An Efficient Chemical and Enzymatic Synthesis of Nicotinamide Adenine Dinucleotide (NAD^+)[1]

David R. Walt, Mark A. Findeis,[2a] Victor M. Rios-Mercadillo,[2b] Jacques Augé, and George M. Whitesides*

Contribution from the Departments of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, and Harvard University, Cambridge, Massachusetts 02138.
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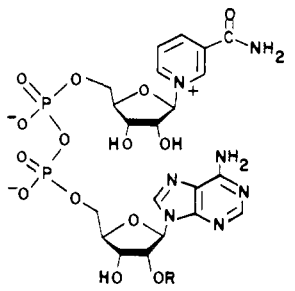
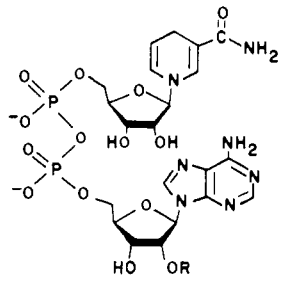
Abstract: This paper describes syntheses of NAD^+ (**1a**) and $NADP^+$ (**1b**) that use traditional synthetic methodology in conjunction with enzyme-catalyzed reactions. The key intermediate, nicotinamide mononucleotide (β-NMN, **4**), is prepared in two steps from ribose 5-phosphate (**5**). NMN is coupled with AMP through the pyrophosphorolysis of ATP (**3**) using the enzyme NAD pyrophosphorylase. The reaction sequence proceeds in 60% overall yield when carried out on 100-mmol scale; on larger scale, yields are lower. The NAD^+ obtained can be used in enzyme-catalyzed syntheses and in recycling schemes. NAD^+ is converted to $NADP^+$ by phosphorylation using NAD kinase and an ATP regenerating cycle.

The nicotinamide cofactors NAD^+ (**1a**), $NADP^+$ (**1b**), NADH (**2a**), and NADPH (**2b**) are required in a variety of enzyme-



1a, R = H, NAD^+

1b, R = PO_3H^-, $NADP^+$



2a, R = H, NADH

2b, R = PO_3H^-, NADPH

catalyzed redox reactions.[3] These compounds are too expensive to be used stoichiometrically: NAD^+ costs approximately \$1500/kg (\$1200/mol) and NADPH costs approximately \$250 000/kg (\$210 000/mol). NAD^+ is isolated from yeast[4] and a variety of other microorganisms.[5] Fermentation yields a range of 1 g/kg of dry cells from *Saccharomyces cerevisiae* to 4.2 g/kg for *Saccharomyces carlsbergensis*. The isolation and purification contributes ~30% to the final cost of the cofactors; the remaining 70% is the cost of the fermentation.[6]

Because these cofactors are useful in enzymology but expensive, we and others have developed NAD(P)H recycling schemes that reuse them. In the reducing direction ($NAD(P)^+ \rightarrow NAD(P)H$), successful strategies for NAD^+ regeneration include enzymatic,[7] electrochemical,[8] chemical,[9] electrochemical–enzymatic,[10] or organometallic–enzymatic[11] procedures. In the oxidizing direction ($NAD(P)H \rightarrow NAD(P)^+$), the procedures include enzymatic,[12] electrochemical,[13] and chemical[14] techniques. The utility of any recycling scheme is, however, finally limited by the fact that the nicotinamide cofactors are inherently unstable in solution. This instability reflects hydrolysis,[15] epimerization,[16] and reactions with

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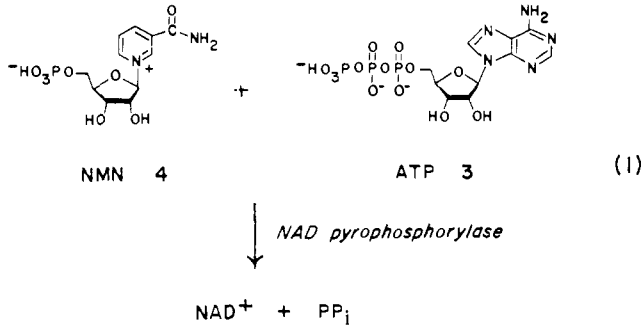
nucleophiles.[17] In working enzymatic reactors, there is an additional instability due to enzyme-induced hydrolysis[18] and nucleophilic additions.[19] Inactive NAD^+ dimers are formed during electrochemical recycling.[20]

The instability of the nicotinamide cofactors in solution requires that a more efficient (less expensive) procedure be developed for their preparation, if they are to be used in large-scale organic synthesis. We set out to develop a method for preparing quantities of these cofactors that would be superior to procedures based on isolation from yeast. This paper describes a synthesis of NAD^+ and $NADP^+$ using a combined chemical and enzymatic approach.[21] This synthesis represents the first stage in a program to develop a source of NAD^+ that is superior to its isolation from yeast. Although it has not yet achieved this objective, this synthesis provides a foundation for further development. Further, it provides a route to NAD which has sufficient chemical flexibility to permit synthesis of research quantities of a number of isotopically and structurally modified analogues of NAD.

Results and Discussion

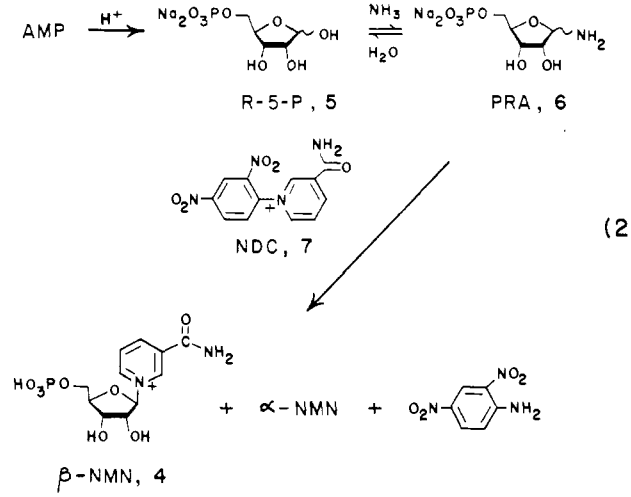
In prokaryotes, the final enzymatic step in the biosynthesis of NAD^+ involves the pyrophosphorolysis of adenosine triphosphate (**3**) (ATP) and nicotinamide mononucleotide (**4**) (NMN) (eq 1).



(1)

This reaction is catalyzed by the enzyme NAD pyrophosphorylase[22] (EC 2.7.7.1). Our synthetic strategy is based on this enzyme. Since we have already described efficient syntheses of ATP from simpler starting materials (RNA, AMP, and adenosine),[23] we faced two problems: first, synthesizing quantities of NMN; second, coupling NMN with ATP enzymatically in vitro.

Synthesis of NMN. NMN (**4**) is a ribofuranose derivative, phosphorylated at the 5-hydroxyl group, and connected to a nicotinamide ring via a β-linkage at C-1. Previous syntheses of NMN have been multistep sequences that used protecting groups, involved low-yield phosphorylations, and required multiple chromatographic separations.[24] The overall yield of the best of these sequences was less than 3%. A difficulty in an efficient synthesis of NMN lies in the need to lock the ribose moiety in the furanose configuration. We obviated this difficulty by taking the route shown in eq 2. Anhydrous ribose 5-phosphate (**5**)



(2)

(R-5-P) in anhydrous ethylene glycol was treated in sequence with anhydrous ammonia and 1-(2,4-dinitrophenyl)-3-carbamoylpyridinium chloride (**7**) (NDC).[25] The requirement for anhydrous conditions results from the reversibility of the ammonolysis reaction in the presence of water. The ribose 5-phosphate was converted quantitatively into a 60:40 mixture of β- and α-anomers of NMN on a small scale;[26] on large scale, yields were poorer, apparently due to difficulties in removing ammonia rapidly and completely from ethylene glycol solution.[27] An alternative procedure, stirring ribose 5-phosphate bis(cyclohexylammonium) salt as a suspension in liquid ammonia with subsequent addition of NDC (**7**) after evaporation of the ammonia, gave yields of β-NMN as high as 45% starting from 100 mmol of ribose 5-phosphate and 35% in one reaction starting with 0.5 mol of ribose 5-phosphate. The β:α ratio in these reactions was also 60:40. While this procedure affords higher yields on a larger scale than the first method, it suffers from a lower overall efficiency. Nonetheless, in one reaction flask and without isolation, we can prepare 0.2-mol quantities of β-NMN. The use of ribose 5-phosphate as a starting material solves two problems that hampered previous synthetic approaches to NMN. First, the ribose moiety is "locked" in the furanose form, because the phosphate group prevents the 5-hydroxyl from participating in pyranose formation. Second, it is unnecessary to carry out a phosphorylation step.

An important aspect in the ammonolysis reaction in ethylene glycol is the order of addition of the reactants. Our original protocol called for bubbling ammonia through a slurry of ribose 5-phosphate (**5**) in ethylene glycol. The yield of β-NMN by use of this procedure rarely exceeded 25%. We postulate that this

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(27) A 60% yield of β-NMN (100% overall) can be obtained routinely by using 5 g of ribose 5-phosphate. In two large-scale reactions (>50 g of R-5-P), residual ammonia in the alcoholic phosphoribosylamine solution prevented the yield from exceeding 25%.

Table I. Stability of Coimmobilized Enzymes Used in the Synthesis of NAD^+ [a]

reaction time, days	relative activity, %[b]		
	NADPP	AcK	AdK
0	100	100	100
20	100	86	92
60	95	74	88
90	90	60	74
120	80	50	61

[a] These activities are those observed under the operating conditions of synthesis. These conditions are described in the Experimental Section. [b] Activity was assayed for the individual enzymes at the end of each cycle of use in synthesis. Thus, the table represents measurements collected with a single group of enzymes, and used in four synthetic reactions.

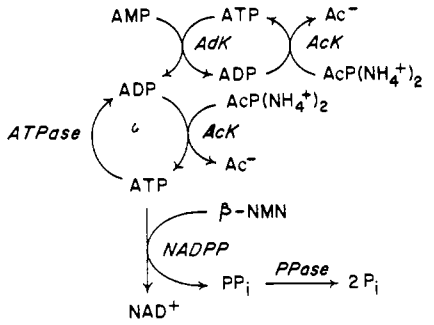
low yield is due to the formation of diphosphoribosylamine in the absence of excess ammonia. This result is probably related mechanistically to the observation that diribosylamine is formed on heating solutions of ribosylamine.[28] By reversing the order of addition (that is, by adding ribose 5-phosphate to a solution containing a large excess of ammonia), we minimized formation of the secondary amine. The ethylene glycol solution of α- and β-NMN can be stored at −20 °C with only slight decomposition: 80% of the original β-NMN remains after 8 months.

Enzymatic Coupling of NMN and ATP—Synthesis of NAD^+.[29] Small-scale coupling experiments using NAD pyrophosphorylase (NADPP, EC 2.7.5.1)[30] immobilized in PAN[31] were carried out according to the reaction of eq 3. The pyrophosphorolysis reaction

$$ATP + NMN \underset{}{\overset{NADPP}{\rightleftharpoons}} NAD^+ + PP_i \xrightarrow{PP_i ase} 2P_i \quad (3)$$

is reversible and has an equilibrium constant of 0.6.[29] It is therefore necessary to couple this reaction to the hydrolysis of the pyrophosphate byproduct in order to drive the reaction which forms NAD^+ to completion. This reaction is catalyzed by inorganic pyrophosphatase (PP_iase, EC 3.6.1.1). By employing these two enzymes, we could routinely obtain nearly quantitative conversions of β-NMN to NAD^+. It is noteworthy that none of the impurities of the NMN synthesis (e.g., α-NMN, ethylene glycol, methanol, dinitroaniline) inhibit the NAD^+-forming reaction.

In order to have large quantities of NAD^+, correspondingly large quantities of ATP are required. Additional ATP may also be necessary since NADPP is claimed to possess ATPase activity.[32] Although we have not independently verified this claim, we employed an ATP generating and regenerating cycle that efficiently allows all the adenine moieties to be used, even in the presence of an ATPase activity (eq 4). This technique regenerated ATP



(4)

from the NADPP-induced hydrolysis product, ADP, using acetyl phosphate (AcP) as the phosphate source. The requisite enzymes NADPP, acetate kinase (AcK, EC 2.7.2.1), adenylate kinase (AdK, EC 2.7.4.3), and PP_iase were coimmobilized in PAN gel[31] to stabilize them. A comparison of the stability of soluble and immobilized NADPP (the most difficult enzyme to obtain in the system) is shown in Figure 1. Table I shows stability for all of the coimmobilized enzymes involved in the large-scale synthesis of NAD^+. The NMN solution, AMP, a catalytic quantity of ATP, acetyl phosphate, dithiothreitol (as an antioxidant), and magnesium chloride were added to the reaction vessel containing the gel. NAD^+ production began immediately. The course of the reaction was followed by periodically sampling and assaying the reaction mixture. When the reaction was complete, as determined by enzymatic assay of β-NMN and NAD^+, the gel was allowed to settle and the solution was decanted. The coupling reaction typically resulted in greater than a 90% yield of NAD^+ based on initial β-NMN. The loss of material is probably due to hydrolysis of NMN and NAD^+ during the course of the reaction.

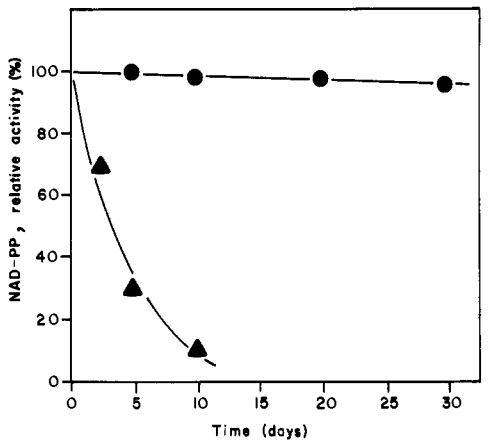
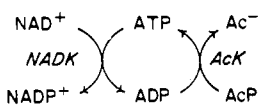


Figure 1. Stability of NADPP in solution (▲) and immobilized (●).

The NAD^+ solution was used directly from the reaction, *without isolation* or purification, for enzyme-catalyzed organic synthesis and in recycling schemes. If a solid preparation of NAD^+ was desired, the reaction mixture was treated with Dowex H^+ to remove cations and the free acid of NAD^+ was precipitated with ethanol. Using this procedure, we isolated a solid containing 60% NAD^+.

A disappointing feature of the large-scale reaction is the unexpectedly low activity of the NADPP under operating conditions. The real activity of NADPP (as measured by the rate of formation of NAD^+) was only 1–5% that expected from small-scale assays of the NADPP. We believe that this low activity reflects precipitation of magnesium ion required for activity of the enzymes (NADPP, AdK, AcK) in the form of magnesium ammonium phosphate. In addition, the precipitate coats the gel particles and blocks access of the substrates to the enzymes. These problems have been solved on a small scale by using disodium ATP in place of ATP generated from diammonium acetyl phosphate (see Experimental Section), but we have not yet tested ammonium-free systems in large-scale syntheses.

Synthesis of $NADP^+$. NAD^+ was converted to $NADP^+$ (**1b**) by phosphorylation with ATP in a reaction catalyzed by NAD kinase (NADK, EC 2.7.1.23); ATP was regenerated using acetyl phosphate and AcK (eq 5). Using this technique, we were able



(5)

to obtain quantitative conversions of NAD^+ to $NADP^+$. The NADP could also be used directly for enzyme-catalyzed organic synthesis and in recycling schemes.

Conclusion

This paper describes a large-scale synthesis of NAD^+ from readily available starting materials. The synthesis combines

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(30) NADPP was isolated using a modification of the procedure from Kornberg.[29]
(31) Pollak, M.; Blumenfeld, H.; Wax, M.; Baughn, R. L.; Whitesides, G. M. *J. Am. Chem. Soc.* **1980**, *102*, 6324–6336.
(32) Grassl, M.; Mollering, H. In "Methods of Enzymatic Analysis", 2nd English ed.; Bergmeyer, H. U., Ed.; Academic Press: New York, 1974; Vol. 4, pp 2073–2077.

conventional organic synthetic methods to prepare the key intermediate—NMN—with an enzyme-catalyzed coupling reaction to prepare the synthetic target—NAD^+. The overall sequence still bears improvement. Our present method for preparing ribose 5-phosphate from AMP could be improved both economically and aesthetically, by replacing it with a method that involves a direct phosphorylation of ribose.[33] A method is required for preventing the precipitation of magnesium ammonium phosphate and thereby maintaining the activity of the NADPP in large-scale reactors. Our preliminary experiments suggest that the solution to this problem may involve nothing more complex than changing to a system that involves little or no ammonium ion, but this suggestion remains to be tested in large scale. Finally, we must develop improved procedures for separating ammonia quickly from the ethylene glycol solution of 5-phosphoribosylamine (**6**). If the ammonia is removed incompletely, it consumes NDC (**7**); if it is removed slowly, the yield of PRA suffers since the water byproduct converts PRA (**6**) back to R-5-P. Because this separation is presently experimentally difficult, the preparation of NMN has proved difficult to scale up.

Despite these present technical shortcomings, the method described is a major improvement over previous syntheses of NMN (and NAD^+) in that it is short, proceeds in high overall yield, and generates useful quantities of material. The reaction sequence avoids the use of protecting groups, chromatographies, separations, phosphorylations, and multiple purifications. It directly produces concentrated solutions of NAD^+ and $NADP^+$, which can be used without additional purification for enzymatic syntheses. Solid preparations of NAD^+ can also be obtained. The method is sufficiently straightforward that we believe it will compete (with further development) with fermentation methods for preparing NAD^+. We are presently using similar methods to prepare NAD^+ analogues.

Experimental Section

General Methods. NAD pyrophosphorylase from hog liver (EC 2.7.7.1, 1.5 U/10 mg)[34] used for NMN assay was purchased from Boehringer Mannheim Biochemicals. Adenylate kinase from rabbit muscle (EC 2.7.4.3, 2000 U/mg), NAD kinase from liver (EC 2.7.1.23, 1 U/72 mg), acetate kinase from *E. coli* (EC 2.7.2.1, 180 U/mg), inorganic pyrophosphatase from yeast (EC 3.6.1.1, 1000 U/mg), Dowex 50W (20-50 mesh), Brewers bottom yeast, and dithiothreitol were from Sigma Chemical Co. Anhydrous ammonia was from Matheson. AMP free acid (99%) was from Kyowa Hakko Kogyo. All other chemicals were of reagent grade from Aldrich Chemical Co. Spectrophotometric measurements were performed on a Perkin-Elmer Model 552 spectrophotometer, equipped with a constant temperature cell compartment. A Chemtrix pH controller with an LKB 12000 Varioperpex peristaltic pump was used to maintain pH during reactions. Yeast autolyses were carried out on a New Brunswick Model G-25 shaker-incubator. Centrifugations were performed on a Sorvall RC-5.

Enzyme Assays. NADPP, NMN, and NAD^+ were assayed by using procedures of Bergmeyer et al.[35] NAD kinase and $NADP^+$ were assayed by the procedure of Apps.[36] Ribose 5-phosphate was assayed by the procedure of Racker.[37] Protein was determined by the method of Lowry et al.[38] AMP and ATP were assayed by the method of Lamprecht et al.[39]

Preparation of Ribose 5-Phosphate (5) from AMP.[40] Dowex H^+ (50W-X8, 1 kg) was added to a 4-L Erlenmeyer flask equipped with a mechanical stirrer. Doubly distilled water was added to the flask to give an approximate combined volume (Dowex + water) of 2 L. AMP free acid (100 g, 0.288 mol) was added to the stirred suspension. The turbid mixture was heated to a final temperature of 100 °C; during this time the AMP dissolved. It took 30 min to reach 100 °C.[41] The mixture was stirred at 100 °C for 6 min and then immediately cooled in an ice bath to 35 °C. The resin was removed by filtration and the solution was cooled to 0 °C. The disodium salt of ribose 5-phosphate was prepared by adjusting the pH of the solution to 7.5 at 0 °C with 10 N sodium hydroxide. This solution (~2 L) was concentrated to 1 L on a rotary evaporator at 35 °C and lyophilized: 77.2 g of a pale yellow, hygroscopic powder was obtained, which assayed for 0.248 mol of ribose 5-phosphate (85% yield base on AMP). The material was 88% pure based on an anhydrous molecular weight of 274 for ribose 5-phosphate disodium salt.

Ribose 5-phosphate bis(cyclohexylammonium) salt was obtained by essentially the same method by adding cyclohexylamine instead of 10 N NaOH. Lyophilization afforded a fine pale yellow powder in 84–91% yield (based on AMP) and 92–96% purity based on an anhydrous molecular weight for 428 g for ribose 5-phosphate bis(cyclohexylammonium) salt.

1-(2,4-Dinitrophenyl)-3-carbamoylpyridinium Chloride (7, NDC).[25] Nicotinamide (100 g, 0.820 mol) and 1-chloro-2,4-dinitrobenzene (400 g, 1.98 mol) were placed in a 4-L Erlenmeyer flask. The mixture was melted in a boiling water bath. After melting, the reaction mixture was heated in the bath an additional 1 h. Methanol (500 mL) was added to the resulting gummy brown mixture. Most of the material dissolved. The insoluble materials were separated by decanting, and ether (3.8 L) was added to the solution to precipitate the product. The ether layer was decanted from the oily byproduct and the precipitation procedure was repeated two times to complete the removal of unreacted chlorodinitrobenzene. The oily residue was dissolved in distilled water (2.5 L) and the solution was treated with activated charcoal (20 g). The charcoal was removed by filtration through Celite and the filtrate was treated once again with activated charcoal (20 g). After filtration, the golden solution was concentrated on a rotary evaporator to a viscous oil, which was poured while still warm into an equal volume of methanol. The yellow crystalline product was collected by filtration and dried in vacuo with a water aspirator and then with a vacuum pump for 24 h. The golden powder (147.5 g, 0.45 mol) was used directly for the NMN synthesis. The yield for the reaction was 55% based on nicotinamide.

Synthesis of Nicotinamide Mononucleotide (4, NMN). Ethylene glycol (125 mL), dried over 3-Å molecular sieves, was added to a 500-mL round-bottomed flask equipped with a magnetic stirring bar. The flask was sealed with a rubber septum and cooled to 0 °C in an ice bath. Anhydrous ammonia was bubbled through the magnetically stirred ethylene glycol for 30 min until the rate of bubbling into and out of the solution appeared to be approximately equal (i.e., until the solution seemed to be saturated). Ribose 5-phosphate disodium salt (5 g, 81% pure, 14.8 mmol) was added to the stirred solution and the flask was quickly resealed. Ammonia was bubbled through the mixture for an additional 15 min, during which time the ribose 5-phosphate dissolved. The solution was stored at 5 °C for 15 h. Ammonia was removed on a vacuum pump for 6 h while the reaction flask was maintained at 0 °C in an ice bath. A cold solution of NDC (13.8 g, 43 mmol[42]) in anhydrous methanol (75 mL) was added to the solution of phosphoribosylamine. The reaction mixture immediately turned a deep red. The reaction flask was sealed and wrapped in aluminum foil, and the mixture was stirred at 5 °C for 14 h. The reaction mixture was assayed by diluting an aliquot of the orange solution with 2 volumes of 0.6 M Hepes buffer (pH 7.4), separating the precipitated dinitroaniline and excess NDC by centrifugation, and subjecting the resulting clear pale yellow solution to enzymatic assay. The solution contained 8.84 mmol of β-NMN (a 60% yield based on R-5-P). Methanol was removed on a rotary evaporator. The reaction mixture could be stored in a freezer at −20 °C with minimal decomposition of NMN. When NMN was required for NAD^+ synthesis, the solution was taken out of the freezer, diluted with two volumes of doubly distilled water at 0 °C, filtered through Celite, and added immediately to the enzyme reactor (see below). If not used immediately, the solution was neutralized at 0 °C with 0.1 N HCl to prevent basic hydrolysis of the NMN.

Large-Scale Synthesis of Nicotinamide Mononucleotide. (a) The procedure used was essentially the same as that described above except that the quantities used were ethylene glycol (1.75 L), ribose 5-phosphate disodium salt (70 g, 81% pure, 207 mmol), and NDC (124 g, 255 mmol) in anhydrous methanol (900 mL). In addition, the removal of ammonia took 2 days and was incomplete. The procedure yielded 50 mmol of β-NMN (or 24% based on ribose 5-phosphate).

(33) AMP costs $200/kg (3 mol).
(34) 1 U = 1 unit (μmol of product formed per min).
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(41) The amount of ribose 5-phosphate can be monitored throughout the reaction sequence by taking an aliquot of the reaction mixture, cooling to 0 °C, diluting with 9 volumes of water, and subjecting the sample to enzymatic assay.[37]
(42) Excess NDC is required to react with residual ammonia.

(b) Ribose 5-phosphate bis(cyclohexylammonium) salt (254 g, 94% pure, 556 mmol) was transferred to a flame-dried 5-L three-necked round-bottomed flask equipped with an overhead stirrer and a cold-finger condenser containing 2-propanol cooled to −60 °C with an immersion cooler. The flask was placed in a cold bath (dry ice/2-propanol), and ca. 1.5 L of ammonia was condensed into it. The cooled mixture was stirred at a moderate rate to evenly suspend the solid in the ammonia. After 36 h the ammonia was pumped off at room temperature with a water aspirator over 1 h. The resulting solid was dried for 4 h in vacuo. NDC (482 g, 1.48 mol) in 3 L of cold methanol–ethylene glycol (1:1) was added and the resulting solution was stirred at 4 °C. After 11 h the solution assayed for 194 mmol of β-NMN (35% based on R-5-P).

Isolation of NAD Pyrophosphorylase—Small Scale. The procedure used was adapted from that of Kornberg.[29] Brewers bottom yeast (100 g) was suspended in 0.1 M Tris buffer (300 mL, pH 7.4, containing 0.01 M dimercaptopropanol)[43] in a 2-L Erlenmeyer flask. The flask was placed in a shaker-incubator at 38 °C and agitated at 110 rpm for 48 h. After autolysis, the suspension was centrifuged at 8000 rpm for 20 min at 3 °C. All subsequent operations were carried out at 3 °C. The autolysis mixture after centrifugation yielded a clear amber supernatant (~230 mL), which was adjusted to 400 mL with the buffer solution. To this mixture, ammonium sulfate (136 g) was added with stirring. After 10 min the precipitate was collected by centrifugation (9000 rpm, 20 min) and the supernatant discarded. The precipitate was dissolved with 20 mL of buffer solution and the preparation was allowed to stand for 12 h, during which time a flocculant precipitate developed. This precipitate was removed by centrifugation (15 000 rpm, 20 min) and discarded. The supernatant (30 mL) was assayed for NADPP activity. To the supernatant (30 mL) was added ammonium sulfate (7.9 g) and the resulting suspension was stirred for 10 min. A precipitate developed which was collected by centrifugation (15 000 rpm, 20 min) and dissolved in 10 mL of buffer solution. The resulting slightly turbid solution was assayed for NADPP activity. This fraction was dialyzed against 1 L of 0.005 M Hepes buffer at pH 7.4 containing 0.25 mL of dimercaptopropanol for 12 h. The dialysate was lyophilized and stored at −20 °C. This material was used routinely for NAD^+ synthesis.

Isolation of NAD Pyrophosphorylase—Large Scale. Brewers bottom yeast (1 kg) was divided into four 250-g portions and each portion was added to a 2-L Erlenmeyer containing 750 mL of the buffer solution. The rest of the purification procedure was the same as that described above. Final enzymatic activities from three large-scale preparations were 46, 60, and 100 U. The specific activity was 0.04, 0.66, and 0.9 U/mg protein, respectively. While the total activity varied by only a factor of 2, the specific activity varied by a factor of 22. Kornberg has pointed out that these values are highly dependent on the particular batch of yeast.[29] One large-scale isolation produced 46 U in 5.4 g of lyophilysate after dialysis. This preparation could be purified 5-fold by suspending the lyophilysate in ice cold water (9.8 mL), centrifuging (10 000 rpm, 10 min), resuspending the pellet in ice cold water (100 mL), and lyophilizing the suspension. All the NADPP activity remained in the lyophilysate (1.13 g).

Small-Scale Immobilization of NAD Pyrophosphorylase. All enzymes were immobilized by the polymerization condensation method.[31] The enzyme solution contained 10 mg of protein (1–10 U). The buffer solution consisted of 0.3 M Hepes (1.79 g/25 mL), pH 7.4, containing 8 mM ATP disodium salt (0.1 g), 1 mM NAD^+ free acid (0.017 g), 1.5 mM $Na_2P_2O_7 \cdot 10H_2O$ (0.017 g), and 8 mM $MgCl_2 \cdot 6H_2O$ (0.041 g). One gram of PAN-1000 was dissolved in 4 mL of the immobilization buffer and was stirred for 1 min, after which time 0.02 mL of 0.5 M dimercaptopropanol and the enzyme solution were added. Triethylenetetramine (0.075 mL of 0.05 M solution) was immediately added to the mixture. Within 2 min the solution set to a transparent gel, which was allowed to stand for 60 min at ambient temperature under an argon atmosphere. The gel was ground in a small mortar for 15 min while four 1-mL aliquots of washing solution (50 mM Hepes, pH 7.4, 10 mM glycerol, 10 mM dimercaptopropanol, and 50 mM ammonium chloride) were added. The gel was then washed with three 15-mL aliquots of the washing solution containing no ammonium chloride. The gel suspension was transferred to a plastic centrifuge tube and agitated for 15 min. The tube was sealed with a serum stopper and oxygen was removed by bubbling argon through the solution. After centrifugation (3000 rpm, 10 min), the washes (a total of three) were combined and were assayed for activity with the gel suspension. The immobilization yield of NADPP was quantitative.

Large-Scale Coimmobilization of NADPP, PPᵢase, AdK, and AcK. PAN-1000 (10 g) was dissolved at 25 °C in 40 mL of 0.3 M Hepes buffer, pH 7.4, containing 10 mM ATP, 1 mM NAD^+, 20 mM ADP, 5 mM $Na_2P_2O_7 \cdot 10H_2O$, 30 mM $MgCl_2 \cdot 6H_2O$, and 10 mM glycerol in a 50-mL beaker equipped with a magnetic stirring bar. After 1 min of vigorous stirring, the polymer dissolved completely. Immediately 1 mL of 0.5 M dimercaptopropanol was added, followed by 1 mL of a solution containing NADPP (50 U), PPᵢase (50 U), AcK (200 U), and AdK (100 U).[44] Finally, 7.5 mL of 0.5 M triethylenetetramine was added. After 2 min the mixture set to a gel. The subsequent procedures were similar to those described above for NADPP immobilization.

Synthesis of Nicotinamide Adenine Dinucleotide (1a, NAD^+). NMN (5.2 mmol in 1.4 L, diluted and filtered as described above) was added to a two-necked round-bottomed flask fitted with a pH electrode. The reaction vessel was charged with ATP disodium salt (4.1 g, 7.5 mmol), NADPP (18 U) immobilized on PAN gel, $MgCl_2 \cdot 6H_2O$ (10 g, 75 mmol, ~25 mM), dithiothreitol (1.5 g, ~5 mM), and inorganic pyrophosphatase (300 U). The solution was adjusted to pH 7.2 and maintained by a pH controller using potassium hydroxide. The reaction vessel was wrapped in aluminum foil to prevent photochemical decomposition of NMN and NAD^+. The suspension was stirred under argon. After 17.5 h the reaction was complete when assayed for NAD^+ (4.9 mmol, 94% yield).

Large-Scale Synthesis of Nicotinamide Adenine Dinucleotide Using ATP Regeneration. The reaction apparatus was set up as described above for the small-scale preparation. A typical reactor was initially charged with 0.5 L of NMN solution (20 mmol), AMP (8.7 g, 25 mmol), ATP disodium salt (1.01 g, 2 mmol), and 100 mL of PAN gel containing NADPP, PPᵢase, AcK, and AdK. Water was added to a volume of 2 L and the solution was adjusted to pH 7.2. The pH was maintained between 7.0 and 7.4 with 1 N NaOH by using a pH controller. Magnesium chloride and 1,3-dimercaptopropanol were added to final concentrations of 25 and 10 mM, respectively. Acetyl phosphate (AcP) solution[45] (1 M, pH 7.0) was kept at 0 °C and added to the reactor via a peristaltic pump. The reaction was monitored by following the formation of NAD^+ by enzymatic assay. NMN, AMP, and AcP were added to the reaction as required until a total of 40 mmol of NMN had been added. A total of 100 mmol of AcP was consumed during the reaction. After the reaction was complete, stirring was stopped and the gel suspension was allowed to settle for 3 h at ambient temperature. AMP and ATP were determined by enzymatic assay.[39] The supernatant was decanted under positive argon pressure with a stainless-steel cannula. The supernatant assayed for 39 mmol of NAD^+, corresponding to a yield of 97% based on β-NMN. The reactor was reloaded to the same conditions as those described above. At the conclusion of the reaction, 110 mmol of AcP had been consumed and 37 mmol of NAD^+ generated (91% based on NMN). The NAD^+ solution was stored in the freezer and could be used as needed for enzyme-catalyzed organic synthesis.

Isolation of Nicotinamide Adenine Dinucleotide. The reaction mixture (3.5 L) from the previous step was concentrated to 0.5 L at 35 °C on a rotary evaporator. The solution was adjusted to pH 1.5 with Dowex H^+ (1 kg) to remove inorganic cations. The resin was removed by filtration and rinsed with distilled water. The filtrate and the combined washes were cooled to 0 °C. A saturated solution of barium hydroxide was added to the ice-cold filtrate to pH 7.0. A precipitate of barium phosphate formed and was removed by filtration and discarded. The filtrate was again adjusted to pH 1.5 with Dowex H^+ and the Dowex was removed by filtration. To the yellow filtrate (1 L) absolute ethanol (5 L) was added with stirring at 0 °C. A fine precipitate developed which was collected by filtration, washed with absolute ethanol, and quickly transferred to a vacuum desiccator. The solid was dried under high vacuum. A pale yellow powder (21 g) was obtained and contained NAD^+ (16 mmol) of 50% purity based on a molecular weight of 663.4. The powder was stored below 0 °C and was used as needed for enzyme-catalyzed organic synthesis.

Synthesis of Nicotinamide Adenosine Dinucleotide Phosphate (1b, $NADP^+$). NAD^+ (3.4 mmol in 300 mL, from the NAD^+ synthesis described above) was added to a 1-L round-bottomed flask equipped with a pH electrode and a magnetic stirring bar. To this solution was added ATP (0.01 mmol), $MgCl_2 \cdot 6H_2O$ (203 mg, 1 mmol), dimercaptopropanol (186 mg, 1.5 mmol, 5 mM), and PAN gel particles (10 mL) containing NAD kinase (0.3 U, 60% immobilization yield) and AcK (10 U, 50% immobilization yield). Solid diammonium acetyl phosphate (0.3 mmol) was added to the reaction mixture daily. The reaction mixture was maintained between pH 6.8 and 7.2 with 1 N sodium hydroxide by using a pH controller. The reaction was carried out at ambient temperature under argon. After 16 days the reaction was stopped and the gel was allowed to settle for 3 h. The supernatant was decanted under positive

(43) This solution will be referred to as "the buffer solution".

(44) AdK and AcK were dialyzed against Hepes buffer to remove ammonium ions that can interfere with condensation polymerization.

(45) The most convenient procedure for preparation of acetyl phosphate is that in Crans and Whitesides: Crans, D. C.; Whitesides, G. M. *J. Org. Chem.* **1983**, *26*, 3130–3132.

argon pressure with a stainless-steel cannula. The solution assayed for 2.85 mmol of $NADP^+$, corresponding to an 84% yield based on NAD^+. The NADP was not isolated.

Registry No. 1a, 53-84-9; **1b**, 53-59-8; **2a**, 58-68-4; **2b**, 53-57-6; **3**, 56-65-5; **4**, 1094-61-7; **5**, 4300-28-1; **5**·2Na, 18265-46-8; 6, 14050-66-9; **7**, 53406-00-1; NADPP, 9032-70-6; NAD kinase, 9032-66-0; AdK, 9013-02-9; 5'-AMP, 61-19-8; nicotinamide, 98-92-0; ribose 5-phosphate bis(cyclohexylammonium) salt, 87763-86-8; PP_iase, 9024-82-2; 2-chloro-2,4-dinitrobenzene, 97-00-7.

Structure of *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine

Scott Rice,[†,‡] Meng Yu Cheng,[†] Roger E. Cramer,[†] Morton Mandel,[‡] Howard F. Mower,*[‡,§] and Karl Seff*[†]

Contribution from the Department of Chemistry, the Department of Biochemistry and Biophysics, and the Cancer Center of Hawaii, University of Hawaii at Manoa, Honolulu, Hawaii 96822.
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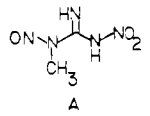
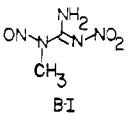
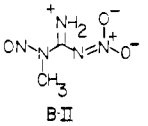
Abstract: *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG, $C_2H_5N_5O_3$) has been shown to be a nitrimine, $ONN(CH_3)C(NH_2)NNO_2$, by crystallographic methods in the solid and by ^{15}N NMR in Me_2SO-d_6 solution. MNNG crystallizes in the space group $P2_1/c$ with a = 11.673 (2) Å, b = 11.260 (3) Å, c = 9.892 (3) Å, β = 114.56 (2)°, V = 1178.9 (5) Å^3, ρ = 1.64 (1) g cm^{-3} by flotation in a bromoform and 1-bromohexane mixture, M_r = 147.09, and Z = 8. The structure was determined by direct methods and was refined with 1019 reflections by full-matrix least-squares procedures to R_1 = 0.075 and R_2 = 0.060. MNNG is nearly planar, indicative of a highly but not fully delocalized electronic structure. An intramolecular amino-to-nitro hydrogen bond is seen. Rapid proton exchange in Me_2SO-d_6 and $CDCl_3$, probably the result of intramolecular hydrogen bonding, is indicated by the uncoupled ^{15}N and proton spins of the amino group and by the presence of only a single resonance signal for the two amino protons despite restrictions to the rotation of the amino group.

The routine use of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) as a mutagen and animal carcinogen has made this compound one of the most widely studied molecules in cancer research.[1] McKay and Wright (1947)[2] first synthesized MNNG by reacting methylamine with nitroguanidine followed by treatment of the resulting methylnitroguanidine with nitrous acid. Without presenting supporting data, they assigned the nitramine structure, A, to the product. Subsequent investigations of the structure of nitroguanidine and related compounds using dipole moment and dissociation constant data,[3-5] as well as ultraviolet[3,6] and infrared spectroscopy,[7] suggested that MNNG should have the tautomeric nitrimine structure, B (see B-I). Ioki and co-


A


B·I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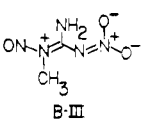
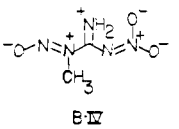
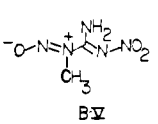

B·III


B·IV


B·V

workers[8] used EPR spectroscopy to investigate the structure of a nitroxide radical resulting from the photolysis of MNNG. Bond lengths reported for the crystal structures of compounds with bonds similar to those in MNNG were used to calculate the coupling constants expected for various possible nitroxides, and it was concluded that this MNNG-derived nitroxide radical is a nitrimine. Indeed, nitroguanidine was found by crystallographic methods to be a nitrimine, with a highly delocalized electronic structure that could be represented as a resonance hybrid of at least eight different forms.[9] Even though it has been shown that nitroguanidine and probably many of its derivatives exist in the nitrimine form, the use of the nitramine, A, to represent MNNG dominates in the literature.[10-13] It thus appears that the controversy over the correct structure of MNNG has remained unresolved.

Realizing the widespread application of MNNG as a model carcinogenic N-nitroso compound, it seemed imperative to confirm its structure in order to evaluate structure–activity relationships in this and related molecules. In particular, we have been employing MNNG as a model compound for determining the structure of nitrosocimetidine, which is similar to MNNG in mutagenic activity and structure.[14-16]

[†] Department of Chemistry.
[‡] Department of Biochemistry and Biophysics.
[§] Cancer Center of Hawaii.

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