

Mapping the role of NAD metabolism in prevention and treatment of carcinogenesis

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Abstract

Studies presented here show that cellular NAD, which we hypothesize to be the relevant biomarker of niacin status, is significantly lower in humans than in the commonly studied animal models of carcinogenesis. We show that nicotinamide and the resulting cellular NAD concentration modulate expression of the tumor suppressor protein, p53, in human breast, skin, and lung cells. Studies to determine the optimal NAD concentrations for responding to DNA damage in breast epithelial cells reveal that DNA damage appears to stimulate NAD biosynthesis and that recovery from DNA damage occurs several hours earlier in the presence of higher NAD or in cells undergoing active NAD biosynthesis. Finally, analyses of normal human skin tissue from individuals diagnosed with actinic keratoses or squamous cell carcinomas show that NAD content of the skin is inversely correlated with the malignant phenotype. Since NAD is important in modulating ADP-ribose polymer metabolism, cyclic ADP-ribose synthesis, and stress response proteins, such as p53, following DNA damage, understanding how NAD metabolism is regulated in the human has important implications in developing both prevention and treatment strategies in carcinogenesis. (*Mol Cell Biochem* **193**: 69–74, 1999)

Key words: skin, lung and breast cancer, p53 expression, cyclic ADP-ribose, poly(ADP-ribose) metabolism, niacin status

Introduction

Considerable evidence now indicates that the NAD content of cells influences cellular responses to genomic damage by multiple mechanisms. NAD is directly consumed for the synthesis of ADP-ribose polymers and cyclic ADP-ribose. The metabolism of ADP-ribose polymers appears to be involved with responses that can lead to normal cellular recovery, apoptosis, or necrosis [1, 2]. Cyclic ADP-ribose is a potent calcium releasing agent that also may mediate signalling pathways leading to apoptosis or necrosis [3, 4]. Finally, recent studies have shown that the NAD content of cells modulates the expression of stress proteins that play important roles in responses to genomic damage [5], including the tumor suppressor protein, p53 [6]. Consequently, NAD metabolism is a target for both prevention and treatment of cancer. The scheme shown in Fig. 1 outlines relationships in NAD metabolism that remain poorly defined. While it is known that dietary nicotinamide and nicotinic acid serve as precursors of NAD in many human tissues,

much less is known about the conversion of tryptophan to NAD. The liver and perhaps the kidney are capable of the latter pathway. Since 60 mg of tryptophan consumed in protein is often assumed to be converted to 1 mg of niacin, tryptophan accounts for nearly one half of calculated niacin consumption in Western diets [7]. However, tryptophan does not appear to be a source of tissue NAD in humans under conditions of restricted niacin intake over a period of a few weeks [8]. Thus, it is likely that niacin intake may be significantly less than reported [7].

Measuring the relationship of dietary intake of NAD precursors and the circulating levels of various precursors that supply the tissues has been technically difficult. Recently, our laboratory has developed methods that allow measurement of nicotinic acid and nicotinamide in fasted serum samples [9]. Preliminary studies that control niacin in animal models suggest that a given concentration of precursor can produce vastly different effects on NAD in the various tissues [10–13]. The optimal intracellular NAD content for eliciting protective biochemical responses following DNA damage

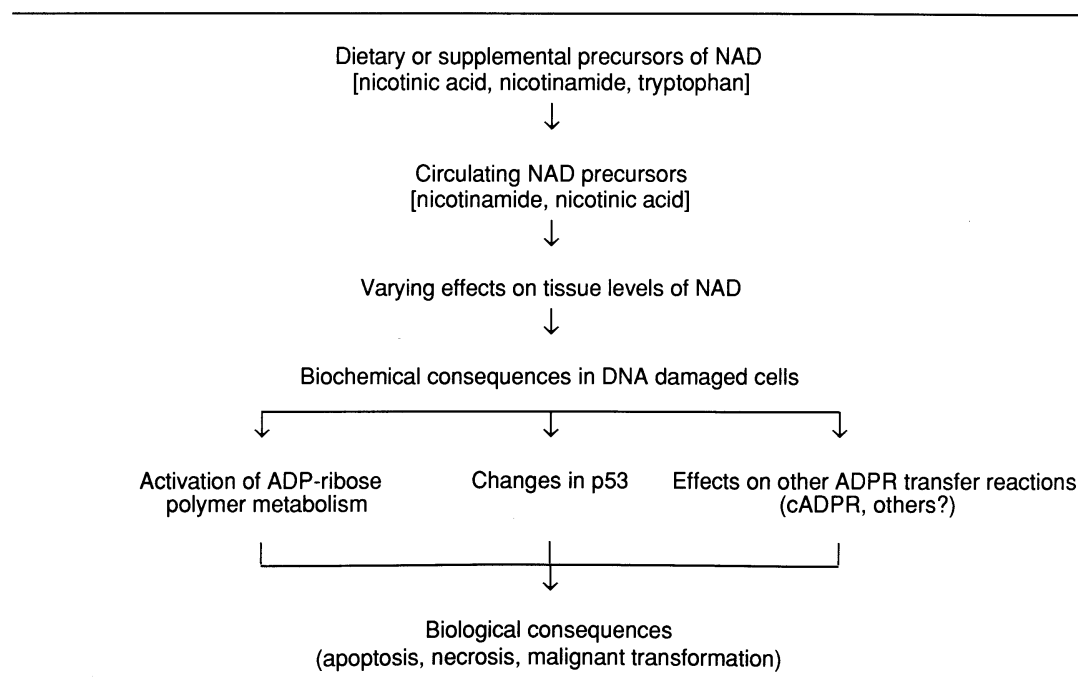


Fig. 1. Mapping the role of NAD metabolism in cancer prevention and treatment.

has not been defined. Given the relationship between NAD content and biochemical pathways involved in carcinogenesis, we are attempting to determine the interrelationships shown in Fig. 1 to understand how niacin metabolism and tissue NAD impact the prevention and treatment of cancer.

Results and discussion

Species and tissue differences in requirements for NAD precursors

The first step in characterizing the relationships of NAD metabolism shown in Fig. 1 for humans is the establishment of reliable methods for assessing niacin status. Biochemical assessment of niacin status has traditionally involved measurement of urinary oxidation products of nicotinamide. However, discovery of reactions that use NAD as a substrate in ADP-ribose transfer reactions and the relationship between NAD content and expression of stress response proteins such as p53, have led us to hypothesize that intracellular NAD may be a more relevant measure of niacin status. A biochemical measure of niacin status where the ratio of NAD to NADP in blood cells reflects dietary intake has been developed from the metabolic ward studies of Fu *et al.* and our observations on the distribution of NAD in whole blood [8, 14, 15]. We refer to this ratio as niacin number.

Since studies of carcinogenesis frequently employ rodents as animal models, we have compared niacin status as measured by blood cell pyridine nucleotides in mice, rats, and humans as a function of niacin intake. The data of Table 1 are compiled from our laboratory and others as indicated. The values are shown as niacin number and as NAD per ml of blood, because many earlier studies have only measured the latter. We have compiled the data in both formats for means of comparison. The NAD content of human blood in an individual consuming the RDA for niacin is less than 50% of that in rats and less than 25% of that in mice consuming proportional dietary niacin. These data indicate that the niacin status of humans is considerably lower than that of rodents. Additionally, analyses from a large Western population within The Malmö Diet and Cancer Study reveal a large variability in the human population. The data in Table 1 represent the values for 95% of a population where n is equal to 1300. A range of NAD content of 3 fold is observed. Approximately 15–20% of individuals in this population have significant niacin deficiency [16]. Because pharmacological doses of niacin are frequently administered as therapy for hypercholesterolemia, we have been able to measure the effect of introducing niacin supplementation on human blood cell NAD. As shown in Table 1, a mean niacin number of 175 in this population increased nearly 4 fold to 663 after 2 months of niacin therapy. It is interesting that this therapy elevates human niacin status to that of mice. However, in contrast to

Table 1. Comparison of blood cell niacin status in humans and rodents

Species	nmol NAD/ml blood	Niacin number (NAD/NADP×100)
Human, controlled intake at RDA ^{a,b,h}	41	175
Human, range in large Western population ^{a,h}		84–236
Human, niacin depletion ^b		62
Human, niacin supplementation ^{c,h}	184	667
Rat	71–75 ^d , 119–122 ^e , 83 ^f	321–357 ^d
Rat, niacin depletion	27 ^d , 71 ^e , 43 ^f	124 ^d
Mouse ^{g,h}	179	702
Mouse, niacin supplementation ^{g,h}	157	683
Mouse, niacin depletion ^d	unchanged	unchanged

^aJacobson, E.L., unpublished observations; ^bFu, *et al.*, J Nutr 119: 1949–1955, 1989 (ref. [8]); ^cJacobson, E.L., Robins, H.I. and The University of Wisconsin Lipid Clinic, unpublished observations; ^dJames Kirkland, unpublished observations; ^eZhang, JZ., *et al.*, J Nutr 123: 1349–1355, 1993 (ref. [11]); ^fShibata, K. and Murata, K, Nutr Int 2: 177–181, 1986 (ref. [13]); ^gJacobson, E.L., *et al.*, Proc Am Assoc Cancer Res 37: 279, 1996 (ref. [10]); ^hNAD and NADP were determined from whole blood as previously described by Jacobson, E.L. and Jacobson, M.K., Meth Enzymol 280: pp 221–230, Academic Press, New York, 1997 (ref. [14]).

humans, mice supplemented with pharmacological doses of niacin maintain the same blood cell NAD content. When all 3 species are placed on diets deficient in niacin, blood cell NAD drops to about 30% of control values in humans and to about 50% of control values in rats. Interestingly, mice are resistant to niacin deficiency [17]. These species differences are likely related to the efficiency with which each converts tryptophan to niacin. Despite the mechanism, the data in Table 1 clearly show that humans maintain a lower niacin status than rodents when each are given tryptophan and preformed niacin at the recommended daily intake for humans. Therefore, humans appear to be at increased risk for niacin deficiency relative to rats or mice. These differences are critically important to interpretation of data derived from model systems in studying the role of niacin in cancer prevention and treatment. Pursuing information regarding dietary intake, circulating precursors, and the resulting NAD in blood cells and other tissues will be essential to optimizing requirements for niacin in humans.

Tissues at risk for NAD depletion include breast, lung and skin

While blood cell NAD can be used as a clinical measure of niacin status, we also have been interested in how different tissues respond to niacin intake. We predict that tissues that undergo cell division and/or are exposed to oxidative stress will be at increased risk for niacin deficiency. Consequently, our initial studies are focusing on human tissues with significant cell turnover and/or exposure to oxidative stress, such as breast, lung, and skin. The data in Table 2 show that human fibroblasts or epithelial cells from each of these tissues become severely NAD depleted if grown for 4 to 5 population doublings in the absence of nicotinamide. This

phenomenon is completely reversible, but requires 6–24 h in excess nicotinamide. Further, nicotinamide depletion has no effect on cell growth rates until NAD content drops to less than 10% of normal. Human breast epithelium undergoes significant cell growth in phase with the menstruation cycle. Skin also undergoes continuous replacement with new cells, and the lung epithelium is believed to turn over, although it is not clear whether this is in response to oxidative stress or is a normal periodic replacement. Thus, growth of these tissues in limiting niacin would be expected to result in depleted NAD pools as is observed in cultured cells from these tissues (Table 2). Studies employing niacin deficiency in growing rats confirm this prediction [11–13].

Modulation of p53 expression by NAD in breast, lung and skin cells

NAD depletion in cultured cells derived from all of the tissues mentioned above is accompanied by decreased expression of the tumor suppressor protein, p53. These data were obtained by Western blot analyses of cell extracts separated by polyacrylamide gel electrophoresis (PAGE), as indicated in the legend to Table 2, and are presented as a ratio of p53 to actin to correct for any quantitative errors in loading of the protein on gels or during transfer to nitrocellulose for blotting. NAD depletion varied between the different cell lines due to varying periods of growth in niacin deficient medium and different growth rates and also due to the fact that in breast cells, nicotinamide was not completely removed from the medium because of a requirement for bovine pituitary extract, which appears to contain nicotinamide. In all cases, p53 expression relative to actin was decreased 36 to 70%, due solely to restriction of nicotinamide in the medium. This represents the first report of nicotinamide modulation of p53 expression in

Table 2. NAD content and p53 expression in nicotinamide depleted human cells

Cell type	Population doublings in limiting nicotinamide	NAD pmol/10 ⁶ cells	p53/actin
IMR-90 human diploid lung fibroblasts	0 4	2164 476	1.73 0.52
CF3 human diploid skin fibroblasts	0 4	1023 384	3.33 1.27
Human primary mammary epithelial cells	0 5	3241 513	0.82 0.53

The normal human lung diploid fibroblast strain IMR-90 (ATCC CCL 186) and the normal human foreskin diploid fibroblast strain CF-3 (gift from Dr. R. Dell'Orco) were cultured in Dulbecco's modified Eagle's medium (Gibco) in the presence of 10% fetal bovine serum (Hyclone) at 37°C in a humidified atmosphere of 5% CO₂. These cell lines were subcultured once per week, employing a 1:4 split, and fresh medium was applied every 4 days. For depletion of nicotinamide, commercially prepared medium free of nicotinamide was used in combination with exhaustively dialyzed fetal calf serum. Human mammary epithelial cells (Clonetics) were grown in a commercially prepared medium (Clonetics) or an optimized formulation of MCDB 170 basal medium containing 14 mM sodium bicarbonate and serum-free supplements (SFS) [20]. For nicotinamide depletion experiments, MCDB 170 lacking nicotinamide was custom prepared in this laboratory from stock solutions according to the procedures of McKeehan [21]. Stock cell cultures were maintained in modified MCDB 170 (Clonetics) with SFS and bovine pituitary extract (BPE), which was purchased from Clonetics. Isoproterenol (Sigma) was added at 1.0 μM in some experiments to maintain HMEC in a continuously dividing state [20]. Dishes of cells to be analyzed were washed with phosphate buffered saline, and NAD, cell number, and protein were determined. NAD was extracted with 0.5 M HClO₄ and neutralized with 1.0 M KOH/0.33 M potassium phosphate, pH 7.3 and quantified as described previously [14]. Protein was quantified from the HClO₄ precipitate by the method of Bradford [19]. Cell number was determined by a particle counter. For Western blot analyses of p53 and actin, cells were detached from the culture dishes using a 0.25% trypsin-EDTA solution (Sigma) for 1 min. The trypsin was then inactivated by adding 3 ml of culture medium containing 10% serum and centrifuged at 500 × g for 5 min at 4°C. After the supernatant was removed, the cell pellet was washed twice with PBS and re-suspended in a lysis buffer, containing 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 0.1% Triton x-100, 0.5% Nonidet P-40, 0.1% EDTA, 1 mM phenylmethylsulfonyl fluoride, and 0.01 mg/ml each of aprotinin and leupeptin, at a ratio of 50 μl per 10⁶ cells. The solution was then sonicated on a Virsonic Cell Disrupter to lyse the cells. Cell lysates were subjected to SDS-12.5% PAGE, followed by transfer of proteins to a nitrocellulose membrane (Schleicher and Schuell). The membrane was then probed with monoclonal antibody Ab-3 (Oncogene Science) against p53 and monoclonal antibody Ab-1 (Oncogene Science) against actin. The secondary antibody was an anti-mouse antibody linked to horseradish peroxidase (Amersham). Final detection was by enhanced chemiluminescence (Amersham) on Hyperfilm-ECL (Amersham). The densities of the resulting bands were determined by an Ambis Model 4000 Optical Imaging System. The ratio of responses (p53/actin) was calculated to control for gel loading differences.

human cells and shows that the phenomenon appears to occur in several different cell types. Such a relationship of nicotinamide, NAD, and p53 expression has been reported previously for cells derived from Chinese hamster ovary cells [6]. In those earlier studies, decreased p53 expression correlated with NAD depletion and also occurred in chemically mutated cells carrying greatly reduced poly(ADP-ribose) polymerase (PARP) activity. Thus, the mechanism by which nicotinamide depletion affects p53 metabolism may involve utilization of NAD by PARP. However, further studies employing the PARP null genotype ongoing in our laboratory should resolve this question. Regardless of the mechanism, the modulation of p53 expression by nicotinamide in these human tissues has important implications, since diminished p53 function is strongly associated with tumorigenesis in breast, lung and skin [18].

NAD utilization and biosynthesis after DNA damage in breast cells

In order to define optimal NAD for responses to DNA damage, we have investigated the effects of various con-

centrations of nicotinamide in the culture medium of primary human mammary epithelial cells (HMEC). Cells were grown for 5 population doublings in medium containing bovine pituitary extract with no added nicotinamide or in the same medium containing 50 or 500 μM nicotinamide. As can be seen in Fig. 2, this resulted in cell populations with 0.75, 2.4, and 3.1 nmol of NAD/10⁶ cells (0 time point), respectively. When cells were grown in the absence of nicotinamide (0.75 nmol NAD/10⁶ cells) and were then replenished with 50 μM nicotinamide 24 h prior to extraction, the NAD increased to 2.27 nmol/10⁶ cells, demonstrating that the depletion is reversible. The four resulting populations of cells (depleted, replenished, 50 and 500 μM nicotinamide cultures) containing a range of NAD were treated with 50 μM N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) for 30 min and analyzed for NAD over the next 24 h. NAD decreased in response to MNNG in all cases except where the cells were grown in the absence of added nicotinamide. In those cells, the small remaining NAD pool appears to be inaccessible to NAD consuming reactions and could represent that sequestered in the mitochondria. A significant increase in NAD occurred between 6 and 9 h after treatment. This represents an apparent stimulation of NAD biosynthesis by

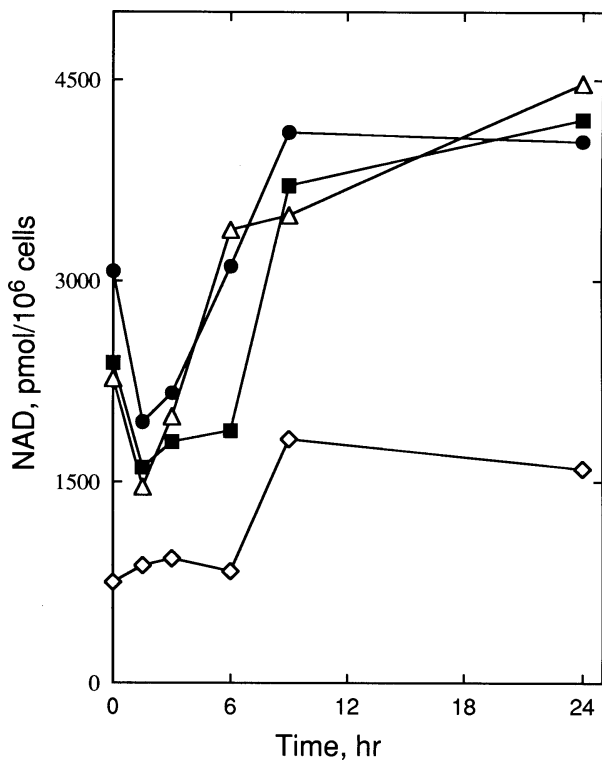


Fig. 2. Effect of various concentrations of nicotinamide in the growth medium on NAD content and recovery following DNA damage. HMEC of passage number 9–10 were seeded into 100 mm dishes (9.3×10^4 cells/dish) in Clonetics medium. Approximately 12 h after plating, the medium was removed and replaced with MCDB 170/SFS (see legend to Table 2) with (50 or 500 μ M) or without nicotinamide. The cells were fed, and cell number, protein, and NAD were measured every 48 h. After 3–4 population doublings (10–12 days), medium containing 50 μ M MNNG in 0.5% DMSO was added to the cells for 30 min at 37°C. Dishes of cells to be analyzed were washed with phosphate buffered saline, and NAD, cell number, and protein were determined. NAD was extracted with 0.5 M HClO₄ and neutralized with 1.0 M KOH/0.33 M potassium phosphate pH 7.3 and quantified as described previously [14]. Protein was quantified from the HClO₄ precipitate by the method of Bradford [19]. Cell number was determined by a particle counter. Closed squares represent cells grown in 50 μ M nicotinamide; closed circles represent cells grown in 500 μ M nicotinamide; open triangles represent cells grown in the absence of nicotinamide until 24 h prior to treatment, at which time 50 μ M nicotinamide was added; and open diamonds represent cells grown in the absence of added nicotinamide.

the DNA damage, since no decrease in NAD occurred in response to MNNG. Cells grown in control medium containing 50 μ M nicotinamide also demonstrated a burst of NAD biosynthesis during this time, while cells grown in 500 μ M nicotinamide or those that had been given nicotinamide after depletion and analyzed 24 h later appeared to initiate NAD biosynthesis earlier, between 3 and 6 h after DNA damage. All cell cultures containing at least 50 μ M nicotinamide at the time of DNA damage showed significantly elevated NAD 24 h later, reaching approximately 4.0–4.4 nmol NAD/10⁶ cells as compared to 2.4 nmol NAD/

10⁶ cells prior to DNA damage. The percent elevation was inversely related to the initial NAD content. Thus, in these four conditions of niacin status, cells actively undergoing NAD biosynthesis and/or those with excess nicotinamide recovered from DNA damage more quickly.

Completion of these studies should identify optimal precursor levels for responding to DNA damage and increase our understanding of the regulation of NAD biosynthesis following DNA damage in breast tissue. Using this data in combination with that obtained from *in vivo* relationships of circulating precursors and breast tissue NAD should allow optimization of dietary niacin intake for prevention of breast cancer. Optimizing niacin status also may be very important during chemotherapy of breast cancer since the most common limitation to aggressive therapy is loss of bone marrow stem cells. It is likely that optimizing the NAD pool will contribute to protection of these cells, while not affecting therapy of breast cancer cells.

Inverse correlation of skin NAD with malignant phenotype

Because we have shown nicotinic acid supplementation to decrease incidence of UV induced skin tumors in mice, concomitantly with elevation of skin NAD [10], we have begun to examine how niacin status in human skin affects carcinogenic processes. We have developed methods sensitive enough to measure NAD in human skin shavings. In collaboration with the Arizona Cancer Center, we have measured NAD in skin samples from subjects diagnosed with actinic keratoses (AK) and squamous cell carcinomas (SCC). Skin from these subjects was obtained from normal, sun protected regions, such as the underarm or buttocks, as well as from the AK or SCC areas. AK commonly occurs in fair skinned individuals overexposed to sunlight and are known to be premalignant lesions. The data in Fig. 3 show the results of these analyses. Normal skin from subjects with AK have significantly higher NAD than the normal skin of SCC subjects (3.7 ± 0.6 vs. 1.7 ± 0.8 pmol/ μ g protein, $p = 0.0006$). These preliminary data correlate a decreased NAD in the skin with the occurrence of the malignant phenotype. This study is now being expanded to a larger population. We have also examined the NAD in AK and SCC tissues and compared them to the normal tissues. Even though AK tissues have a higher average NAD than SCC tissues, the difference is not statistically significant within this sample size. Also, when the NAD of affected tissue is compared to normal tissue of the same individual, no significant difference exists within this population. The association of lower NAD with malignancy in skin supports the hypothesis that niacin maybe an important preventive factor in cancer.

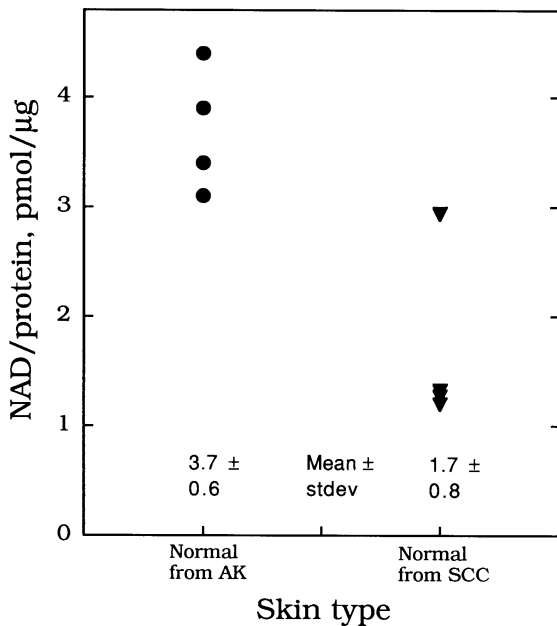


Fig. 3. NAD from human skin shavings. Skin shavings were shipped on dry ice and stored at -80°C . The skin sample was added to a mortar containing 1–2 ml of liquid nitrogen and quickly ground to a powder, which was transferred to a preweighed vial. The vial was weighed again to obtain the sample weight. NAD was extracted using 0.1 ml of ice-cold 1 M NaOH with mechanical agitation. Within 2 min, the alkaline solution was neutralized with 0.027 ml of 2.0 M phosphoric acid, 0.013 ml of 2 mM PES was added to the sample, and the solution was allowed to stand at room temperature in the dark for 5 min to oxidize NADH. The protein was precipitated with 0.047 ml of 2.0 M HClO_4 and allowed to stand on ice for 15 min. The insoluble fraction was collected by centrifugation at 14,000 rpm for 5 min. The supernatant (0.175 ml) was transferred to another vial and neutralized with 55 μl of 2.0 M KOH. Following centrifugation, the supernatant was assayed for NAD [14].

Acknowledgements

This work was supported in part by NIH grants CA 65579 and 43984.

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