7.1), and the first reaction has been shown to be rate limiting [6]. The reactions are dependent on 5-phosphorylribose-1-pyrophosphate (PRPP)/Mn^{2+} and ATP/Mg^{2+}, respectively [7]. There are other pathways for the production of NAD involving synthesis from tryptophane or directly from nicotinic acid [4] but they have been considered of minor importance under normal growth conditions [1].

NAD plays a major role in cellular metabolism [1, 8]. It is essential for the synthesis of ATP, the phosphorylation to NADP and to maintain the cell's redox potential via NAD(P)/NAD(P)H ratios. NAD is also an important co-factor in different enzyme reactions, and it is consumed as a substrate by the NAD-catabolizing enzymes, (ADP-ribosyl)transferases, NAD-glycohydrolases (NADases) and ADP-ribosyl cyclase. Mono(ADP-ribosyl)transferases are mainly localized in the cytoplasm or in the plasma membranes and have a signal transduction function via ADP-ribosylation of G-proteins and elongation factor 2 [9, 10]. Poly(ADP-ribosyl)transferase is involved in nuclear processes like DNA repair, cell differentiation and gene expression (for review see Ref. 11) [12–14]. NADases hydrolyse NAD to ADP-ribose (ADPR) and NAM, and they represent a diverse group of enzymes showing a wide variation in regard to cellular localization (e.g. soluble and membrane bound), size and tissue specificity (for review see Ref. 15). A group of NADases also possess ADP-ribosyl cyclase activity. They have been purified [16–18] and in one case cloned [19], and the NAD hydrolysing properties are coupled to synthesis of cyclic ADP-ribose (cADPR), which in turn, has been suggested to be a second messenger involved in the induction of Ca^{2+} release [20, 21].

The intracellular turnover of NAD is very rapid. The main pathway for NAD catabolism is via ADP-ribosylation reactions and the formation of poly(ADP)ribose [22–24]. This reaction is dependent on DNA strand breaks and is stimulated by DNA damage coming from both endogenous and exogenous sources; e.g. gamma radiation or H_2O_2 [11]. The central role of NAD in cellular metabolism, coupled to its dependence on ADP-ribosylation reactions involved in signal transduction and cell nuclear events, have suggested to us that the widely accepted main pathway for NAD synthesis via cellular uptake and transport of NAM may not be a passive process but a highly regulated cellular event. Earlier work from our laboratory has indicated that both high and low doses of NAM have a radiosensitizing effect on mammary adenocarcinomas transplanted into C3H mice [25–27].

† Corresponding authors. Molecular Ecogenetics, Wallenberg Laboratory, University of Lund, Box 7031, S-220 07 Lund, Sweden.

§ Abbreviations: NAM, nicotinamide; NMN, nicotinamide mononucleotide; 5-HT, 5-hydroxytryptamine; 3-AB, 3-amino benzamide; BAM, benzamide; ADPR, ADP-ribose; MCA, metoclopramide; 3-acetylpyridine-AD, 3-acetylpyridine adenine dinucleotide; cADPR, cyclic ADP-ribose.

This could not be attributed to inhibition of DNA repair via direct effects on ADP-ribosylation, since NAM at doses of 10 mg/kg was able to radiosensitize whereas benzamide (BAM), an even more potent inhibitor of ADP-ribosylation could not. These results led us to raise the question of whether extracellular NAM could affect (ADP-ribosyl)transferases and/or NAD synthesis by altering uptake and transport. Using the human leukemic cell line K-562 as a model system, we collected data that indicated an active uptake of extracellular NAM, and the cellular transport mechanism could not be separated from NAD glycohydrolase or nuclear (ADP-ribosyl) transferase activities [28].

These data have inspired us to further investigate and characterize the nature of the NAM uptake. Here we report on the further characterization of a NAM uptake system, which we show to be via specific binding of extracellular NAM to the plasma membrane, which is in turn coupled to NAD synthesis, and thereby to the turnover of the NAD pool. The NAM binding and uptake can be competitively inhibited by extracellular NAM, NAD and their structural analogs, but it is poorly competed for by drugs known to bind to well established plasma membrane receptors such as the adenosine-, dopamine-, 5-hydroxytryptamine (5-HT) (serotonin) and β-adrenergic-receptors.

MATERIALS AND METHODS

Chemicals. Radioactive [^{14}C]NAM and [^{14}C]-NAD were obtained from Amersham International (Amersham, U.K.). NAM was purchased from E. Merck (Darmstadt, Germany), NAD from Boehringer (Mannheim, Germany), metoclopramide (MCA) from Delagrange (Paris, France) and chlorpromazine from AB LEO (Helsingborg, Sweden). 1-Methyl-NAM, N^5-methyl-NAM, nicotinic acid, NMN, ADPR, adenosine, 3-amino benzamide (3-AB), BAM, 3-acetylpyridine, 3-acetylpyridine adenine dinucleotide (3-acetyl-pyridine-AD), 5-HT (serotonin), sulpiride, dopamine and noradrenaline were all obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.).

Cell culture. Cells from the human leukemic cell line K-562 were used in our experiments. The cells were grown in RPMI 1640 medium (Gibco), supplemented with 10% fetal calf serum in a 5% CO_2 atmosphere at 37°. The cells were subcultured after 2–3 days and the cell viability was always >98% when determined by trypan blue exclusion. The cells were washed in 25 mL RPMI 1640 and resuspended in 20 mL NAM free RPMI 1640 medium (Integro b.v. Zaandam/Holland) supplemented with Hepes buffer (10 mM, pH 7.4), before they were used in any kind of experiments. Viable cell studies were performed in NAM-free RPMI 1640 diluted to half the normal concentration with physiological saline (50% RPMI).

NAM uptake. Cells suspended in 50% RPMI/50% physiological saline at a cell density of 1×10^6 cells per 100 μL, were incubated for 30 min at 37° with micromolar concentrations of ^{14}C-labeled NAM (carbonyl-[^{14}C]NAM, 56 mCi/mmol). In order to obtain an estimate of the non-specific background uptake of NAM, cultures were incubated in parallel at 4°. The reaction was stopped by washing the cells with 2×5 mL ice-cold physiological saline (400 *g*, 5 min and 4°), and thereafter, the pellets were lysed in 0.5% sodium dodecyl sulfate (w/v) and counted in 10 mL scintillation fluid (Ready Safe, Beckman). The NAM uptake was expressed as pmol [^{14}C]NAM/10^6 cells/30 min.

NAM binding. NAM binding studies were performed in membrane fractions of K-562 cells, prepared by homogenization of 3×10^6 cells in a 50 mM Tris–HCl buffer with 10 mM magnesium chloride and 0.5 mM EDTA (pH 8.1 at +4°). The membranes were centrifuged for 10 min at 25,000 *g*, followed by a 1 mL wash of the membrane pellet at 25,000 *g* with homogenization buffer. The binding assay was performed by incubating the 25,000 *g* pellet in 150 μL homogenizing buffer for 45 min, unless stated otherwise, with [^{14}C]NAM (56 mCi/mmol) at concentrations in the micromolar range. In the competition experiments, varying concentrations of competitors were added before incubation with NAM as stated above at a [^{14}C]NAM concentration of 12 μM (56 mCi/mmol). The reaction was terminated by placing the cultures on ice and immediately adding 2 mL ice-cold homogenizing buffer followed by rapid vacuum filtration through Whatman GFB-glass fiber filters. The filters were rapidly washed with 3×5 mL aliquots of washing buffer. After drying, the filters were counted in 5 mL scintillation fluid. The non-specific binding was determined by incubating in the presence of 10 mM NAM, and as in Fig. 5 10 mM 3-acetylpyridine. The specific binding was defined as total binding minus the non-specific binding and was expressed as pmol [^{14}C]NAM bound per mg protein. The 25,000 *g* pellet contained about 470 ± 39 μg protein, which is equal to 157 ± 13 μg protein per 10^6 cells.

TLC analysis. The intracellular NAD content was estimated by two different TLC systems. In both systems PEI-cellulose F-plates (E. Merck) were used with either 2 M ammonium chloride in 0.12 M sodium citrate, pH 5.3: 95% (v/v) ethanol (1:3) or 1 M ammonium acetate, pH 7.4: 99% ethanol (7:3) as solvents [29, 30]. Briefly, the cells collected after incubation and washing were dissolved in 1 mL 70% ethanol (0°) and after freezing at −20° and thawing, the homogenate was centrifuged for 2 min at 14,000 *g* (0°), and the supernatant was then evaporated to dryness under vacuum. The dried sample was redissolved in a small volume (25 μL) of 70% (v/v) ethanol and then it was used for TLC analysis. The standards were unlabeled and hence they were visualized by UV fluorescence; NAD ($R_f = 0.06$ and 0.02), NAM ($R_f = 0.78$ and 0.82), nicotinic acid ($R_f = 0.63$), 1-methyl-NAM ($R_f = 0.53$ and 0.62), N^5-methyl NAM ($R_f = 0.85$) and NMN ($R_f = 0.04$ and 0.11), where the first given R_f values are for the ammonium acetate : ethanol system.

Since neither of the two solvent systems gave complete separation of NMN from NAD, the plates in the ammonium chloride/sodium citrate/ethanol system were rerun in the same system to improve the separation of NMN from NAD. This improved the separation ability, and R_f values of 0.265 for

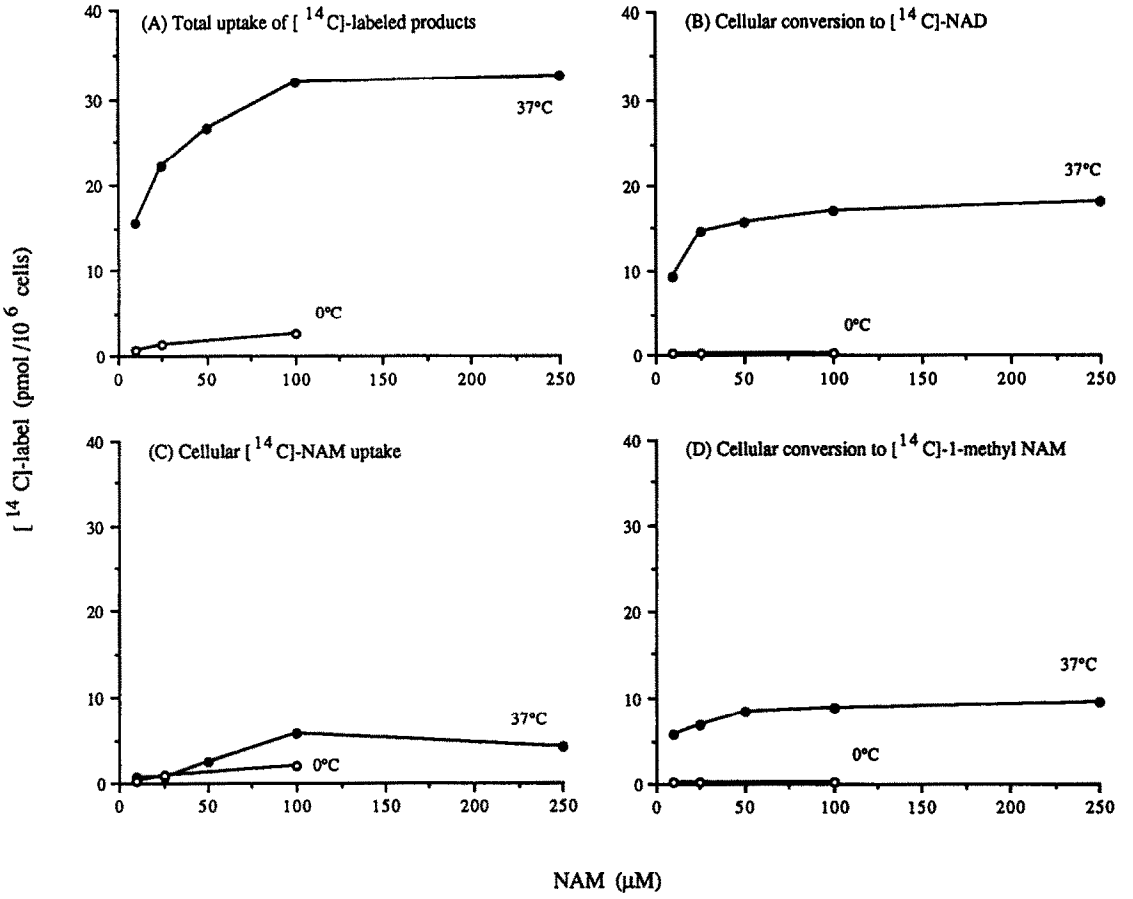

Fig. 1. Metabolic fate of [^{14}C]NAM supplied to K-562 cells, under conditions of specific uptake (i.e. uptake at 37°; uptake at 0°). Panel (A) represents the total amount of ^{14}C-label uptake, (B) conversion to NAD, (C) uptake of NAM and (D) conversion to 1-methyl NAM. Cells per culture (2×10^6) were incubated for 30 min in NAM-free RPMI with varying concentrations of [^{14}C]NAM at 37° or 0°. The incubation was stopped by washing the cells with 2×5 mL ice-cold physiological saline at 400 *g*, followed by freezing and thawing the cells at −70° in 1 mL 70% (v/v) ethanol. The lysed cells were centrifuged at 14,000 *g* and the supernatant was then taken to dryness and redissolved in 25 μL 70% ethanol before TLC analysis (PEI-cellulose; 1 M ammonium acetate: ethanol, 3:7).

NMN and 0.12 for NAD were calculated. It was then possible to state that there is no accumulation of intracellular NMN and that the detectable radioactivity in the NAD region was always moving together with NAD.

RESULTS

When K-562 cells were first incubated in culture in the presence of [^{14}C]NAM and then exhaustively washed with physiologic saline, we observed that the uptake was saturable and temperature dependent (Fig. 1A). TLC analysis of the various metabolic intracellular pyridine pools, after incubation with 12.5–250 μM [^{14}C]NAM concentrations, showed that the main portion of the NAM that was taken up by the cells at 37° had been converted to NAD (i.e. 50%, Fig. 1B). The remaining cell uptake could be accounted for in the form of NAM (12%, Fig. 1C) and 1-methyl NAM (38%, Fig. 1D).

In order to verify if there is a selective intracellular uptake of NAM and a subsequent conversion to NAD, a time study was carried out. K-562 cells were incubated with 50 μM [^{14}C]NAM for different time periods between 2 and 30 min at 37°, and then washed twice with ice-cold saline. The radioactive cell content was prepared and analysed by TLC. Already after 2 min incubation at 37°, the greater portion of radioactivity was in the form of NAD (50%) rather than NAM (35%) (Fig. 2A). The NAM portion of total radioactivity found in the cells remained constant at a level of 2–3 pmol/10^6 cells (Figs 1C and 2A). To further prove that NAM is taken up, and that radioactivity does not derive from some NAM metabolite bound to plasma membranes, the same type of experiment was done, but the cells were first preincubated at 0° until the [^{14}C]NAM was added and then transferred to 37° for incubation at the various time periods indicated (Fig. 2B). The results showed that conversion of NAM to NAD

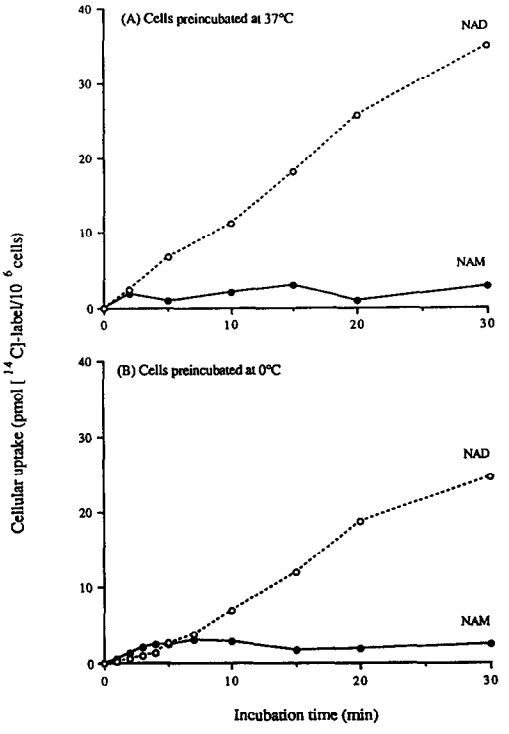

Fig. 2. Time course of [^{14}C]NAM uptake after preincubation of K-562 cells at 37° (A) or 0° (B). The cells were batchwise preincubated in 50% RPMI at 37° or 0°, before 50 μM [^{14}C]NAM (56 mCi/mmol) was added. The cells were immediately transferred to 37°, and portions of 1 or 2×10^6 cells were taken out at indicated time points. After two washes with 5 mL ice-cold saline, the cells were either analysed for [^{14}C]NAM uptake (10^6 cells) or treated with 70% (v/v) ethanol before TLC-analysis (2×10^6 cells). The per cent ^{14}C-label that was found in the 1-methyl NAM region was about 25% of the total uptake. Each data point represents the average of two independent observations from separate experiments.

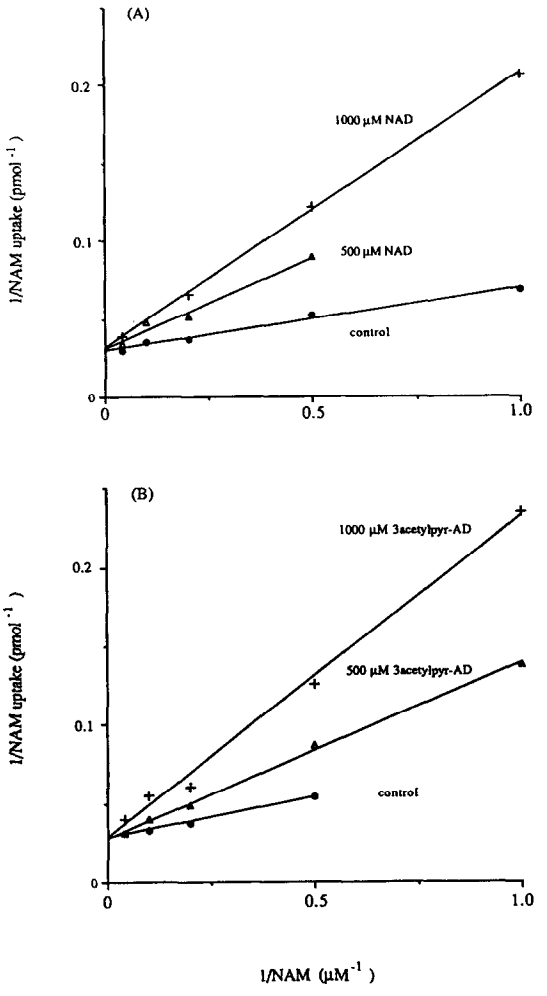

Fig. 3. Competitive inhibition of NAM uptake in K-562 cells by extracellular supplied (A) NAD or (B) 3-acetylpyridine-AD. Cells (1×10^6) were incubated with [^{14}C]NAM at concentrations between 1 and 25 μM for 30 min at 37° in the absence (○), or presence of inhibitor at 500 μM (△) or 1000 μM (+). The reaction was terminated by washing the cells with 2×5 mL ice-cold physiological saline at 400 g, and the non-specific uptake was estimated by parallel cultures at 0°. Four separate control experiments gave a K_m value of 2.3 ± 1.0 μM and a V_{max} of 1.5 ± 0.5 pmol/10^6 cells. The K_i values for NAD and 3-acetylpyridine-AD were calculated to 315 and 255 μM, respectively. Each data point represents an average of two independent observations.

could be delayed, so that the cells could accumulate NAM (2–3 pmol/10^6 cells) before any substantial NAD synthesis occurred (i.e. <5 min incubation, Fig. 2B).

Furthermore, when K-562 cells were incubated in the presence of extracellular NAD or the NAD derivative 3-acetylpyridine-AD, NAM uptake was inhibited at concentrations between 0.5 mM and 1 mM (Fig. 3A and B). An average K_m value from four separate control experiments where no inhibitor was added, was 2.3 ± 1.0 μM with a calculated V_{max} of 1.5 ± 0.5 pmol/10^6 cells/min. The double-reciprocal plots were linear and demonstrated a competitive inhibition for both substances with K_i values for NAM uptake of 315 μM for NAD and 255 μM for 3-acetylpyridine-AD, respectively (Fig. 3A and B). As phosphorylated pyridine nucleotides are known not to be freely diffusable through the cell membrane, these data are taken as evidence for the ectoplasmic membrane location of the initial NAM binding. Therefore, the data are consistent with a mechanism whereby NAM (<100 μM) first binds to the plasma membranes, and then it is transported and converted to intracellular NAD and 1-methyl NAM.

This interpretation of our data suggested to us that free unbound NAM may never exist in the cell. To test this hypothesis, binding studies of ^{14}C-labeled NAM to membrane fractions (25,000 g pellet) prepared from homogenized K-562 cells were performed. The results presented in Table 1 establish that only NAM binds to the membranes, and thus, NAM must bind to the plasma membranes before it

Table 1. Comparison between [^{14}C]NAM uptake by viable cells versus bound [^{14}C]NAM to a 25,000 *g* membrane pellet

	NAM content (pmol/10^6 cells)	TLC-analysis (^{14}C-label in % of total radioactivity) NAM	NAD	1-Methyl NAM
Viable cells	2.25	12	50	38
25,000 *g* membranes on GFB-filters	1.56 ± 0.39	>99	0	0

The incubation was performed for 45 min at 37° with a [^{14}C]NAM concentration of 50 μM (56 mCi/mmol).

The cell pellet was analysed for ^{14}C-uptake after two washes and bound [^{14}C]NAM to 25,000 *g* membrane pellets by collection onto GFB filters as described in Materials and Methods. TLC-analysis of NAM metabolism was performed as described in Materials and Methods.

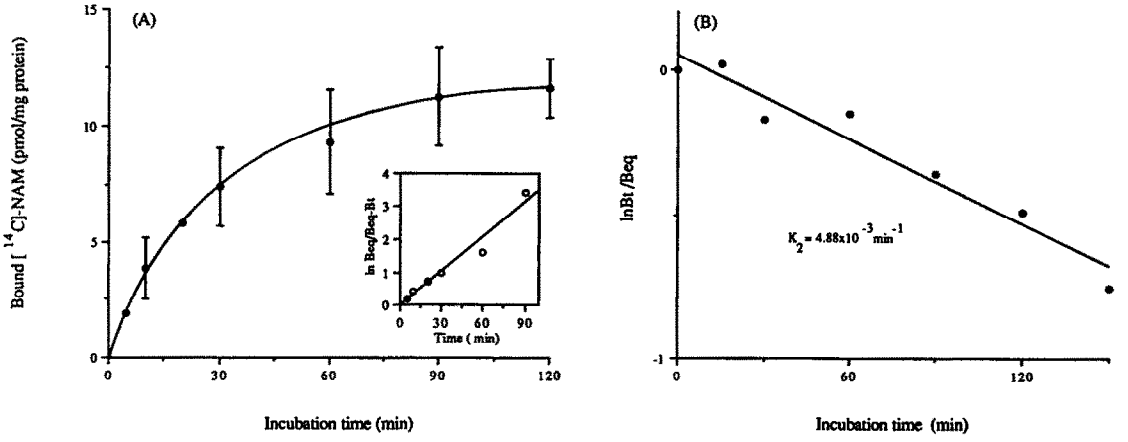

Fig. 4. Kinetic characteristics of the specific binding of [^{14}C]NAM to K-562 cell membrane (25,000 *g* pellet) fractions. (A) Time course for the association reaction. Membrane pellets from 3×10^6 cells (470 μg protein) were incubated with 20 μM [^{14}C]NAM at 37° for the times indicated. The non-specific binding was determined by including 10 mM NAM and 10 mM 3-acetylpyridine in the reaction mixture. Inset shows the pseudo first order kinetic plot of $\ln B_{eq}/(B_{eq} - B_t)$ versus time which gave a k_{obs} value of 3.53×10^{-2} min^{-1} and a k_1 value of 1.52×10^3 min^{-1} μM^{-1}. (B) The dissociation of specific bound [^{14}C]NAM from the membrane fraction (25,000 *g* pellet). Plot of $\ln(B_t/B_{eq})$ versus time, where B_{eq} is the specific bound [^{14}C]NAM at equilibrium and B_t is the binding at time t, after addition of 10 mM NAM and 10 mM 3-acetylpyridine to initiate dissociation of bound [^{14}C]NAM at equilibrium ($t = 0$ min in Fig. 4B). The first order dissociation constant k_2 was 4.88×10^{-3} min^{-1} which resulted in a K_d value of 3.2 μM. Error bars in Fig. 4A are the standard deviations derived from three independent measurements with triplicate cultures, and each point in Fig. 4B represents the mean of two independent measurements with triplicate cultures.

can be converted to NAD and 1-methyl NAM. Moreover, specific binding of NAM was demonstrated by incubating membrane fractions at 37° with 20 μM [^{14}C]NAM for the time periods indicated in Fig. 4, and then removing non-specifically bound [^{14}C]NAM by immobilization and washing on glass fiber filters as described in Materials and Methods. The NAM binding reached equilibrium between 60 and 90 min (Fig. 4A). The initial binding rate constant (k_{obs}) for the association reaction was calculated by a pseudo first order kinetic plot of $\ln(B_{eq}/B_{eq} - B_t)$ versus time (Fig. 4A, inset), where B_{eq} was the specific bound [^{14}C]NAM at equilibrium and B_t was the binding at time t. The slope of the line (k_{obs}), was estimated by linear regression ($r = 0.979$) and gave a $k_{obs} = 3.53 \times 10^{-2}$ min^{-1} [31].

The [^{14}C]NAM binding was displaceable when a mixture of 10 mM NAM and 10 mM 3-acetylpyridine was added to the reaction mixture after 120 min (Fig. 4B). At 150 min after addition of NAM and 3-acetylpyridine, 47% of the specific binding was replaced. A first order kinetic plot of $\ln[B_t/B_{eq}]$ versus time was made, and the negative slope, calculated by linear regression ($r = 0.972$) is equivalent to the first order dissociation constant k_2 and gave a value of 4.88×10^{-3} min^{-1}. From the equation $k_1 = (k_{obs} - k_2)/[NAM]$, where k_2 is the rate constant for the dissociation (Fig. 4B), the

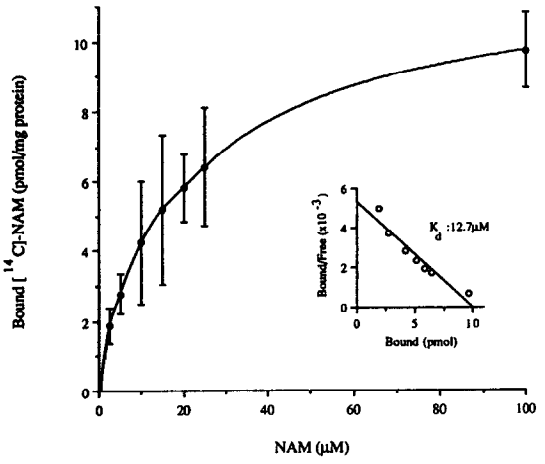

Fig. 5. Saturation plot of [^{14}C]NAM binding to 25,000 g membrane pellets (470 μg protein = 3×10^6 cells). The incubation was performed at 37° for 45 min with indicated concentrations of [^{14}C]NAM (56 mCi/mmol) as described in Materials and Methods. The non-specific binding was determined by including 10 mM unlabeled NAM in the reaction mixture. The inset plot shows the Scatchard analysis of the data, which gave a K_d value of 12.7 μM and a B_{tot} of 10.1 pmol/mg protein. Each data point represents an average of two to four independent experiments ± SD.

second order rate constant for the association reaction could be calculated ($k_1 = 1.52 \times 10^3\ \mu M^{-1}\ min^{-1}$). From these rate constants the equilibrium dissociation constant $K_d = k_2/k_1$ was calculated which gave a K_d value of 3.2 μM. This is in good agreement with the K_m value for NAM uptake in whole cells (i.e. 2.3 μM, Fig. 3).

The NAM-binding reaction carried out on the 25,000 g membrane pellets, was saturated at a NAM concentration of 50 μM with a calculated K_d of 12.7 μM and a B_{tot} of 10.1 pmol/mg protein (1.56 pmol/10^6 cells, Fig. 5, inset). The size of the B_{tot} value strongly supports the conclusion that all the NAM uptake observed when K-562 cells are incubated in the presence of <100 μM NAM for 30 min, is in bound form (i.e. 1.58 pmol vs 2–3 pmol/10^6 cells, Table 1, Figs 1 and 2) and not free in the cytosol. An estimation of the number of receptor sites/cell calculated from B_{tot} gave 9.5×10^5 receptor sites per cell. This was determined using the formula: receptors/cell = (mol bound NAM/cell number) $\times 6.02 \times 10^{23}$ molecules/mol.

As the time study and the saturation plot of [^{14}C]-NAM binding to membrane fractions (Figs 4 and 5) was performed using a crude 25,000 g pellet from homogenized cells without removal of the nuclear components, an experiment was designed (Table 2) to determine if the observed NAM binding to membranes was associated more to the plasma membranes than to the nuclear components. For this purpose, the cells were gently homogenized in Tris–HCl buffer (see Materials and Methods) and then divided into four parallel samples. Two samples were first centrifuged at 300 g for 10 min (4°) to remove nuclear components and then centrifuged a second time at 25,000 g for 10 min (3×10^6 cells = 172 μg protein, Fraction II). Two samples were just centrifuged at 25,000 g for 10 min (3×10^6 cells = 460 μg protein, Fraction I). The resulting membrane pellets were washed and redissolved in Tris buffer (50 mM) and one sample of fractions I and II were sonicated for 3 × 5 sec on ice (MSE, amplitude 12 microns) before incubation with [^{14}C]NAM. The total binding to membrane fraction II, which had the nuclear components removed, was reduced from 3.2 to 1.7 pmol (50% reduction), but the specific bound [^{14}C]NAM/μg protein was increased from 7.1

Table 2. NAM binding to nuclear and membrane fractions of K-562 cells

Membrane fraction	Total protein in sample (μg)	Protein on filter (μg)	Total ^{14}C-binding (pmol)	Specific binding on filter (pmol/mg protein)
Ia. (with nuclear components)	460	450	3.2	7.1
Ib. (with nuclear components + sonicated)	506	470	0	0
IIa. (without nuclear components)	172	169	1.7	10.2
IIb. (without nuclear components + sonicated)	146	140	0.15	1.0

Cells (72×10^6) were homogenized with a glass–teflon homogenizer in homogenizing buffer (Tris–HCl 50 mM, $MgCl_2$ 10 mM, EDTA 0.5 mM, pH 8.1 at 4°). The homogenate was divided into four equal fractions (Ia, Ib and IIa, IIb). Two fractions (IIa and IIb) were first centrifuged at 300 g for 10 min and the pellets were discarded. All the fractions were then centrifuged at 25,000 g for 10 min. The fractions were resuspended in 150 μL homogenizing buffer and after sonication of fractions Ib and IIb (3 × 5 sec), all fractions were incubated in the presence of 50 μM [^{14}C]NAM (56 mCi/mmol) at 37° for 45 min. The reaction was terminated by collecting and washing the membranes on GFB-filters followed by analysis for radioactivity. The non-specific binding was monitored by adding 10 mM unlabeled NAM to three out of six samples from each fraction. The protein content on the filters gave an average yield of 96 ± 2%, determined by a modified Lowry method [32].

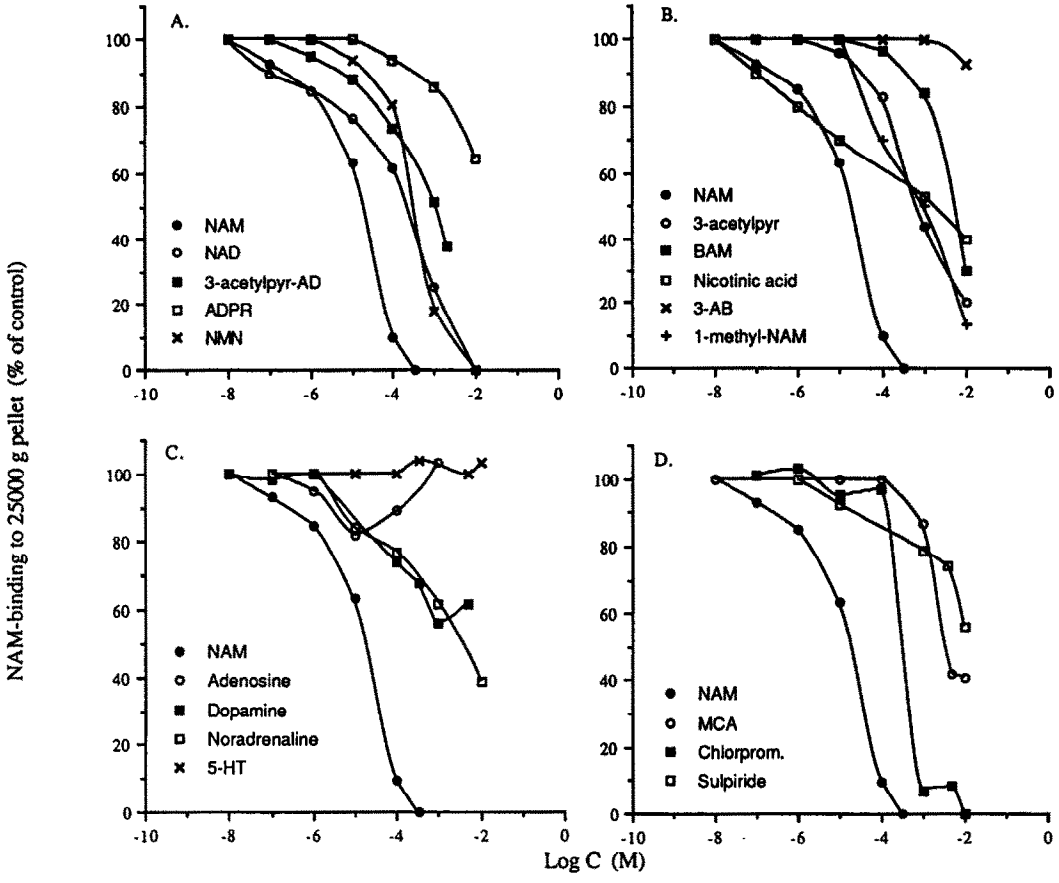

Fig. 6. Effect of NAM binding to K-562 cell membranes (25,000 *g* pellet) by (A) phosphorylated NAD analogs, (B) NAM derivatives, (C) agonists to the adenosine, β-adrenergic, dopamine D_1 and D_2 and 5-HT receptors, all located in the plasma membranes, and (D) antagonists to the β-adrenergic and the dopamine receptors. Membranes from 3×10^6 cells were incubated for 45 min at 37° with 12 μM [^{14}C]-NAM in presence or absence of added agonist/antagonist at indicated concentrations. Each data point represents the percentage bound [^{14}C]NAM in presence of the inhibiting drug compared to the control level in the same experiment, and each point is an average of two to four different determinations.

to 10.2 pmol/mg protein (43% increase, Table 2). Sonication had a devastating effect on the membrane's ability to bind NAM (0–0.15 pmol, Table 2). These data, together with the fact that NAM uptake to whole cells was inhibited by extracellular NAD or 3-acetylpyridine-AD, were taken as a clear indication that the specific NAM binding is associated with the plasma membrane fraction and not the nuclear fraction.

The specificity of NAM binding was also characterized by competitive inhibition experiments using NAD, NAM derivatives, and agonists or antagonists of well known plasma membrane receptors such as dopamine D_1 and D_2 receptors, adenosine receptor, 5-HT receptor and the β-adrenergic receptor (Fig. 6). The competition experiments were run at a [^{14}C]NAM concentration of 12 μM, which was above the K_d value for NAM binding to K-562 cells. NAD and the NAM analogs competed for the [^{14}C]NAM binding with the IC_{50} and K_i values [33] presented in Table 3. NAD was the best inhibitor followed by 3-acetylpyridine whereas the other NAM analogs were less effective as competitors to NAM binding with 50–100 times higher K_i values. Moreover, 3-AB, a structural analog of NAM that is characterized as a potent inhibitor of poly(ADP-ribosyl)transferase [34], did not show any effect on NAM binding. Agonists of the plasma membrane receptors, except for noradrenaline, only showed a tendency to compete for NAM binding at very high doses of dopamine, adenosine or serotonin (Fig. 6C). MCA and chlorpromazine with known affinity to the dopamine D_2 and 5-HT_3 receptors [35–37] competed significantly for NAM binding with K_i values of 1300 and 120 μM, respectively (Table 3 and Fig. 6D). Taken together these data demonstrate that there is a high degree of structural specificity to the NAM binding site, and the specific binding of NAM could occur at a unique receptor site and not via affinity for other well recognized plasma membrane receptors.

DISCUSSION

Human serum levels of NAM are about 0.5 μM

Table 3. IC_{50} and K_i values for inhibition of [^{14}C]NAM binding to 25,000 *g* membrane pellet from K-562 cells

Competing substance	IC_{50} (μM)	K_i (μM)
NAD	230	92
3-Acetylpyridine-AD	1160	465
3-Acetylpyridine	550	220
1-Methyl nicotinamide	1000	400
Benzamide	4200	1680
Nicotinic acid	630	250
Metoclopramide	3200	1300
NMN	750	300
Chlorpromazine	300	120
Noradrenaline	3200	1280

The inhibition constants (K_i) for the competing substances were calculated from the equation $K_i = IC_{50}/(1 + [NAM]/K_d)$ [33]. IC_{50} values were calculated from the inhibition curves in Fig. 6, and where a [^{14}C]NAM concentration of 12 μM was used. The K_d value originates from the NAM binding studies shown in Figs 5 and 6, and was based on the concept of only one binding site with a median K_d value of 8 μM (3.2–12.7 μM).

for normal asymptomatic individuals [38]. Therefore, it seems quite unlikely that serum levels as high as 100 μM NAM could ever be achieved from the diet or from the currently recommended levels of niacin supplementation [39]. As serum nicotinamide is accepted as the major regulatory factor for maintaining cellular NAD levels and adequate niacin status [1], the data presented in this study strongly support that under normal physiological conditions all the intracellular transport of nicotinamide occurs via a binding to a specific membrane site, and not by simple passive diffusion through the plasma membrane. Our evidence for this conclusion is based on the following data presented in this study. (a) Only 2–3 pmol/10^6 cells of the total [^{14}C]NAM uptake (30 pmol/10^6 cells) remains in the form of free NAM, the rest is rapidly converted to NAD and 1-methyl NAM (Figs 1 and 2). (b) The NAM uptake was competitively inhibited by extracellular NAD or 3-acetylpyridine-AD (Fig. 3), indicating that the binding of NAM occurs initially as an ectoplasmic extracellular event. (c) NAM is bound preferentially to the plasma membrane fraction, not to the nuclear membrane containing fraction (Table 2). (d) Specific binding of NAM to plasma membranes (1.56 pmol/10^6 cells) did not involve formation of NAD or 1-methyl NAM, which clearly indicated that NAM first bound to membranes and then was internalized and converted to NAD and 1-methyl NAM. (e) The specific binding of NAM was saturable with time and it was displaceable by addition of high concentrations of NAM and 3-acetylpyridine (Fig. 4). (f) The NAM binding was temperature dependent (i.e. no binding at 0°) and saturable at concentrations above 50 μM (Fig. 5). (g) The specificity of NAM binding could be demonstrated by competition with structural analogs and agonists or antagonists of other plasma membrane receptors (Table 3, Fig. 6).

Another key factor under normal physiological conditions is the metabolic coupling between uptake of NAM and its conversion to NAD. For example, after 2 min exposure of K-562 cells to 50 μM NAM at 37°, more than 50% of the intracellular NAM has been converted to NAD (Figs 1 and 2). This becomes accomplished even though all the cellular NAM can be accounted for in the bound form (i.e. compare Fig. 5 to Fig. 1C and 2A and also Table 1). These data indicate that NAD synthesis is tightly associated to the plasma membrane and not to the cytosol or the nucleus. Furthermore, sonication without any loss of protein, had a profound effect on the membrane's ability to specifically bind NAM (Table 2), and this might be explained by disrupture of the membrane structure relating to the NAM binding protein, or solubilization of a receptor as in the case of other membrane receptors [40, 41]. The difference in the K_d values, 3.2 μM compared with 12.7 μM, calculated from the two different sets of experiments (Figs 4 and 5), also indicates that a postulated NAM receptor could be sensitive to the homogenizing procedures (i.e. whole cells versus homogenizing and/or sonication) [40, 41]. However, this could also be explained by technical difficulties to remove the non-specific bound [^{14}C]NAM component at higher concentrations (>50 μM) when only 10 mM NAM was used to displace bound [^{14}C]NAM (saturation plot Fig. 5), instead of both 10 mM NAM and 10 mM 3-acetylpyridine where a lower K_d value was calculated (Fig. 4, [40]).

There are four enzymes which might be involved in the transport of NAM, namely NAD glycohydrolase, ADP-ribosyl cyclase, NAM phosphoribosyltransferase and (ADP-ribosyl)transferases. Many NAD glycohydrolases have an ectoplasmic location in the membranes [15, 42–44], which are known to bind NAM and NAD analogs with good affinity [15, 30], and under appropriate conditions have been shown to synthesize NAD instead of hydrolysing it [45, 46]. Reported K_m values for membrane bound mammalian NAD glycohydrolases range between 20 and 220 μM [15], which corresponds well to the K_i value for inhibition of NAM uptake (K_i = 315 μM) and binding (K_i = 92 μM) by NAD. In addition, 3-acetylpyridine-AD acts as a substrate for NADase with a K_m of 150 μM [15], which is in the same concentration range we observed for inhibiting NAM uptake and binding (i.e. 255 and 465 μM).

The enzyme ADP-ribosyl cyclase, originally identified as a NAD glycohydrolase [20, 47], has recently been purified from dog brain [16] and purified and cloned from the ovotestis of the mollusk, *Aplysia californica* [17, 19]. The enzyme synthesizes cADPR from NAD, generating NAM as a byproduct [17, 20]. The K_m for this enzymatic reaction has been reported to be 700 μM [18], which is comparable to, although slightly higher than, the K_i value for inhibition of NAM uptake by NAD (K_i = 315 μM, Fig. 3B). Hence our data are consistent with the possibility that NAD glycohydrolase, having ADP-ribosyl cyclase activity, could be involved in the affinity binding and intracellular transport of NAM.

There is also evidence that implicates the involvement of NAM phosphoribosyltransferase in the intracellular transport of NAM. The K_m of this enzyme is 1–2 μM [48, 49] which is very close to the

K_d for NAM binding (Fig. 4). Furthermore, NAM phosphoribosyltransferase has a high affinity NAM binding site which is influenced by a lower affinity phosphate binding site [48]. The K_i values for inhibitors such as NAD, 3-acetylpyridine-AD and 3-acetylpyridine were 40, 100 and 7 μM for NAM phosphoribosyltransferases [48], where our results gave 92 μM for NAD, 465 μM for 3-acetylpyridine-AD and 220 μM for 3-acetylpyridine with NAM binding (Table 3). In fact, these are among the most potent inhibitors for both NAM phosphoribosyltransferase [48] and NAM binding. On the other hand, the K_i value for NAD inhibition of NAM uptake was higher (315 μM). Another inconsistency that does not link NAM phosphoribosyltransferase activity to the active transport of NAM is the fact that the enzyme is reported to be cytosolic [48, 49]. Whether there is a membrane form of NAM phosphoribosyltransferase involved in the NAM binding and uptake is currently under investigation in our laboratory.

There are (ADP-ribosyl)transferases that consume NAD as a co-substrate during the monoribosylation of acceptor proteins in membranes and cytosol as well as mono- and poly(ADP-ribosyl)transferase located in the nucleus [50]. These enzymes are responsible for the rapid turnover of NAD because of their catabolic activity, and as such, they may indirectly influence NAM uptake because they provide a need for NAD synthesis which is coupled to the intracellular transport of NAM (Figs 1 and 3). However, a direct involvement of (ADP-ribosyl)transferases is unlikely because the vast majority of NAD consumption is via nuclear poly(ADP-ribosyl)transferase activity where NAM binding would be irrelevant as a transport mechanism. If membrane (ADP-ribosyl)transferases were involved in NAM transport, it would require binding of NAM ectoplasmically and its conversion intracellularly to NAD. To our knowledge, the synthesis of NAD by (ADP-ribosyl)transferases has not been demonstrated. It should be noted that potent inhibitors of poly(ADP-ribosyl)transferase such as BAM and 3-AB [34] are poor inhibitors of NAM binding (Fig. 6).

Acknowledgements—This study was supported by grants from the Swedish Cancer Society (1728-B88-04XB), the King Gustav V Jubilee Fund, Stockholm (89:513), the Medical Faculty, University of Lund and Oxigene, Inc. in New York City.

REFERENCES

1. Bernofsky C, Physiologic aspects of pyridine nucleotide regulation in mammals. *Mol Cell Biochem* **33**: 135–143, 1980.
2. Dietrich LS, Regulation of nicotinamide metabolism. *Am J Clin Nutr* **24**: 800–804, 1971.
3. Carson DA, Seto S and Wasson BD, Pyridine nucleotide cycling and poly(ADP-ribose) synthesis in resting human lymphocytes. *J Immunol* **138**: 1904–1907, 1987.
4. Jacobson EL, Lange RA and Jacobson MK, Pyridine nucleotide synthesis in 3T3 cells. *J Cell Physiol* **99**: 417–426, 1979.
5. Hillyard D, Reichsteiner M, Manlapaz-Ramos P, Imperial JS, Cruz LJ and Olivera BM, The pyridine nucleotide cycle. *J Biol Chem* **256**: 8491–8497, 1981.
6. Berger SJ, Manory I, Sudar DC and Berger NA, Induction of the pyridine nucleotide synthesis pathway in mitogen-stimulated human T-lymphocytes. *Exp Cell Res* **169**: 149–157, 1987.
7. Berger SJ and Berger NA, An enzymatic method for the measurement of nicotinamide mononucleotide pyrophosphorylase in cells and tissues. *Anal Biochem* **154**: 64–70, 1986.
8. Berro AM and Olivera BM, Intracellular pyridine nucleotide degradation and turnover in pyridine nucleotide coenzymes. In: *Pyridine Nucleotide Coenzymes: Chemical, Biochemical and Medical Aspects* (Eds. Dolphin D, Poulson R and Avramovic O), Vol. 2, Part B, pp. 25–77. Wiley-Interscience, New York, 1987.
9. Gilman AG, G proteins and dual control of adenylate cyclase. *Cell* **36**: 577–579, 1984.
10. Lee H and Iglewski WJ, Cellular (ADP-ribosyl)-transferase with the same mechanism of action as diphtheria toxin and *Pseudomonas* toxin A. *Proc Natl Acad Sci USA* **81**: 2703–2707, 1984.
11. Cleaver JE, Borek C, Milan K and Morgan WF, The role of poly (ADP-ribose) synthesis in toxicity and repair of DNA damage. *Pharmacol Ther* **31**: 269–293, 1985.
12. Althaus FR, Lawrence SD, Sattler GL and Pitot HC, (ADP-ribosyl)transferase activity in cultured hepatocytes. Interactions with repair. *J Biol Chem* **257**: 5528–5535, 1982.
13. Farzaneh F, Zahlin R, Brill D and Shall S, DNA strand breaks and (ADP-ribosyl) transferase activation during cell differentiation. *Nature* **300**: 262–266, 1982.
14. Althaus FR, Lawrence SD, He YZ, Sattler GL, Tsukaka Y and Pitot HC, Effects of altered [ADP-ribose]$_n$ metabolism on expression of fetal functions by adult hepatocytes. *Nature* **300**: 366–368, 1982.
15. Price SR and Pekala PH, Pyridine nucleotide-linked glycohydrolases: In: *Pyridine Nucleotide Coenzymes: Chemical, Biological and Medical Aspects* (Eds. Dolphin D, Poulson R and Avramovic O), Vol. 2, Part B, pp. 513–548. Wiley-Interscience, New York, 1987.
16. Rusinko N and Lee HC, Widespread occurrence in animal tissues of an enzyme catalysing the conversion of NAD^+ into a cyclic metabolite with intracellular Ca^{2+}-mobilizing activity. *J Biol Chem* **264**: 11725–11731, 1989.
17. Lee HC and Aarhus R, ADP-ribosyl cyclase: an enzyme that cyclizes NAD^+ into a calcium-mobilizing metabolite. *Cell Regul* **2**: 203–209, 1991.
18. Hellmich MR and Stumwasser F, Purification and characterization of a molluscan eggspecific NADase, a second-messenger. *Cell Regul* **2**: 193–203, 1991.
19. Glick DL, Hellmich MR, Beushausen S, Tempst P, Bayley H and Strumwasser F, Primary structure of a molluscan egg-specific NADase, a second-messenger enzyme. *Cell Regul* **2**: 211–218, 1991.
20. Lee HC, Walseth TF, Bratt GT, Hayes RN and Clapper DL, Structural determination of a cyclic metabolite of NAD^+ with intracellular Ca^{2+}-mobilizing activity. *J Biol Chem* **264**: 1608–1615, 1989.
21. Galione A, Ca^{2+}-induced Ca^{2+} release and its modulation by cyclic ADP-ribose. *Trends Pharmacol Sci* **13**: 304–306, 1992.
22. Wielckens K, Schmidt A, George E, Bredehorst R and Hiltz H, DNA fragmentation and NAD depletion. Their relation to the turnover of endogenous mono(ADP-ribosyl) and poly(ADP-ribosyl) proteins. *J Biol Chem* **257**: 12872–12877, 1982.
23. Carson DA, Seto S, Wasson BD and Carrera CJ, DNA strand breaks, NAD metabolism and programmed cell death. *Exp Cell Res* **164**: 273–281, 1986.
24. Schraufstatter IU, Hinshaw DB, Hyslop PA, Spragg

RG and Cochrane CG, Oxidant injury of cells. *J Clin Invest* **77**: 1312–1320, 1986.
25. Kjellén E, Jonsson GG, Pero RW and Christensson PI, Effects of hyperthermia and nicotinamide on DNA repair synthesis, (ADP-ribosyl)transferase activity, NAD and ATP pools and cytotoxicity in gamma irradiated human mononuclear leukocytes. *Int J Radiat Biol* **49**: 151–158, 1986.
26. Kjellén E, Pero RW, Cameron R and Ranstam J, Radiosensitizing effects of nicotinamide on a C3H mouse mammary adenocarcinoma. A study on per os drug administration. *Acta Radiol Oncol Radiat Phys Biol* **25**: 281–284, 1986.
27. Kjellén E, Pero RW and Nilsson P, Comparison of low dose nicotinamide versus benzamide, administered per os, as radiosensitizers in a C3H mammary carcinoma. *Radiother Oncol* **12**: 327–331, 1988.
28. Pero RW, Olsson A, Olofsson T and Kjellén E, Active transport of nicotinamide and the adenine moiety of NAD by metabolically controlled NAD catabolism. In: *ADP-ribose Transfer Reactions: Mechanisms and Biological Significance* (Eds. Jacobson MK and Jacobson EL), pp. 378–385. Springer, New York, 1989.
29. Witholt B, A bioautographic procedure for detecting TPN, DPN, NMN and NR. *Methods Enzymol* **18**: 813–816, 1971.
30. Kempson SA, NAD-glycohydrolase in renal brush border membranes. *Am J Physiol* **249**: 366–373, 1985.
31. Weiland AG and Molinoff PB, Quantitative analysis of drug receptor interaction: I. Determination of kinetic and equilibrium properties. *Life Sci* **29**: 313–330, 1981.
32. Bensadoun A and Weinstein D, Assay of proteins in the presence of interfering materials. *Anal Biochem* **70**: 241–250, 1976.
33. Cheng Y-C and Prusoff WH, Relationship between the inhibition constant (K_I) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem Pharmacol* **22**: 3099–3108, 1973.
34. Rankin PW, Jacobson EL, Benjamin RC, Moss J and Jacobson MK, Quantitative studies of inhibitors of ADP-ribosylation *in vitro* and *in vivo*. *J Biol Chem* **264**: 4312–4317, 1989.
35. Harrington RA, Hamilton CW, Brogden RN, Linkewich JA, Romankiewicz JA and Heel RC, Metoclopramide. An updated review of its pharmacological properties and clinical use. *Drugs* **25**: 451–494, 1983.
36. Blower PR, The role of specific 5-HT receptor antagonism in the control of cytostatic drug-induced emesis. *Eur J Cancer* **26** (Suppl 1): S8–S11, 1990.
37. King FD and Sanger GJ, 5-HT_3 receptor antagonists. *Drugs Future* **14**: 875–889, 1989.
38. Clark BR, Halper RM and Smith RA, A fluorometric method for quantification in the picomole range of N^1-methylnicotinamide and nicotinamide in serum. *Anal Biochem* **68**: 54–61, 1975.
39. Horwitt MK, Harvey CC, Rothwell WS, Cutler JS and Haffron DJ, Niacin supplementation. *Nutrition* **60** (Suppl 1): 43, 1956.
40. Meurs H, Van den Bogaard W, Kauffman HF and Bruynzeel PLB, Characterization of (−)-[^{3}H]-dihydroalprenolol binding to intact and broken cell preparations of human peripheral blood lymphocytes. *Eur J Pharmacol* **85**: 185–194, 1982.
41. Ransom RW, Young GS, Schneck K and Goodman CB, Characterization of solubilized bradykinin B_2 receptors from smooth muscle and mucosa of guinea pig ileum. *Biochem Pharmacol* **43**: 1823–1827, 1992.
42. Kim UH, Rockwood SF, Kim HR and Daynes RA, Membrane associated NAD^+ glycohydrolase from rabbit erythrocytes is solubilized by phosphatidylinositol specific phospholipase C. *Biochim Biophys Acta* **965**: 76–81, 1988.
43. Amar-Costesec A, Prado-Figueroa M, Beaufay H, Nagelkarke JF and van Berkel TJC, Analytical study of microsomes and isolated subcellular membranes from rat liver. IX. Nicotinamide adenine dinucleotide glycohydrolase: A plasma membrane enzyme prominently found in kupffer cells. *J Cell Biol* **100**: 189–197, 1985.
44. Artman M and Seeley RJ, Nicotinamide adenine dinucleotide splitting enzyme: A plasma membrane protein of murine macrophages. *Arch Biochem Biophys* **195**: 121–127, 1979.
45. Lötscher HR, Winterhalter KH, Carafoli E and Richter C, Hydroperoxide-induced loss of pyridine nucleotides and release of calcium from rat liver mitochondria. *J Biol Chem* **29**: 9324–9330, 1980.
46. Kaplan NO and Ciotti MM, Chemistry and properties of the 3-acetylpyridine analogue of diphosphopyridine nucleotide. *J Biol Chem* **221**: 823–832, 1956.
47. Lee HC, Specific binding of cyclic ADP-ribose to calcium-storing microsomes from sea urchin eggs. *J Biol Chem* **266**: 2276–2281, 1991.
48. Dietrich LS and Muniz O, Inhibition of nicotinamide phosphoribosyltransferase by pyridine nucleotides. *Biochemistry* **11**: 1691–1695, 1972.
49. Elliot GC and Rechsteiner HC, Evidence for a physiologically active nicotinamide phosphoribosyltransferase in cultured human fibroblasts. *Biochem Biophys Res Commun* **104**: 996–1002, 1982.
50. Poirier GG and Moreau P (Eds.), *ADP-Ribosylation Reactions*. Springer, New York, 1992.