THE EFFECT OF NAD^+ AND NAD^+ ANALOGS ON PROTEIN SYNTHESIS IN RABBIT RETICULOCYTES: REPLACEMENT OF THE ENERGY REGENERATING SYSTEM

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SUMMARY: The addition of NAD^+ to a cell-free protein synthesizing system derived from lysed rabbit reticulocytes replaces the requirement for exogenously added energy regenerating systems. NAD^+ is effective at a concentration of 5 μM and maximal stimulation is achieved above 25 μM NAD^+. NADH can completely replace NAD^+. NAD^+ analogs substituted in the adenosine moiety, i.e., with 8-bromoadenosine, tubercidin, 3'-deoxyadenosine, 2'-deoxyadenosine, formycin, 1,N^6-ethenoadenosine, and inosine, show the following activities compared to NAD^+: 110, 82, 27, 18, 14, 9, and 5%. $NADP^+$, NADPH, and analogs with substitutions in the nicotinamide moiety can not replace NAD^+; thionicotinamide NAD^+ has about 34% activity relative to NAD^+. The lysate contains less than 1 μM NAD^+. Evidence for an enzyme system capable of rapidly converting NAD^+ to ATP is presented.

Maximum rates of protein synthesis in rabbit reticulocyte lysates require the addition of certain factors. In addition to ATP and GTP, an energy regenerating system containing CP[1] and CPK or PEP and PK is generally used (1). The necessity of such systems in maintaining a high energy charge for chain initiation has recently been reported (2,3). At temperatures above 25°C, hemin addition is also needed to inhibit the formation of a translational repressor (4-10). This repressor has recently been shown to be associated with a cyclic AMP-dependent protein kinase activity (11,12). It is reported here that addition of NAD^+ and NAD^+ analogs replaces exogenously added energy regenerating systems. A preliminary report of this work has appeared (13).

[1]Abbreviations used: CP, creatine phosphate; CPK, creatine phosphokinase (EC 2.7.3.2); PEP, phosphoenol pyruvate; PK, pyruvate kinase (EC 2.7.1.40); Br^8NAD^+, $NTuD^+$, $3'dNAD^+$, $2'dNAD^+$, NFD^+, εNAD^+, and NID^+ are NAD^+ analogs substituted in the adenosine moiety by 8-bromoadenosine, tubercidin, 3'-deoxyadenosine, 2'-deoxyadenosine, formycin, 1,N^6-ethenoadenosine, and inosine, respectively.

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MATERIALS AND METHODS

Chemicals and biological compounds of the highest purity were purchased from Sigma, P.L. Biochemicals, Eastman Kodak, and Boehringer-Mannheim. D,L-[4,5-^{3}H(N)]leucine was from New England Nuclear; [adenine-^{14}C]NAD^{+} and [carbonyl-^{14}C]NAD^{+} from Amersham/Searle; [U-^{14}C]ATP from Schwarz/Mann. Nicotinamide ribose was prepared from nicotinamide ribonucleotide by hydrolysis with 5'-nucleotidase from *Crotalus adamantus* (Ross Allen Reptile Institute) and purified by paper chromatography using solvent 1. Solvents used were: solvent 1, *iso* butyric acid: ammonium hydroxide: water (66:1:33, v/v/v); solvent 2, 95% ethanol: 1 M ammonium acetate, pH 7.5 (7:3, v/v); solvent 3, 1 M lithium chloride; solvent 4, 0.01 M potassium phosphate, monobasic, pH 4.8; solvent 5, 1 M ammonium acetate, pH 5.0: 95% ethanol (3:7, v/v); solvent 6, acetonitrile: water (4:96, v/v).

NAD^{+} analogs were synthesized as reported (14). Lysates were prepared as described using phenylhydrazine-treated rabbits (15). Cell-free incubations were performed in 100 µl aliquots containing 40 µl lysate, 40 µl master mix, and 20 µl additions. The master mix contained the following components to yield the indicated concentrations in the final reaction mixture: KCl (80 mM), $MgCl_2$ (1.5 mM), ATP (1 mM), GTP (0.2 mM), $HEPES_3$ (20 mM, pH 7.4), 19 unlabeled amino acids (50 µM), and 8 µCi D,L-[4,5-^{3}H(N)]leucine (specific activity 60 Ci/mmole). CP (15 mM), CPK (45 units/ml), PEP (5 mM), PK (50 units/ml) and other additions were made as indicated. Hemin was prepared by the procedure of Adamson et al. (5).

Incorporation of tritium labeled leucine into protein was determined by removing 10 or 20 µl aliquots, precipitating with 1 ml 5% trichloroacetic acid in micro test tubes; heating at 80°C, 20 min.; cooling in ice; 0.1 ml bovine serum albumin (20 mg/ml) was added to insure good pellet formation. The mixture was centrifuged at approx. 17,000 x g for 2 min. in a Brinkmann 3200 clinical centrifuge. The pellet was washed twice, dissolved in NCS solubilizer (Amersham/Searle) and counted in a Packard liquid scintillation spectrometer using toluene scintillation solution (14). Nicotinamide adenine nucleotide concentrations in the lysates were determined by the method of Klingenberg (16). [Adenine-^{14}C]NAD^{+} or [carbonyl-^{14}C]NAD^{+} was incubated with lysate and at various times;10 µl aliquots were removed and added to 5 µl of 15% TCA and centrifuged; 5 µl of the resulting supernatant was used for analysis.

[Adenine-^{14}C]NAD^{+} was analyzed by three separate methods: (1) chromatography on polyethyleneimine-cellulose(Machery-Nagel, polygram cel 300 PEI) using solvent 3. The radioactivity associated with NAD^{+}, AMP, ADP, and ATP (R_f = 0.83, 0.35, 0.23, 0.1) was determined by cutting out the corresponding spots and counting in 10 ml Bray's scintillation solution (17); (2) two-dimensional chromatography on cellulose plates (No. 13254, Eastman Kodak), first dimension -- solvent 1(R_f of AMP, ADP, ATP = 0.45, 0.30, 0.20); second dimension -- solvent 2 (R_f of NAD^{+}, ADPR, and AMP = 0.33, 0.27, 0.15); (3) high pressure liquid chromatography (Waters Corp. Model 440 Chromatograph, µC-18 Bondapak column 4 mm ID x 30 cm) -- solvent 4, pressure 1500 psi, flow rate 2 ml/min; retention times (min) of AMP, ADP, ADPR, ATP, NAD^{+} = 5.0, 5.0, 5.6, 7.0, 10.3; five µl supernatant was mixed with 10 µl of 0.2 M phosphate buffer, pH 7.0, and injected into the column. Eluates corresponding to NAD^{+}, AMP, and ATP were collected in a calibrated centrifuge tube and 0.5 ml was counted in 10 ml Aquasol (New England Nuclear).

[Carbonyl-^{14}C]NAD$^+$ was analyzed by either: (1) chromatography on cellulose plates using solvent 5 (R_f of nicotinamide, NMN, NAD$^+$ = 0.8, 0.27, 0.13); (2) high pressure liquid chromatography (μC-18 Bondapak column, solvent 6, 1500 psi, flow rate 2 ml/min), retention times for NAD$^+$, nicotinic acid, NMN, nicotinamide = 4.4, 4.4, 6.2, 8.8 min., respectively.

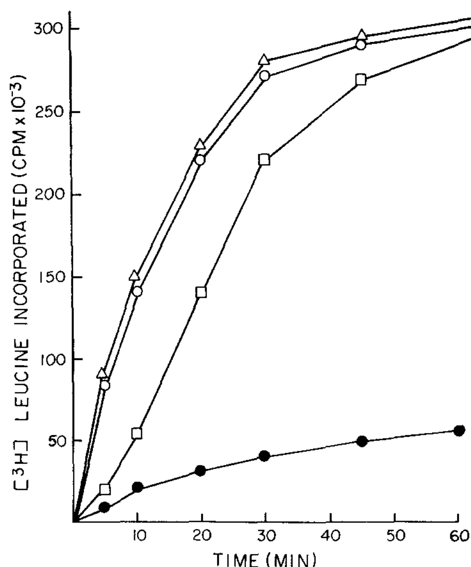

Figure 1. Effect of NAD$^+$ and CP/CPK on protein synthesis at 26°C in the presence of 30 μM hemin. Assay as described in Materials and Methods. NAD$^+$ and/or CP/CPK were added as indicated: ●, no addition; △, CP/CPK + NAD$^+$; o, CP/CPK; □, 50 μM NAD$^+$.

RESULTS

The energy requirement for maximal rates of protein synthesis is presented in Fig. 1. Addition of CP/CPK (or PEP/PK), NAD$^+$, or a combination of NAD$^+$ and CP/CPK give the same maximal radioactive amino acid incorporation after 60 min. A significant lag is consistently observed with NAD$^+$ at earlier time points. Experiments with NAD$^+$ plus CP/CPK performed in the absence of hemin gave similar results (data not shown). These data suggest that NAD$^+$ may be replacing the CP/CPK system as a source of energy rather than interacting with the translational repressor. These findings are in agreement with results obtained by Legon *et al.* (18). A

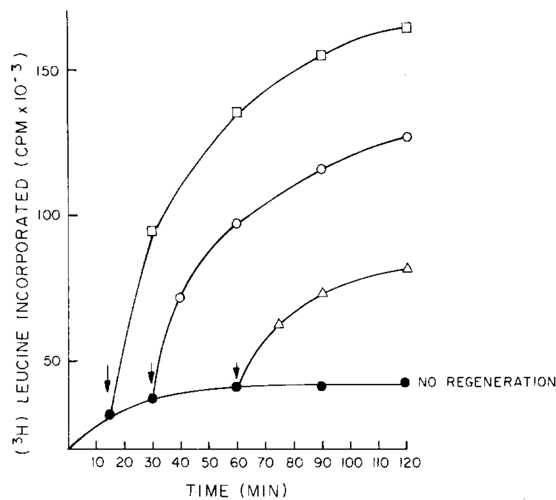

Figure 2. Stimulation of protein synthesis by NAD^+. 50 μM NAD^+ was added at the time points indicated by the arrows: ●, no addition; □, 15 min; o, 30 min; △, 60 min. Assay was conducted at 26°C as described in Materials and Methods.

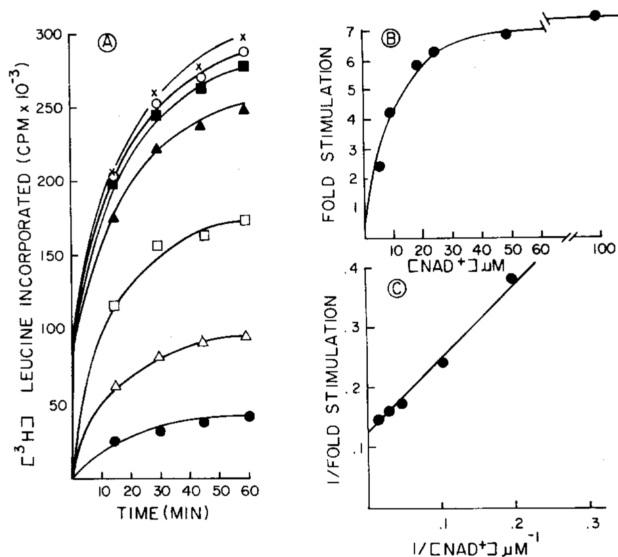

Figure 3. A. Stimluation of protein synthesis by various concentrations of NAD^+. ●, no NAD^+; △, 5 μM NAD^+; □, 10 μM NAD^+; ▲, 20 μM NAD^+; ■, 25 μM NAD^+; o, 50 μM NAD^+; x, 100 μM NAD^+. B. Fold stimulation of protein synthesis by different concentrations of NAD^+ at 15 min. C. Double reciprocal plot of 3B.

relatively high adenylate energy charge is maintained when NAD^+ is present while in the absence of NAD^+ or CP/CPK, there is a rapid decline in the adenylate charge during protein synthesis (data not shown).

Stimulation of protein synthesis occurs when NAD^+ is added to the lysate at various times (Fig. 2). This stimulation is significant at 5 μM NAD^+ and maximal at 20-30 μM (Fig. 3A). A stimulation constant, K_{stim} (the concentration required for half maximal stimulation), of approx. 8 μM can be calculated from the data in Figs. 3B and 3C.

The effect of NAD^+ analogs and metabolites on protein synthesis is shown in Table 1. Only NADH completely replaces NAD^+ and gives a K_{stim} identical to NAD^+. The stimulation of protein synthesis by NAD^+ and NADH

TABLE I: EFFECT OF ANALOGS AND METABOLITES OF NAD^+ ON STIMULATION OF PROTEIN SYNTHESIS [a]

ADDITION	ACTIVITY RELATIVE TO NAD^+	ADDITION	ACTIVITY RELATIVE TO NAD^+
NAD^+	100 %	3-Acetylpyridine NAD^+	< 5 %
NADH	100	3-Acetylpyridine NID^+	0
$NADP^+$	0	3-Pyridine aldehyde NAD^+	0
NADPH	0	3-Pyridine aldehyde NID^+	0
$NTuD^+$	82	Adenosine diphosphoribose	< 5
NFD^+	14	NMN	0
εNAD^+	9	Nicotinamide ribose	< 5
NID^+	5	Nicotinamide	0
8-BromoNAD^+	110	Adenosine	0
2'dNAD^+	18	Inosine	< 5
3'dNAD^+	27	Ribose-5-phosphate	0
Thionicotinamide NAD^+	34		

[a]All concentrations were 10 μM. Stimluation was measured at 5 minutes.

are not additive at saturating concentrations indicating that NAD^+ and NADH probably act by the same mechanism. NAD^+ analogs, substituted in the adenosine moiety of NAD^+, show varying degrees of activity at 10 μM. At 200 μM, all NAD^+ analogs except NID^+ approach the level of stimulation observed with NAD^+. With the exception of thionicotinamide NAD^+, analogs modified in the nicotinamide portion of NAD^+ show little stimulation of protein synthesis at concentrations up to 200 mM. None of the NAD^+ metabolites tested gave significant stimulation.

The NAD^+ concentration of the lysate is less than 1 μM. This compares with values of approximately 30 μM reported for whole blood (19). Addition of $[^{14}C]NAD^+$ to the incubation mixture showed 60% disappearance of the NAD^+ after 10 minutes and greater than 90% after 30 min. The decrease in NAD^+ is directly proportional to the formation of $[^{14}C]ATP$ or $[^{14}C]$nicotinamide (Fig. 4).

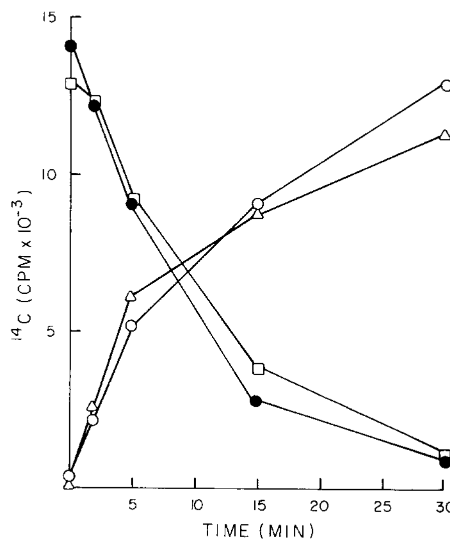

Figure 4. Metabolic fate of labeled NAD^+ in rabbit reticulocyte lysates. The disappearance of [adenine-$^{14}C]NAD^+$ and [carbonyl-$^{14}C]$-NAD^+ and the appearance of $[^{14}C]ATP$ and $[^{14}C]$nicotinamide were measured at 26°C as described in Materials and Methods; ●, [adenine-$^{14}C]NAD^+$; □, [carbonyl-$^{14}C]NAD^+$; △, $[^{14}C]ATP$; o, $[^{14}C]$nicotinamide. $[^{14}C]NAD^+$ concentrations were 10 μM in both cases.

DISCUSSION

The experiments described here show that NAD^+ effectively substitutes for the energy regenerating systems commonly used in rabbit reticulocyte *in vitro* translation. When the molar ratio of ATP:NAD^+ in the reaction mixture is 50:1, there is a marked stimulation of protein synthesis by NAD^+.

Several possibilities can be offered to account for the stimulation of translation by exogenously added NAD^+. NAD^+ may relieve a metabolic block in an energy regenerating system imposed by its absence. A block at the glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) and/or lactate dehydrogenase (EC 1.1.1.27) steps in glycolysis could explain why either NAD^+ or NADH stimulates protein synthesis. This could account for the catalytic nature of their action via enzymatic recycling. It is interesting that the stimulation of translation by the various NAD^+ analogs is similar to their catalytic activities with certain dehydrogenases (15,20,21). It is also possible that the oxidation of NADH may be coupled to ATP synthesis via electron transport by mitochondrial particles. Another possibility is that the conversion of NAD^+ to ATP is occurring at a localized site essential to protein synthesis. Finally, there may be a direct effect of NAD^+ on some aspect of the translational machinery. It has recently been suggested that NAD^+ turnover may be involved in some aspect of mRNA metabolism (22) such as modifications at the 5' end (23). Precedence for additional roles of NAD^+ in reactions other than oxidation-reductions is now well known (24,25).

The rapid disappearance of [carbonyl-^{14}C]NAD^+ and concomitant appearance of [^{14}C]nicotinamide confirms the presence of an active NADase. The requirement for exogenously added NAD^+ in the assay of UDP-glucose-4'-epimerase (EC 5.1.3.2) in hemolysates has been attributed to such an NADase (26). However, since ^{14}C is rapidly found in ATP following incu-

bation with [^{14}C-adenine]NAD^+, another pathway for the metabolism of NAD^+ in the lysate must be considered. Mamaril and Green reported an NAD^+ to ATP conversion in Ehrlich Ascites tumor cells (27). Another possibility is the formation of ATP from ADPR either directly by the action of an NAD pyrophosphorylase (EC 2.7.7.1) like enzyme or from AMP via pyrophosphatase cleavage (28,29,30,31). In addition, Gholson and coworkers have proposed a pyridine nucleotide cycle for NAD^+ (32,33). However, this cycle appears unlikely in these lysate preparations since resynthesis of NAD^+ has not been observed.

A logical extension of these studies would be to test NAD^+ as a source of energy regeneration in protein synthesizing systems from wheat embryo and Ehrlich Krebs II Ascites tumor cells. These studies are currently under investigation in this laboratory.

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REFERENCES

1. Todd, P., and Campbell, P. (1969) in Protein Biosynthesis (Campbell, P., and Sargeant, J., eds.), New York, Academic Press, p. 251-256
2. Walton, G., and Gill, G. (1976) Biochim. Biophys. Acta 418, 195-203
3. Rupniak, H., and Quincey, R. (1975) FEBS Letters 58, 99-101
4. Rabinovitz, M., Freedman, N., Fisher, J., and Maxwell, C. (1969) Cold Spring Harbor Symp. Quant. Biol. 34, 567-578
5. Adamson, D., Herbert, E., and Godchaux, W. (1968) Arch. Biochem. Biophys. 125, 671-683
6. Zucker, W., and Schulman, H. (1968) Proc. Natl. Acad. Sci. 59, 582-589
7. Maxwell, C., and Rabinovitz, M. (1969) Biochem. Biophys. Res. Commun. 35, 79-85
8. Howard, G., Adamson, S., and Herbert, E. (1970) Biochem. Biophys. Res. Commun. 213, 237-240
9. Maxwell, C., Kamper, C., and Rabinovitz, M. (1971) J. Mol. Biol. 58, 317-327
10. Hunt, T., Vanderhoff, G., and London, I. (1972) J. Mol. Biol. 66, 471-481
11. Levin, D., Ranu, R., Ernst, V., Fifer, M., and London, I. (1975) Proc. Natl. Acad. Sci. 72, 4849-4853
12. Ernst, V., Levin, D., Raner, R., and London, I. (1976) Proc. Natl. Acad. Sci. 73, 1112-1116.

13. Lennon, M. B., Lichtenwalner, D. M., and Suhadolnik, R. J. (1976) Fed. Proc. 35, 2049
14. Suhadolnik, R. J., Lennon, M. B., Monahan, J. E., Uematsu, T., and Baur, R. (1976) J. Biol. Chem., submitted.
15. Palmiter, R. (1973) J. Biol. Chem. 248, 2095-2106
16. Klingenberg, M. (1974) in Methods of Enzymatic Analysis, 2nd edition (Bergmeyer, H., ed.) New York, Academic Press, p. 2045-2059
17. Bray, G. A. (1960) Anal. Biochem. 1, 279-285
18. Legon, S., Grayley, A., Hunt, T., and Jackson, R. (1974) Biochem. Biophys. Res. Commun. 56, 745-752
19. Pennell, R. (1974) in The Red Blood Cell (Surgenon, D., ed.) New York, Academic Press, p. 93-146
20. Everse, J., and Kaplan, N. O. (1973) in Advances in Enzymology, Vol. 28 (Meister, A., ed.) New York, John Wiley and Sons, p. 61-133
21. Colowick, S. P., vanEys, J., and Park, H. J. (1964) in Comprehensive Biochemistry, Vol. 14 (Florkin, M., and Stolz, E., ed.) New York, Elsevier Publishing Co., p. 1-98
22. Elliott, G., and Rechsteiner, M. (1975) J. Cell Physiol. 86, 641-652
23. Rottman, F., Shatkin, A., and Perry, R. (1974) Cell 3, 197-199
24. Hayaishi, O. (1976) Trends in Biochemical Science 1, 9-10
25. Suhadolnik, R. J., Baur, R., Lichtenwalner, D. M., Monahan, J. E., Uematsu, T., Smulson, M., Roberts, J. H., and Sudhakar, S. (1976) J. Biol. Chem., submitted.
26. Isselbacher, K., Anderson, E., Kurahaski, K., and Kalckar, H. (1956) Science 123, 635-636
27. Mamaril, F. P., and Green, S. (1973) Fed. Proc. 32, 1439
28. Jacobson, K. B., and Kaplan, N. O. (1957) J. Biol. Chem. 226, 427-437
29. Morrison, J. F., and Doherty, M. D. (1961) Biochem. J. 79, 433-446
30. Doherty, M. D., and Morrison, J. F. (1962) Biochim. Biophys. Acta 65, 364-366
31. Bachorik, P. S., and Dietrich, S. (1972) J. Biol. Chem. 247, 5071-5078
32. Gholson, R. (1968) J. Vitaminol. 14, 114-122
33. Andreoli, A., Grover, T., Gholson, R., and Matney, T. (1969) Biochim. Biophys. Acta 192, 539-541