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Title Page

NAD^+ and Vitamin B3: From metabolism to therapies

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Abbreviations

Aβ :plaque forming proteolysis product of APP.
ADPR: adenosine diphosphate ribose
APP: amyloid precursor protein
NaAD: nicotinic acid adenine dinucleotide
NaMN: nicotinic acid mononucleotide
nampt: nicotinamide phophoribosyltransferase
NMN: nicotinamide mononucleotide
nmnat: nicotinamide/nicotinate mononucleotide adenylyltransferase
NR: nicotinamide riboside
PARP: poly-ADPribosylpolymerase
PBEF: pre-B-cell colony enhancing factor
PRPP: 5-phosphoryl-ribose-1-pyrophosphate
QA: quinolinic acid
Sir2: silencing information regulator 2
SIRT1: mammalian sirtuin 1

Abstract

The role of NAD^+ metabolism in health and disease is of increased interest as the use of niacin (nicotinic acid) has emerged as a major therapy for treatment of hyperlipidemias and with the recognition that nicotinamide can protect tissues and NAD^+ metabolism in a variety of disease states including ischemia/reperfusion. In addition, a growing body of evidence supports the view that NAD^+ metabolism regulates important biological effects including lifespan. NAD^+ exerts potent effects through the poly-ADP-ribosyl polymerases (PARPs), mono-ADP-ribosyltransferases (ARTs) and the recently characterized sirtuin enzymes. These enzymes catalyze protein modifications such as ADP-ribosylation and deacetylation, leading to changes in protein function. These enzymes regulate apoptosis, DNA repair, stress resistance, metabolism and endocrine signaling suggesting that these enzymes and/or NAD^+ metabolism could be targeted for therapeutic benefit. This review considers current knowledge of NAD^+ metabolism in humans and microbes, including new insights into mechanisms that regulate NAD^+ biosynthetic pathways, current use of nicotinamide and nicotinic acid as pharmacological agents and opportunities for drug design that are directed at modulation of NAD^+ biosynthesis for treatment of human disorders and infections.

Vitamin B3 (nicotinamide and nicotinic acid) is essential to all living cells. Vitamin B3 is biosynthetically converted to nicotinamide adenine dinucleotide (Figure 1, NAD^+), a versatile acceptor of hydride equivalents to form the reduced dinucleotide, NADH. The phosphorylated forms of the nicotinamide dinucleotides (NADP/NADPH) perform similar chemical functions within cells, although these are generally utilized in biosynthetic pathways and in cell protection mechanisms against reactive oxygen species. NAD(P)H provides reducing equivalents for cellular biochemistry and energy metabolism. Within eukaryotic cells energy metabolism is largely mediated by electron transport chains found within the mitochondrion, and NADH plays a vital role in furnishing reducing equivalents to fuel oxidative phosphorylation. Thus, cellular energy metabolism is substantially mediated by Vitamin B3 derived co-factors, and a large fraction of anabolic and catabolic pathways incorporate these co-factors within them.

Nicotinamide dinucleotides also react as electrophiles to transfer the ADP-ribose (ADPR) moiety to a nucleophile. ADPR-transfer to small nucleophiles forms ADPR (nucleophile: water), cyclic-ADPR (nucleophile N1 adenine) and nicotinic acid adenine dinucleotide-phosphate (derived from $NADP^+$, nucleophile: nicotinic acid). These compounds have been shown to regulate processes such as channel opening and calcium release (Pollak et al., 2007). Additionally, ADPR-transfer modifies protein nucleophilic side chains (Hassa et al., 2006). The ADPR-transfer enzymes fall into three distinct families, the mono-ADPribosyl-transferases (Hassa et al., 2006), the poly-ADP-ribosyl-transferases (PARPs) (Virag and Szabo, 2002; Hassa et al., 2006) and the sirtuins (Sauve et al., 2006). The sirtuins transiently ADP-ribosylate acetyllysines of proteins causing

protein deacetylation, releasing an acetyltransfer product, acetyl-ADP-ribose (Sauve et al., 2006). However, some sirtuins are not deacetylases and appear to catalyze protein ADPRtransfer in the ordinary sense. Collectively, these enzymes regulate numerous signaling pathways and respond to changes in NAD^+ metabolism. They exert profound influences on apoptosis, metabolism, proliferation, DNA repair, senescence, endocrine signaling and lifespan (Guarente, 2006).

In addition to being nutrients, nicotinamide and nicotinic acid are clinically applied pharmacological agents. Nicotinic acid is administered in large doses to lower serum lipids and cholesterol (Schachter, 2005). Nicotinamide has recently been used for prevention of type 1 diabetes (Gale et al., 2004) and is being evaluated for prevention of neurotoxicity and for treatment of ischemia. High dose nicotinic acid and nicotinamide enter metabolism and increase NAD^+ pools, but also bind to proteins in cells to elicit their effects. For example, nicotinic acid has a cognate receptor, which is implicated in some of its anti-lipid effects (Soudijn et al., 2007). Nicotinamide inhibits PARP, leading to decreased NAD^+ turnover, to provide beneficial effects in degenerative states where PARP activity is overactivated (Virag and Szabo, 2002).

The multiplicity of functions attributed to nicotinamide, nicotinic acid and the dinucleotides, as well as the linkage of powerful signaling components to NAD^+ metabolism via the ADPR-transferases has provided a surge of interest in the therapeutic possibilities inherent to targeting NAD^+ metabolism for therapy (see Table 1). NAD^+ metabolism has been a topic of several recent reviews (Magni et al., 2004a; Yang et al., 2006; Yang and Sauve, 2006; Belenky et al., 2007; Revollo et al., 2007). Herein, we survey knowledge of NAD^+ metabolism in humans and microbes. We examine the

properties of nicotinamide and nicotinic acid as nutrients and as pharmacologic agents. We consider other precursors of NAD^+ distinct from nicotinamide and nicotinic acid, such as nicotinamide riboside. Finally we explore current and potential applications of therapeutics that target NAD^+ metabolism and consider how future therapies could develop.

NAD^+ METABOLISM

The biosynthetic and recycling metabolism of Vitamin B3 converges on synthesis of the dinucleotides. Nicotinamide and nicotinic acid are synthesized as moieties within nucleotides or dinucleotides before they are available as free species within cells. Synthesis of NAD^+ is achieved via both recycling and de novo pathways in most microbes and in human cells. Database searches as well as biochemical studies indicate that the de novo pathways of microbes and humans form the biosynthetic product, NAMN, via the decarboxylative coupling of PRPP and quinolinic acid (QA) (Kurnasov et al., 2003).

DE NOVO PATHWAYS IN HUMANS MICROBES AND BACTERIA

The de novo pathway in most bacteria and plants starts from aspartate. The aspartate pathway is anaerobic and molecular oxygen is not required (Kurnasov et al., 2003). The reaction of aspartate and dihydroxyacetone-phosphate leads to efficient synthesis of QA, catalyzed by aspartate oxidase and QA synthase (Figure 2). Alternatively, yeast, humans and some bacterial microbes make QA via an aerobic pathway from tryptophan (Kurnasov et al., 2003). Molecular oxygen as a substrate oxidizes tryptophan to downstream metabolites of the kynurenine pathway, 3-hydroxyanthranilate and finally QA (Figure 3) (Kurnasov et al., 2003). The respective de novo pathways are important

sources of Vitamin B3 in most bacteria and in humans. In humans, 1 out of 67 mg of tryptophan eventually ends up as nicotinamide if another B3 source is not available in the diet (Fukuwatari et al., 2004).

RECYCLING IN BACTERIA

In addition to the de novo pathways, most organisms have recycling pathways capable of synthesizing NAD^+ from B3 obtained via diet, or obtained metabolically from decomposition of NAD^+ or related nucleotides in cells. Within bacteria, decomposition of NAD^+ can occur via ADPRtransfer to form nicotinamide. Bacteria encode sirtuins, and these enzymes cleave nicotinamide from NAD^+ (Blander and Guarente, 2004). Once formed, nicotinamide is converted to nicotinic acid with nicotinamidase. Hydrolysis of nicotinamide is an obligate step of recycling in most bacteria, and a nicotinic acid phosphoribosyltransferase couples nicotinic acid to PRPP to form NAMN (Figure 2). The conversions of nicotinic acid to NAD^+ are known as the Preiss-Handler pathway. NAMN adenylyltransferase catalyzes formation of NAAD (Figure 2) and NAD^+ synthetase completes synthesis of NAD^+ by converting the carboxylate to the amide (Figure 4). Most plants and eukaryotes (except mammals) catalyze resynthesis of NAD^+ from nicotinamide similarly to bacteria, via obligate breakdown of nicotinamide to nicotinic acid followed by synthesis NAMN (Kurnasov et al., 2003).

A second major pathway of NAD^+ decomposition in bacteria involves breakage of the phosphate anhydride bond to form NMN and AMP (Foster et al., 1979). This latter reaction is catalyzed by pyrophosphatases as well as bacterial NAD^+ dependent ligases (Figure 2)(Wilkinson et al., 2001). NMN is resynthesized into NAD^+ via NMN adenylate transferases, or further converted to nicotinamide.

RECYCLING IN HUMANS

In humans, the dominant pathways that decompose NAD^+ are catalyzed by ADPribosyltransferases. Studies show that NAD^+ has a halflife of 10 hours in liver (Ijichi et al., 1966). Nicotinamide formed upon NAD^+ turnover is not hydrolyzed, but is coupled directly to PRPP to form NMN by nicotinamide phosphoribosyltransferase (nampt, also known as PBEF)(Figure 3). This enzyme activity is found only in mammals and some classes of bacteria. It is distinct from the enzyme that couples nicotinic acid to PRPP (Yang et al., 2006; Revollo et al., 2007). Although an activity that converted nicotinamide to NAD^+ independently of nicotinic acid was indicated for sometime, the enzyme was only recently identified (Rongvaux et al., 2002).

The human genome also encodes a Preiss-Handler pathway which converts nicotinic acid to NAD^+ via NaMN, and NAAD (Figure 3). Humans use both nicotinic acid and nicotinamide recycling to synthesize NAD^+, but utilize different pathways to achieve this. An enzyme in common between the pathways is the adenylation enzyme nmnat (Figure 3). This enzyme has three isoforms in humans (nmnat-1, nmnat-2, nmnat-3). nmnat-1 is localized to nuclei as determined by immunofluorescence and was recently shown to stimulate PARP-1 (Schweiger et al., 2001; Berger et al., 2007). nmnat-2 is in golgi, and nmnat-3 is in mitochondria (Berger et al., 2005). All isoforms exhibit dual specificity for both NaMN and NMN as a substrate (Raffaelli et al., 2002; Magni et al., 2004b; Berger et al., 2005). nmnat-1 is the most proficient catalyst as determined by catalytic velocity (V_{max}) and efficiency (V_{max}/K_m). The distribution of NAD in cells, and the locations of NAD^+ synthesis have recently received new consideration. Implied from the fact that nmnat activity is required to complete all salvage and de novo pathways of

NAD^+ biosynthesis, mammalian cell NAD^+ synthesis is compartmentalized and there are stable NAD^+ pools within distinct subcellular compartments. Evidence to support this idea is available from cell fractionation studies which confirm that mitochondria maintain relatively high NAD^+ concentrations, and mitochondrial NAD^+ does not readily leak across the inner mitochondrial membrane (Di Lisa and Ziegler, 2001). On the other hand, the majority of cytosolic NAD^+ is probably made within the nucleus of cells, and then redistributed to the cytosol by passive diffusion through nuclear pores(Berger et al., 2005). It has been asserted that the relative distribution of total NAD^+ in cells is largely mitochondrial (Di Lisa et al., 2001; Di Lisa and Ziegler, 2001), although this premise derives mostly from data obtained on myocytes (Di Lisa et al., 2001) which are rich in mitochondria . In contrast, in hepatocytes 30-40 % of total cellular NAD^+ is mitochondrial, whereas the majority is cytosolic (Tischler et al., 1977). On the extreme, erythrocytes have reasonably high concentrations of NAD^+, but have no mitochondria at all. It is apparent that relative NAD^+ contents in cellular compartments are likely to be cell and tissue specific. It is important to point out that while whole cell NAD^+, nicotinamide and nicotinic acid measurements can monitor NAD^+ metabolism at a gross level, knowledge of the metabolite concentrations in subcellular compartments, like the nucleus, cytoplasm and mitochondria, is crucial to gauge how NAD^+ metabolism affects sirtuin and PARP functions at different cellular loci. Technical and experimental progress in this area is needed before it will be possible to describe just how NAD^+ metabolism is coupled to NAD^+ dependent signaling processes.

PATHWAYS INVOLVING NICOTINAMIDE RIBOSIDE

Recently, a recycling pathway independent of nicotinamide and nicotinic acid that

forms NAD^+, was found to be broadly conserved in bacteria, yeast and mammals (Bieganowski and Brenner, 2004). The pathway leads from the metabolite nicotinamide riboside (NR), the dephosphorylated form of NMN. A highly biologically conserved nicotinamide riboside kinase is able to utilize NR as a substrate and can convert NR to NMN in cells (Bieganowski and Brenner, 2004). This activity allows NR to enter NAD^+ metabolism via NMN and then to NAD^+. Thus, NR is converted to NAD^+ in only two metabolic steps (Figure 2, 3) (Bieganowski and Brenner, 2004). Yeast deficient in de novo or B3 recycling pathways but retaining an intact nmnat activity survive with NR as their only source of B3, indicating that yeast can efficiently salvage this nucleoside and synthesize adequate amounts of NAD^+ via this pathway (Bieganowski and Brenner, 2004). In humans two isoforms of the kinase (Nrk1 and Nrk2) have been cloned, although very little is known about the biochemical properties of these enzymes.

THE ROLE OF NAD in ENERGY METABOLISM and OXIDATION PROCESSES

Vitamin B3, in the form of the dinucleotides, plays a central role in energy metabolism, in both oxidative phosphorylation, and in the redistribution of electron equivalents from catabolism redirected toward biosynthetic pathways. NADH, formed from glycolysis and from the TCA cycle reacts at the point of Complex I, the NADH:Coenzyme Q reductase of the mitochondrial electron transport chain (Pollak et al., 2007). Each mole NADH consumed by the mitochondria can furnish energy for the formation of three moles ATP from 3 moles ADP (Pollak et al., 2007). NADH formation in cells is tightly regulated, and typically represents approximately 10% of total cellular NADH and NAD^+ content (Williamson et al., 1967). NADH is highly depleted in the cytosol, where it typically represents less than 1 % of the combined total of NADH and

NAD^+ that is not in complexation with proteins (Williamson et al., 1967). In the mitochondria, NADH represents approximately 15% of the dinucleotide content uncomplexed to proteins ((Williamson et al., 1967; Tischler et al., 1977). The uncomplexed dinucleotides represent the cellular pool able to interact with unliganded proteins in cells (Williamson et al., 1967). As such, the relatively low concentrations of NADH available in uncomplexed form, particularly in the cytosol, suggest that direct NADH interactions with enzymes such as sirtuins (such as nuclear SIRT1) may be insufficient to explain changes in activity attributed to NADH/NAD^+ ratio (Guarente, 2006).

The phosphorylated dinucleotide $NADP^+$ in the reduced form plays important roles in biosynthesis. In contrast to NADH/ NAD^+, the uncomplexed and total (complexed and uncomplexed) NADPH/NADP ratios in cells are maintained high in the cytosolic and mitochondrial compartments (Tischler et al., 1977). This appears to stem from the importance of NADPH to biosynthesis and because NADPH provides several cell protective functions. For instance, NADPH is an important co-factor for P-450 enzymes, that detoxify xenobiotics (Pollak et al., 2007). In oxidative defense, NADPH acts as a terminal reductant for glutathione reductase which maintains reduced glutathione. Enhanced formation of NADPH, via upregulation of glucose-6-phosphate dehdrogenase appears to increase reduced glutathione concentrations. Conversely, deletion of G6PD causes increased sensitivity of cells to oxidative stress (Pollak et al., 2007). Finally, NADPH serves as a substrate for NADPH oxidase, which generates peroxides for release in oxidative burst processes of the immune system (Pollak et al., 2007).

CLINICAL MANIFESTATIONS OF NIACIN DEFICIENCY

Because of the complexity and diversity of effects attributed to niacin in metabolism, significant redundancy in the NAD^+ biosynthetic pathways of humans make modern Vitamin B3 deficiency rare in industrialized nations(Graham, 1993). However, poor diets, alcoholism, AIDS and other diseases can cause niacin deficiency, or pellagra. Symptoms of pellagra include dermatitis, dementia and diarrhea (Revollo et al., 2007). Niacin deficiency is also associated with an increased risk of cancer (Kirkland, 2003) and has been shown to increase toxicity caused by ROS (Pollak et al., 2007).

PROSPECTS FOR DRUGS TARGETED TO INHIBITION OF NAD^+ BIOSYNTHESIS

Antimicrobials

Opportunities exist for development of antimicrobial agents that target NAD^+ metabolism. Because most pathogenic bacteria have a unique aspartate-based pathway for NAD^+ biosynthesis, it would seem logical that drugs could be targeted to this pathway. Bacterial mutations in the aspartate pathway to QA restrict growth of bacteria if the growth media is deficient in a source of preformed nicotinamide or nicotinic acid (including free pyridines, nucleosides, nucleotides or dinucleotides)(Foster et al., 1979). Problematically, nicotinamide is relatively abundant in mammalian tissues (Qin et al., 2006; Yang and Sauve, 2006), and it is not certain how an inhibition of upstream steps of NAD^+ biosynthesis would impact infectivity or virulence if recycling pathways in bacteria can biochemically salvage nicotinamide and nicotinic acid from the host. The effect of targeting this pathway on bacterial growth in a mammalian host is still undetermined.

Targeting nicotinamide/nicotinic acid recycling for antibiotics may be effective

because some human pathogens (e.g. *Borrelia burgdorferei, P. falciparum)* do not seem to encode a de novo NAD^+ biosynthetic pathway. In these cases salvage of host nicotinamide and nicotinic acid pools to complete NAD^+ biosynthesis is likely to be required for parasite viability. It is undetermined if small molecule inhibition of nicotinamide recycling reduces virulence or infectivity in microbial infections, and to date no potent inhibitors of nicotinamidases have been reported. On the other hand, genetic studies have validated the importance of nicotinamidases for infectivity in pathogens that cause human disease. *Borrelia burgdorferei* (Purser et al., 2003) and *Brucella abortus* (Kim et al., 2004) have been shown to be less infective and less pathogenic if their nicotinamidase genes are deleted. In *leishmania* nicotinamide is able to restrict growth in vitro (Sereno et al., 2005). Interestingly, deletion of nicotinamidase causes abnormally high nicotinamide levels in yeast (Anderson et al., 2003; Gallo et al., 2004; Sauve et al., 2005). Thus, disruption of nicotinamidase in leishmania may have an anti-leishmanial effect if it causes elevated intracellular nicotinamide concentrations.

Because bacteria must use NAMN adenylation and NAD^+ synthetase activity to complete both recycling and de novo pathways to NAD^+, (except for recycling of NMN) it is likely that each of these two enzymes could be targeted for drug design with the prospect of antibiotic effects. These enzyme activities are essential for growth of most bacteria and have been identified as broad spectrum drug targets (Gerdes et al., 2002). With respect to the adenylating enzyme humans require their own versions (nmnat1, nmnat2, nmnat3) in both recycling and de novo pathways. Surprisingly, the sequence similarity of the human and bacterial enzymes is quite low, suggesting that small molecule inhibitors could be developed that are specific toward the bacterial forms

(Gerdes et al., 2002). Finally, NAD^+ synthetase activity is not required to recycle nicotinamide in humans and its central role in recycling in microbes suggests it may be an excellent target for antimicrobials. NAD^+ synthetase inhibitors have proven antibiotic properties, killing gram positive bacteria (Velu et al., 2003).

Anticancer agents

NAD^+ metabolism plays a vital role in maintaining the genome, via PARPs and sirtuins, and proliferating cells appear to have higher demands for NAD^+ biosynthesis and greater turnover of NAD^+. The role of PARP as a protector of genomic stability has stimulated investigation of its inhibition as a way to make cancer cells more susceptible to genotoxicity (Virag and Szabo, 2002). Alternatively, compounds directed specifically to inhibition of human NAD metabolism have recently been developed. Specifically an inhibitor of nampt/PBEF (FK-866) has recently been shown to have potent anticancer activity in cell culture and causes acute sensitivity to alkylating agent and increased apoptosis (Pogrebniak et al., 2006). It is currently in early clinical trials as an anticancer therapy.

REGULATION OF NAD IN A MODEL MICROBE: YEAST

Bakers yeast has been a useful model organism for studying the link between NAD^+ metabolism and the biological effects of NAD^+ dependent sir2 (a yeast sirtuin). In yeast, the NAD^+ recycling pathway is subject to regulation and changes in this pathway have profound consequences for lifespan as well as gene silencing. The gene *PNC1* when overexpressed increases gene silencing at the genetic loci *HM*, *TEL*, and *rDNA* and increases replicative lifespan (Anderson et al., 2003; Gallo et al., 2004). Conversely, *pncΔ* strains exhibit defective gene silencing and decreased replicative lifespan

(Anderson et al., 2003; Gallo et al., 2004). *PNC1* expression levels are subject to transcriptional regulation in response to stress, and *PNC1* regulates nicotinamide concentrations (Anderson et al., 2003; Gallo et al., 2004; Sauve et al., 2005). Nicotinamide is a potent sirtuin inhibitor, implying that sirtuin catalytic activity is regulated by nicotinamide concentrations in yeast, which are in turn, controlled by *PNC1* expression levels (Anderson et al., 2003). Stimuli that increase *PNC1* expression also extend lifespan (Anderson et al., 2003; Gallo et al., 2004), an effect that can be reproduced genetically by overexpression of *SIR2*. Thus, changes in NAD^+ metabolism represent a mechanism for regulating heterochromatin formation and lifespan mediated by sirtuins.

REGULATION OF NAD^+ METABOLISM IN HUMANS

NAD^+ consuming reactions are tightly regulated in mammalian cells and NAD^+ depletion can occur rapidly in cells exposed to genotoxic stress. Genotoxins damage DNA and cause DNA strand breaks. These DNA breaks are sensed by a DNA repair system, which includes PARPs and a sirtuin, SIRT6. The activation of PARP, in particular, causes a rapid synthesis of poly-ADP ribose at the site of the strand break, and when this system is overactivated, it can significantly deplete cellular NAD^+. On the other hand, NAD^+ forming reactions are apparently subject to regulation as well. Cellular NAD^+ concentrations are linked to organism nutritional status and physiologic state (Guarente, 2006). For example, NAD^+ concentrations in liver increase 30% with fasting (Guarente, 2006). Thus, NAD^+ metabolism is dynamically regulated by organism nutrient intake and genotoxic stress. Changes in NAD^+ metabolism are now thought to initiate signaling events coupled to sirtuins or other NAD^+ consuming enzymes, such as PARPs, via

concentration changes of NAD^+ and its metabolites, such as NADH and nicotinamide (Guarente, 2006).

The mechanisms that regulate NAD^+ biosynthesis in mammalian cells have recently come under increased investigation. Since the human genome does not encode a nicotinamidase, the regulation of NAD^+ metabolism must be different from that of yeast. Interestingly the nicotinamide recycling enzyme, nampt/PBEF, is a likely regulator for both nicotinamide and NAD^+ levels in cells. This enzyme is transcriptionally regulated in various conditions and studies show that expression levels of nampt/PBEF are correlated to NAD^+ concentrations in cultured cells (Revollo et al., 2007). The generality of nampt/PBEF as a determinant for NAD^+ concentrations in tissues of the body, and its role in activating signaling via sirtuins and other ADP-ribosyltransferases is still poorly determined to date. nampt/PBEF does upregulate SIRT1 catalytic function in cultured cells (Revollo et al., 2007). It remains to be determined if nampt/PBEF regulates NAD^+ concentrations in liver where increased NAD^+ concentrations associated with fasting stimulate SIRT1 and PGC1-α mediated gluconeogenesis (Guarente, 2006). In general the mechanisms that alter human NAD^+ metabolism are likely to include multiple processes, but the understandings of these mechanisms are currently very unclear, and a considerable effort in this area is required before we know how NAD^+ metabolism is controlled, how changes in NAD^+ metabolism influence physiology and how NAD^+ metabolism might be manipulated for therapeutic benefit.

PHARMACOLOGY OF NAD^+ INCREASING AGENTS

Nicotinamide Nicotinamide is a therapeutic agent that has been evaluated in several clinical studies. It is rapidly ingested and circulated into blood, and is rapidly cleared to

all tissues. It has a high hepatic extraction as well. Recommended intake is 0.3 mg kg^{-1} (20 mg for an adult), but recent clinical studies have examined ranges of 25 –50 mg kg^{-1} per day (1.5 –3 g day)(Knip et al., 2000). Nicotinamide at high doses has been reported to be protective of beta cell functions prior to the onset of type I diabetes. However, a large clinical study in Europe failed to show decreases in incidence of onset of type I diabetes with long term nicotinamide dosing (Gale et al., 2004).

High doses of nicotinamide administered orally or through injection are transiently metabolized in liver to increase NAD^+. However, nicotinamide at elevated doses can cause hepatotoxicity. Nicotinamide is methylated to form 1-methylnicotinamide and downstream oxidized pyridones as metabolic end products (Knip et al., 2000). Large doses of nicotinamide cause methyl donor depletion (Knip et al., 2000). A large portion of nicotinamide administered to rats at 500 mg/kg is excreted unchanged within 12 hours after injection. The remainder of nicotinamide is generally excreted as methylated or oxidized forms of the dinucleotide (Knip et al., 2000). At non-pharmacological doses, nicotinamide is lost mostly be excretion of the catabolic products, rather than as the free intact vitamin.

Nicotinic acid Nicotinic acid is widely used in high doses to lower serum cholesterol, and it also lowers serum triglyceride levels (Capuzzi et al., 2000; Kamanna and Kashyap, 2000). This effect is unique to nicotinic acid, and is not observed with high-dose nicotinamide. The doses required typically cause uncomfortable flushing in immediate release formulations (Capuzzi et al., 2000; Kamanna and Kashyap, 2000). Slow release formulations of nicotinic acid have been developed which provide less discomfort from

flushing, but retain the desired lipid-lowering effects (Capuzzi et al., 2000). Nicotinic acid is rapidly metabolized by the liver and can be catabolized by glycine conjugation to nicotinuric acid (Capuzzi et al., 2000). Nicotinic acid increases NAD^+ content in liver but is generally no more effective than nicotinamide in this respect (Jackson et al., 1995) indicating that NAD^+ biosynthesis in liver is not a likely explanation for nicotinic acid correction effects in hyperlipidemia.

The principle effects of nicotinic acid in lowering cholesterol have been proposed to stem from 4 basic causes, 1) inhibition of lipolysis in fat 2) increased HDL levels, 3) lowering of lipoprotein(a) and 4) inhibition of synthesis and secretion of VLDL in liver (Capuzzi et al., 2000). Some of nicotinic acids effects could be from a described interaction with the G protein HM74a (Capuzzi et al., 2000; Soudijn et al., 2007). This affinity was recently shown to be quite potent, (100-200 nM) and nicotinic acid binding antagonizes forskolin mediated increase of cAMP production and inhibits lipolysis in differentiated 3T3L adipocytes (Capuzzi et al., 2000). The decrease in adipose lipolysis is hypothesized to limit liver uptake of FFAs, which reduces synthesis of VLDL, IDL and LDLs (Capuzzi et al., 2000). Nicotinic acid interferes with HDL-ApoA1 mediated uptake by hepatocytes, without interfering with uptake of cholesterol esters (Capuzzi et al., 2000). This inhibition of removal of HDL-ApoA1 has been proposed to increase cholesterol efflux from peripheral tissues (increased reverse cholesterol transport), mediated by an increased amount of HDL particles (Capuzzi et al., 2000). The relative importance of these mechanisms in explaining the beneficial effects of nicotinic acid, as well as the exact molecular mechanisms that explain these effects are still under investigation. Nevertheless, it is known that nicotinic acid dose response profiles are

different for different serum lipotypes, suggesting different pharmacological mechanisms for the effects seen. Clinically, high dose nicotinic acid leads to reduced lipidemias, reduced progression of coronary heart disease and reduced mortality (Capuzzi et al., 2000; Kamanna and Kashyap, 2000).

EFFECTS NA AND NAM ON NAD^+ IN TISSUES

Nicotinamide and nicotinic acid obtained at low doses are readily absorbed and retained by the body, whereas at high doses they are transiently absorbed, and rapidly eliminated from the body, albeit with transient increases in NAD^+ levels in tissues such as the liver. Two week treatment of rats with high doses of nicotinic acid and nicotinamide (500 mg kg^{-1} and 1000 mg kg^{-1}) has been evaluated on NAD^+ levels in various tissues (Jackson et al., 1995). Both blood (packed red blood cells) and liver were responsive to increased dosages of nicotinamide or nicotinic acid, leading to increases of 40-60 % in NAD^+ content for both tissues for either B3. Smaller increases in NAD^+ concentrations not exceeding 15% were observed for 1000 mg kg^{-1} doses of nicotinamide in heart, lung and kidneys. These findings on the one hand, appear to confirm that nampt/PBEF activity, which is responsible for recycling nicotinamide to NAD^+, is typically not rate limited by nicotinamide concentrations in some, but not all, tissues. In cell culture, nampt/PBEF controls NAD^+ concentrations independent of exogenous nicotinamide concentrations (Revollo et al., 2004). nampt/PBEF has a very low K_m for nicotinamide, <2μM, suggesting it is readily saturated by endogenous nicotinamide concentrations (Revollo et al., 2004). On the other hand, the ability of nicotinamide to stimulate NAD^+ synthesis in liver and blood suggests that nicotinamide is convertible to alternative forms of B3 that ultimately increase nicotinamide bioavailability, and/or that nicotinamide treatment

causes cellular adaptations that lead to improved NAD^+ biosynthesis. Why nicotinamide is efficiently utilized in some but not all tissues for NAD^+ biosynthesis is currently unexplained.

Jackson et al also showed that nicotinic acid increased NAD^+ concentrations in liver and blood similar to nicotinamide. In addition, NAD^+ biosynthesis was increased in heart (50%) and kidney (100%) as well. These results show that nicotinic acid generally has a broader effect than nicotinamide for NAD^+ increases in the body. These results also indicate that the Preiss-Handler pathway is typically operating below saturation in most tissues.

GENOME STABILITY

A considerable body of evidence implicates NAD^+ metabolism as important for the maintenance of genome stability (Kirkland, 2003). Of particular importance in this respect is the involvement of PARP-1 as a DNA damage sensor, which co-operates in the DNA damage and repair process. The importance of NAD^+ and PARP is highlighted by studies that show that vitamin B3 deficiency is associated with reduced tissue NAD^+ concentrations, and a reduced ability of tissues to maintain poly-ADPR concentrations at normal levels (Boyonoski et al., 2002a). In the absence of toxins, B3 deficient bone marrow showed a 6.2 fold increase in micronucleus formation, and a 2.8 fold increase in sister chromatid exchange (Boyonoski et al., 2002a) . With DNA damaging agents, animals show reduced ability to synthesize poly-ADPR in bone marrow, and were more susceptible to formation of DNA strand breaks, as measured by comet assay (Boyonoski et al., 2002a). B3 deficiency also results in reduced latency to leukemia in animals treated with ethylnitrosourea, which is used as a model for secondary carcinogenesis

arising from chemotherapies (Henning et al., 1997). Conversely, pharmacological doses of nicotinamide or nicotinic acid supplementation increase NAD^+ in bone marrow, and also increase poly-ADPR levels (Boyonoski et al., 2002b). This latter result provides evidence against the idea that PARP-1 is efficiently inhibited by nicotinamide concentrations at high doses, as is widely assumed, since observed poly-ADPR levels in marrow, and even liver, were increased by nicotinamide and nicotinic acid similarly compared to controls. B3 treatments were able to retard ethylnitrosourea induced carcinogenesis, and led to increased lifespan for versus animals on a normal diet (Boyonoski et al., 2002b).

In vitro results indicate that PARP-1 inhibition leads to delayed DNA repair, particularly base excision repair (Hassa et al., 2006). Consistent with a role for PARP in DNA repair, PARP -/- animals exhibit hypersensitivity to alkylating agents and ionizing radiation (Hassa et al., 2006). Some data appears to indicate that a normal if not an augmented NAD^+ level in tissues aids DNA repair and may reduce carcinogenesis. Some hints that this may be true are found in epidemiological studies that show that PARP-1 activity levels are lower in families predisposed to cancer (Decker and Muller, 2002), and some cancers are found to have reduced PARP activities (Decker and Muller, 2002). Another finding of interest is that PARP activity may be generally higher in long-lived people, suggesting that PARP activity levels may have an anti-aging effect (Decker and Muller, 2002).

Interestingly, there is growing evidence that the body naturally adapts to genotoxic, hypoxic and caloric restriction stress by increasing NAD^+ biosynthesis. These evidences suggest that physiological responses to stress may be partly cued by increased

NAD^+ levels. Consistent with this view, sirtuin signaling has been shown to respond to increased physiological NAD^+ concentrations (Guarente, 2006). Although increased Vitamin B3 intake may seem beneficial, higher dosages of nicotinamide or nicotinic acid have undesirable side effects. In addition to hepatotoxicity, nicotinamide at high doses can adversely affect thymine biosynthesis and cause an increase of DNA damage caused by depleted thymidine levels in the cell (ApSimon et al., 1995).

ISCHEMIA AND STROKE

NAD^+ metabolism is centrally involved in damage that accompanies stroke. Stroke injury is caused by an acute blockage of arterial bloodflow to the brain, which causes starvation of affected tissues for oxygen. Upon removal of the blockage, the tissue that was deprived becomes reperfused with oxygen. This oxygenation of the tissue has serious negative consequences and causes production of oxygen reactive species, such as superoxide anion, peroxide and hydroxyl radicals. In addition, NO produced in the brain is converted to peroxynitrite, which has potent oxidizing power. The burst of oxidative stress upon reperfusion leads to extensive tissue damage. The mechanism of loss of tissue is not strictly oxidative in nature per se, but rather linked to oxidation of important cellular components such as the genetic material, DNA. The damage to DNA sets off the PARP-1 activation cascade, which if highly upregulated, can deplete most of cellular NAD^+, because of hyper-polymerization of APDR in the nucleus (Hassa et al., 2006). In turn, NAD^+ must be resynthesized using ATP, PRPP, and other high energy precursors. It is believed that the demands of resynthesizing NAD^+ in large quantities places serious strains on energy resources in the cell, causing the cell to die from energy depletion (Hassa et al., 2006). Evidence that this model of cell death is considerably accurate has

come from many sources. Among the data include observations that PARP -/- mice experience significantly reduced tissue damage in cerebral ischemia, with corresponding protection of NAD^+ metabolism (Hassa et al., 2006). PARP inhibitors appear to have similar effects (Virag and Szabo, 2002). Nicotinamide, which is a μM inhibitor of PARP is also protective. There remain questions about the mechanisms of action of nicotinamide in this respect, since some studies show that nicotinamide may not inhibit PARP activities, as determined by ADP-ribosyl-polymer measurements (Boyonoski et al., 2002b). Nicotinamide effects may also play a role in enhancing NAD^+ synthesis. Nevertheless the effects of nicotinamide are distinctly more beneficial than those of nicotinic acid in ischemia models, suggestive of its effect as a PARP-1 inhibitor (Virag and Szabo, 2002).

NICOTINAMIDE IN FETAL ISCHEMIA AND FETAL ALCOHOL SYNDROME

The fetal brain is particularly sensitive to genotoxicity, alcohol and oxidative stress. The fetal brain must undergo pattern forming connections to other neurons, forming synapses that lead to proper information processing. It is in the early period of development and synapse formation that apoptosis becomes a susceptibility of the immature neural cells, particularly those damaged during development (Ieraci and Herrera, 2006). From a medical perspective this issue is an important one, since alcohol abuse is considered the leading cause of mental retardation in children (Ieraci and Herrera, 2006). Few interventions are known that mitigate this damage. Recent studies have looked at nicotinamide as a potential intervention, as a means to protect the fetal brain cells during this developmental stage. In fetal mice whose mothers were treated with alcohol, a single nicotinamide treatment of the mother provided protection against oxidative stress markers

in the fetal brain such as lipid peroxidation, and also prevented apoptosis (Ieraci and Herrera, 2006). When assessed for behavior, offspring whose mothers were administered nicotinamide performed better in a number of tests for anxiety, a typical side effect of fetal alcohol syndrome in mice, than their nicotinamide untreated controls (Ieraci and Herrera, 2006). In fetal ischemia, similarly, nicotinamide treatment has been shown to prevent neural damage versus untreated controls, suggesting that nicotinamide could represent a reasonable intervention for early neuron damage during development (Feng et al., 2006).

ALZHIEMERS and NEURODEGENERATIVE DISORDERS

Increased interest in the involvement of NAD^+ metabolism in neurodegenerative processes has hinged partly on observations that preservation of NAD^+ levels protects neurons subject to either genotoxicity or trauma (Araki et al., 2004). Of recent note, Milbrandt showed that the process of axon degeneration, which occurs when an axon is severed, can be significantly slowed when NAD^+ or other NAD^+ precursors are present (Araki et al., 2004). Subsequent work by other laboratories has verified that NAD^+ metabolism can protect severed neurons from degeneration. Interestingly, a mouse with slowed axon degeneration has a triplicate chimeric gene consisting of a partial ubiquitin ligase gene fused to a full nmnat-1 gene (Mack et al., 2001). nmnat-1 is nuclear localized, and couples NMN or NaMN with ATP to form NAD^+ or NaAD respectively (Figure 5). Some controversy has emerged as to the significance of the nmnat-1 biochemical activity in slowing axon degeneration, since nmnat-1 overexpression has not been shown to increase intracellular NAD^+ concentrations (Mack et al., 2001), and overexpression of nmnat-1 does not reproduce the slow degeneration phenotype in a transgenic mouse that

overexpressed this enzyme (Conforti et al., 2007). Nevertheless, NAD^+ appears to be protective to neural cells, and it has been reported that NAD^+, NR, NMN, nicotinamide and NA all protect neurons under different conditions in cell culture experiments.

Chronic disease states like Parkinsons and Alzheimers are still somewhat poorly understood. Nevertheless, recent evidence is starting to suggest that chronic neurodegenerative disorders affect NAD^+ metabolism adversely, and may respond favorably to interventions that target NAD^+ metabolism. For example, it has been known that Parkinsons results in increased methyl-nicotinamide excretion, suggesting enhanced NAD^+ breakdown. Recently, the author participated in a study in which NAD metabolism was examined in transgenic mice that have a gene encoding a human amyloid precursor protein (APP). These animals develop some neuropathology of Alzhiemers, such as plaque formation. Upon assay of brain tissue, NAD^+ levels were decreased and nicotinamide levels were increased in animals affected severely by disease who were on normal diets as compared with animals on calorie restriction diets where the neuropathology was less severe (Qin et al., 2006). NAD^+ itself was implicated in mitigating disease, and exogenous NAD^+ redirected how cells process amyloid precursor protein (Qin et al., 2006). It was shown that NAD^+ treated cells produced less plaque-associated forms of processed APP (Aβ), through a mechanism involving upregulation of alpha secretase, which cleaves APP competitively with beta and gamma secretases preventing Aβ formation (Qin et al., 2006). Increased sirtuin (SIRT1) catalytic activity was also implicated in mediating the enhanced protection from neuropathology in cell culture and in mouse brains (Qin et al., 2006). SIRT1 is transcriptionally upregulated in neurons by calorie restriction, and is activated directly by NAD^+. Although the work in

the area of Vitamin B3 effects in neurodegenerative disorders is still very preliminary, it invites the question of how NAD^+ metabolism affects long-term neurodegenerative processes, and whether enhancements/modulations to NAD^+ metabolism can provide therapeutically meaningful changes in long-term outcomes in these notoriously difficult to treat diseases.

CONCLUSIONS

The emergence of knowledge recognizing the potent role of ADPRtransferases as regulators of lifespan of diverse organisms, and their coupling to NAD^+ metabolism has stimulated a current interest in the possibilities inherent to targeting NAD^+ metabolism for therapeutic purposes. This review suggests that opportunities exist for the development of anti-microbials and anti-cancer drugs that inhibit the basic transformations of NAD^+ metabolism. In addition, expanded use of agents like nicotinamide and nicotinic acid in light of their beneficial characteristics in enhancing NAD^+ levels in tissues deserves consideration. Agents like nicotinamide riboside, which can also enhance NAD^+ concentrations, have barely been investigated for this purpose. In the broad area of neuropathology (stroke, neurodegenerative disorders, fetal brain damage) a surge of new data has pointed to enhancement of NAD^+ metabolism and attenuation of NAD^+ depletion as having potentially protective effects. For new therapies to emerge continued progress will be needed to understand the complex regulatory mechanisms that govern NAD^+ metabolism in cells and tissues and how changes in NAD^+ metabolism affect tissue and organism physiology in health and disease.

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Legends for Figures

Figure 1 Depiction of nicotinic acid and nicotinamide chemical structures and the structure of nicotinamide adenine dinucleotide.

Figure 2 (Top) A schematic representation of de novo NAD^+ biosynthesis in bacteria starting from aspartate. Nicotinate recycling intersects the de novo pathway at NaMN. (Bottom) A schematic representation of recycling of nicotinamide and NMN as observed for microbes. The reaction to generate NMN is catalyzed by bacterial NAD^+ dependent DNA ligases. The salvage of nicotinamide is achieved by obligate nicotinamidase catalyzed hydrolysis to form nicotinate. Nicotinamidase in yeast (*PNC1*) is subject to stress-regulated transcription. Nicotinamidase levels appear to regulate nicotinamide levels in the yeast, causing increased sirtuin activity, as explained in the text.

Figure 3 (Top) A schematic representation of de novo NAD^+ biosynthesis in organisms that use tryptophan as a source for NAD^+. Like the asparatate-based pathway, formation of quinolinate is the crucial step that leads to the nicotinate ring system, via formation of NaMN. Nicotinate recycling is shown to intersect the NAD^+ biosynthetic pathway at NaMN. (Bottom) A schematic representation of paths of metabolism for human nicotinamide, nicotinic acid and nicotinamide riboside that lead to formation of NAD^+. Nicotinamide is converted directly within cells by nicotinamide phosphoribosyltransferase (nampt). This enzyme was first named pre-B cell colony enhancing factor (PBEF). Evidence suggests that this enzyme is regulated by cell stress

and can dramatically alter intracellular NAD^+ concentrations. Humans do not appear to have a nicotinamidase enzyme, which means that nicotinic acid and nicotinamide are incorporated into NAD^+ via non-overlapping pathways. As explained in the text the enzyme nicotinamide/nicotinate mononucleotide adenylyltransferase (nmnat1, nmnat2, nmnat3) serves in both pathways by virtue of its ability to accept either NMN or NaMN as a substrate.

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Table 1. NAD related metabolites and their relationship to disease states in humans

Metabolite	Nutritional or Pharmacologic Condition	Disease	Associated Effects	References
Nicotinamide	High Dose (25-50 mg kg^{-1})	Diabetes	Protection of Islets	Gale et al., 2004 Knip et al., 2000
Nicotinamide	High Dose	Cardiac Disease and Stroke	Protection of Tissues	Virag and Szabo, 2002
Nicotinamide	High Dose	Fetal Ischemia Fetal Alcohol Syndrome	Neuroprotection	Ieraci and Herrera, 2006 Feng et al., 2007
Nicotinamide riboside	No proven use	Bacterial Infection (*H influenzae*)	Precursor for Bacterial NAD^+	Belenky et al., 2007
Nicotinic acid	High Dose (Fast release or slow release 1-20 g day^{-1})	Hypercholesterolemia	Reduced lipids (LDLs, fatty acids and cholesterol) Increased HDL Reduced mortality Reduced cardiac incidents	Capuzzi et al., 2000 Carlson, 2004 Kamanna and Kashyap, 2000
Tryptophan	Nutritional supplement	Niacin deficiency	Restoration of NAD^+ levels in tissues	Fukuwatari et al., 2004
NAD^+	No proven use	Stroke Cardiac Ischemia	Depletion of NAD^+ Cell and Tissue Death	Hassa et al., 2006 Virag and Szabo, 2002
Vitamin B3 (all forms and tryptophan)	Nutritional deficiency	Pellagra or B3 deficiency	Low NAD^+ levels Dermititis Dementia Diarrhea	Belenky et al., 2007; Revollo et al., 2007
Vitamin B3 (all forms and tryptophan)	Nutritional deficiency	Cancer (in B3 deficiency)	UV sensitivity DNA damage sensitivity Mutations Carcinogenesis	Kirkland, 2003

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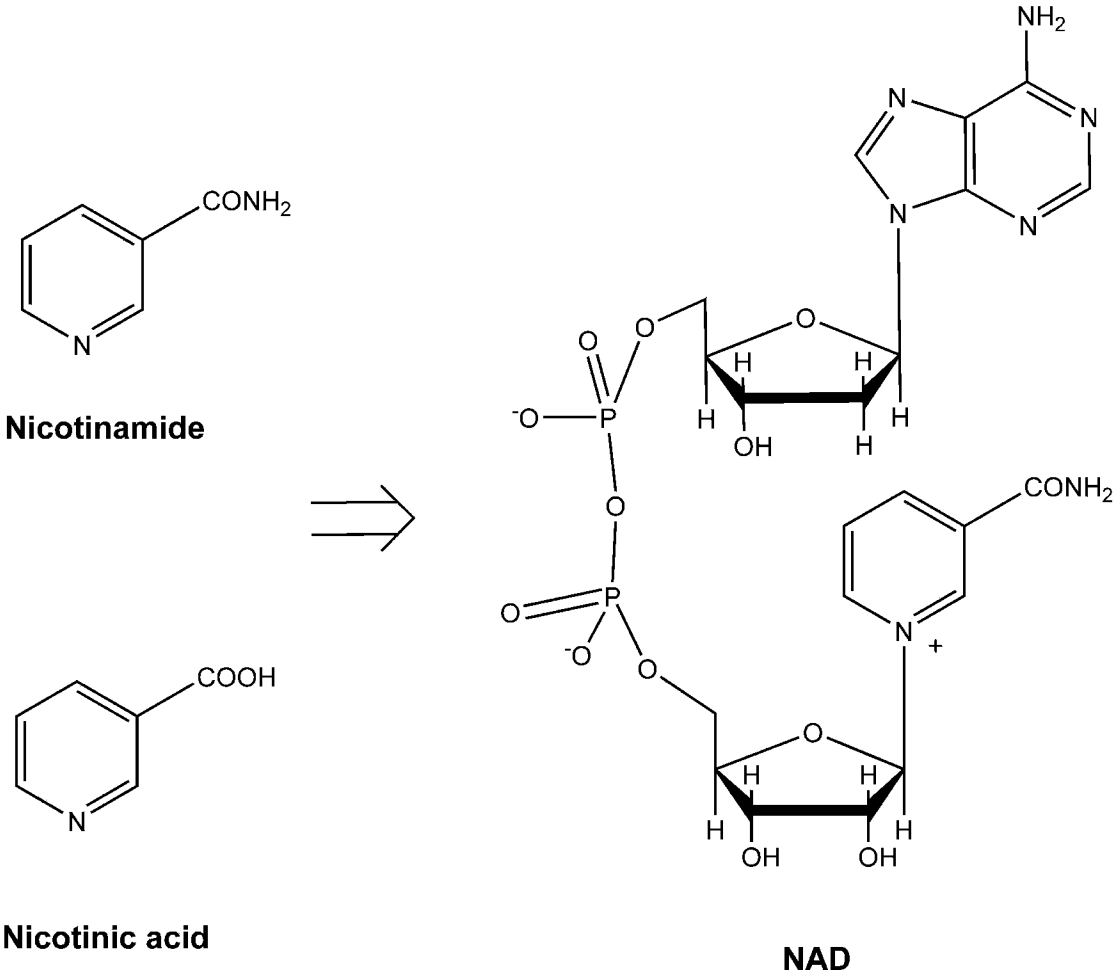

Figure 1

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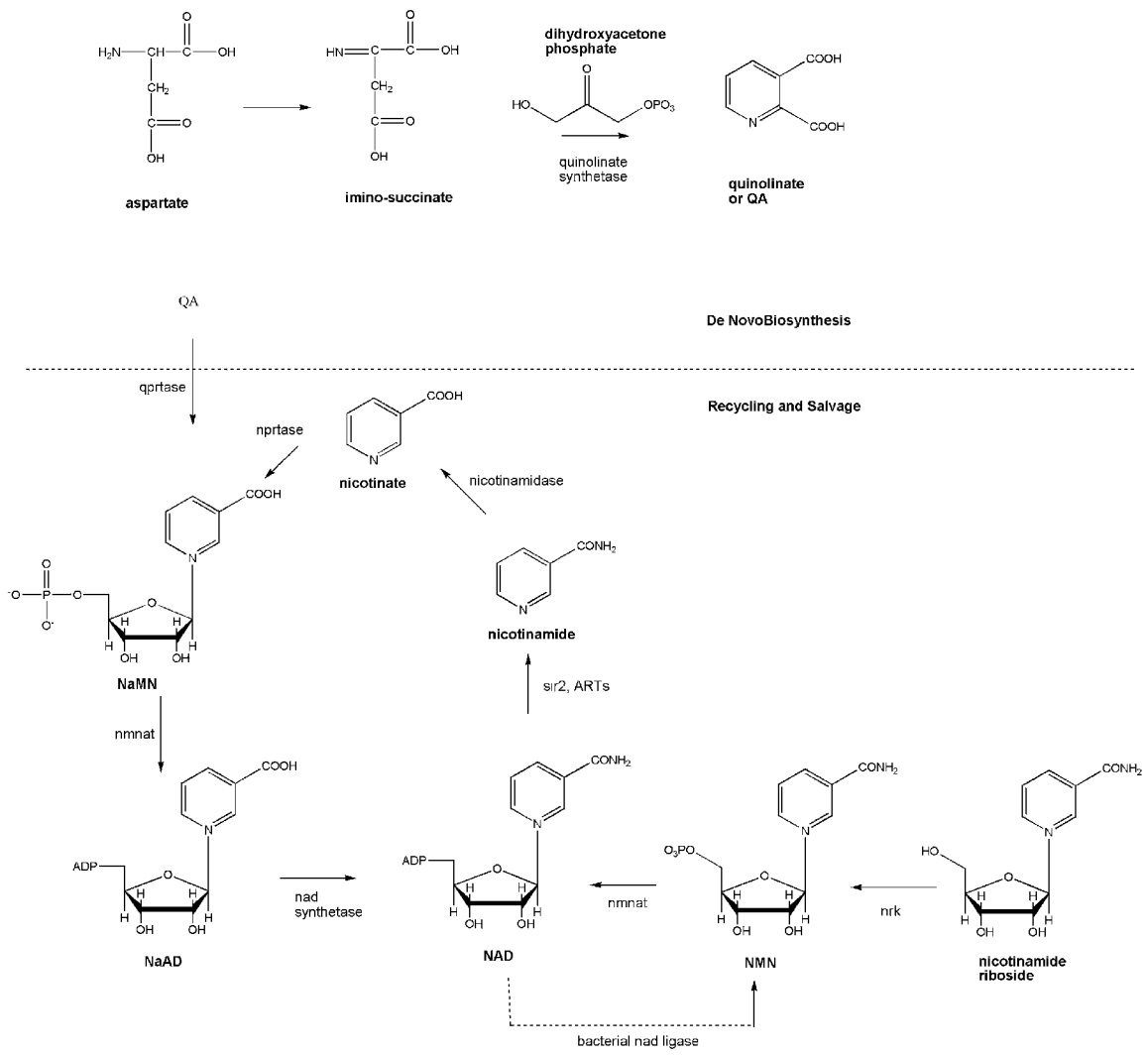

Figure 2

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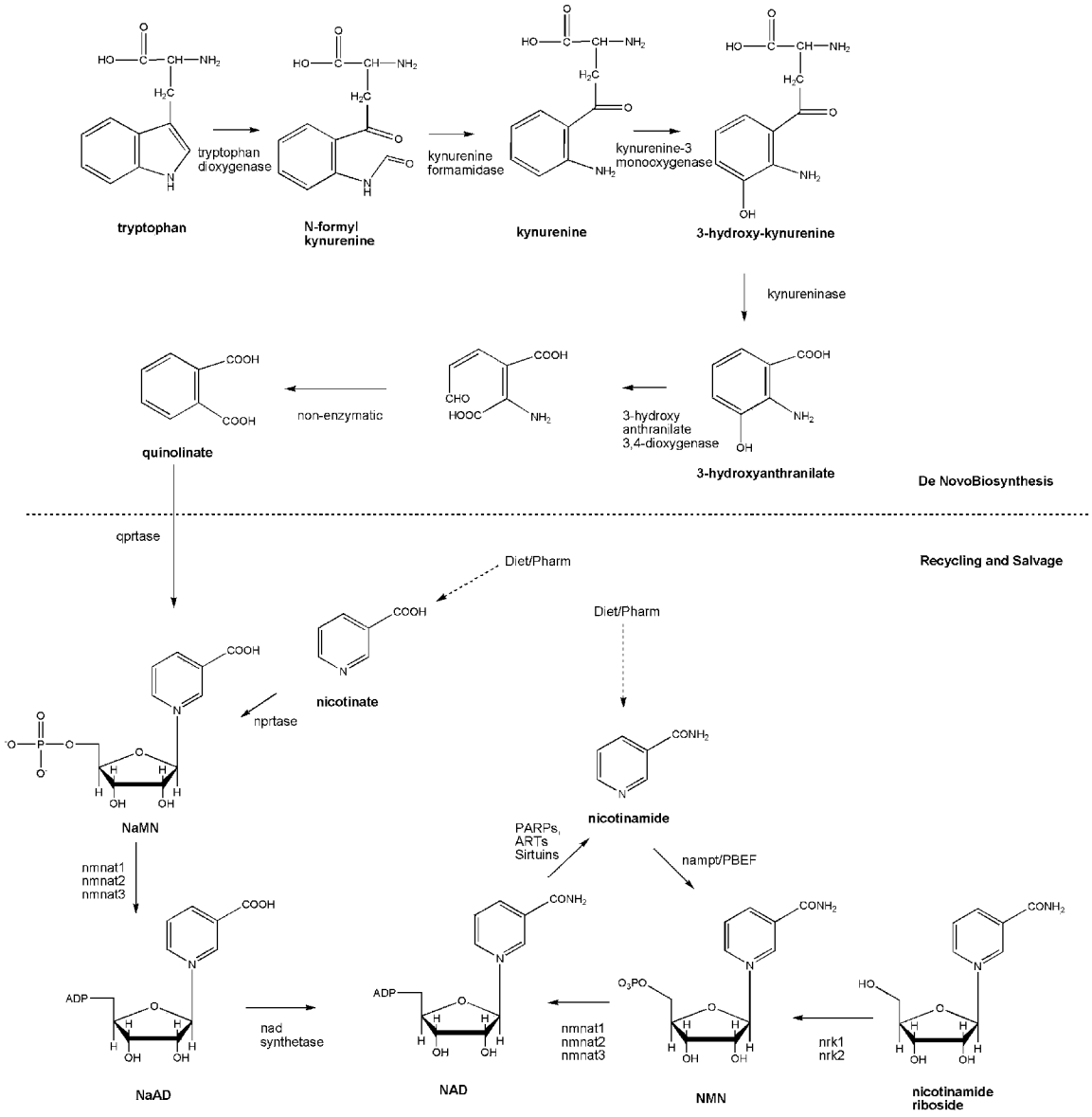

Figure 3