"Coulomb strain" one would have expected tighter ion pairing) behaves in a more normal fashion than **2**.[11] Similarly, the bridge carbons of **2**, not those of **3** and **5**, resonate at extremely low field. One might therefore conclude, in accordance with the above results, that the inner carbons of **2** are under the influence of a paramagnetic ring current effect.

Acknowledgment. Financial support by the Deutsche Forschungsgemeinschaft is gratefully acknowledged.

Supplementary Material Available: ^{1}H NMR spectra of acepleiadylene dianion and tetraanion and π-charge distribution (2 pages). Ordering information is given on any current masthead page.

(11) The fact that **3** shows the typical behavior of a π-delocalized anion certainly demands further consideration of the ion-pair situation. Since the above NMR results are not particularly revealing in this respect, additional techniques, e.g., UV spectroscopy, will have to be applied.

Bernd Ch. Becker, Walter Huber, Klaus Müllen*

Institut für Organische Chemie der Universität zu Köln
D-5 Köln 41, West Germany

Received August 18, 1980

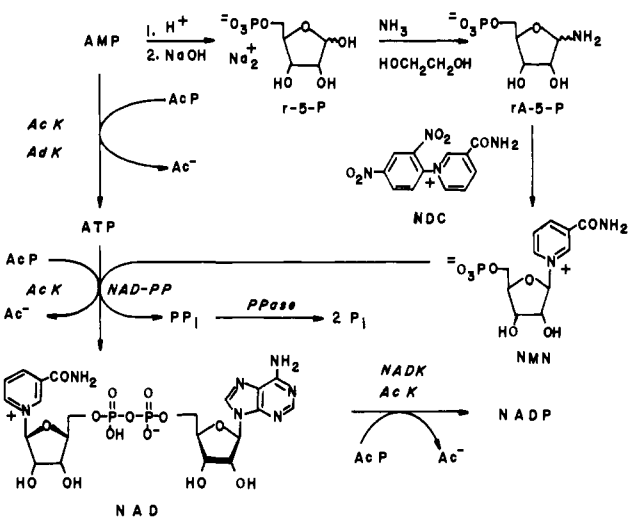
Synthesis of Nicotinamide Adenine Dinucleotide (NAD) from Adenosine Monophosphate (AMP)[1]

Sir:

The acceptance of oxoreductases as catalysts in organic synthesis[2] has been slowed by the expense of the nicotinamide cofactors required by many of these enzymes. Effective procedures for nicotinamide cofactor recycling have decreased the effective cost of these substances by allowing them to be regenerated in situ.[3] The nicotinamide cofactors are, however, intrinsically unstable in solution,[4] and the economic advantage to be gained by any recycling scheme is limited. It is thus also necessary to develop methods for producing them less expensively[5] and for stabilizing them during use. Here we report a combined cell-free enzymatic and chemical synthesis of NAD starting from readily available AMP (Scheme I). This synthesis is a step toward the development of a practical nonfermentation route to NAD and NADP.[6] It also illustrates the utility of enzymatic methods for the synthesis of useful quantities of complex substances and provides a flexible route to derivatives of NAD.

The key intermediate in this synthesis, nicotinamide mononucleotide (NMN), was prepared from AMP in three steps.[7]

Scheme I



Ribose 5-phosphate (r-5-P) was obtained by acid-catalyzed hydrolysis of AMP.[8] Treatment of r-5-P with anhydrous ammonia[9] in dry ethylene glycol provided a solution of ribosylamine 5-phosphate (rA-5-P). This substance was not isolated, but was condensed with N^1-(2,4-dinitrophenyl)-3-carbamoylpyridinium chloride (NDC)[10] to afford NMN in 25% yield based on r-5-P. The NMN (also not isolated) was coupled with ATP[11] in a step catalyzed by NAD pyrophosphorylase[12] (EC 2.7.7.1) immobilized in PAN gel.[13] This enzymatic coupling is an equilibrium reaction and was driven to completion by hydrolyzing the pyrophosphate formed by using pyrophosphatase (EC 3.6.1.1) in PAN.[13] The yield of NAD was 90–97% based on NMN.

A typical reaction sequence follows: Disodium ribose 5-phosphate (156 g, 88% pure, 500 mmol) was added to ethylene glycol (780 mL, dried over 3A molecular sieves). The slurry was cooled to 0 °C, and anhydrous NH_3 was bubbled through it for 1 h. The yellow reaction mixture was stoppered and stored for 1 week in the refrigerator at 4 °C. Excess ammonia was removed (first by using a rotary evaporator and then a vacuum pump). NDC (162 g, 0.5 mol, in 250 mL of methanol) was added as a slurry, and the reaction mixture stirred in the dark for 18 h at 25 °C. Water (1.5 L) was added, and precipitated 2,4-dinitroaniline was removed by filtration. Excess NDC was removed by adsorption on activated charcoal (Darco, 25 g) and filtration. The resulting solution contained 125 mmol of β-NMN by enzymatic assay.[14,15]

For the enzymatic coupling, a 5-L flask was charged with 20 mmol of NMN, 25 mmol of AMP, 2 mmol of ATP, and 100 mL of PAN gel containing coimmobilized NADPP (50 U), PPase (50 U), AcK (100 U), and AdK (100 U).[16] The reaction was adjusted

(1) Supported by the National Institutes of Health, Grant GM 26543, and by grants from the Monsanto Co.

(2) Jones, J. B. In "Enzymic and Non-Enzymic Catalysis"; Dunill, P., Wiseman, A., Blakebrough, N., Eds.; Ellis Horwood: Chichester, England, 1980; pp 54–81. Wong, C-H.; Whitesides, G. M. *J. Am. Chem. Soc.*, submitted for publication. Suckling, C. *Chem. Soc. Rev.* **1977**, *6*, 215–233.

(3) Wang, S. S.; King, C.-K. *Adv. Biochem. Eng.* **1979**, *12*, 119–146. Baricos, W.; Chambers, R.; Cohen, W. *Enzyme Technol. Dig.* **1975**, *4*, 39–53. Shaked Z.; Whitesides, G. M. *J. Am. Chem. Soc.* **1980**, *102*, 7104–7105.

(4) Lowry, O. H.; Passonneau, J. V.; Rock, M. K. *J. Biol. Chem.* **1961**, *236*, 2756–2759. Bernofsky, C. *Arch. Biochem. Biophys.* **1975**, *166*, 645–650. Oppenheimer, N. J.; Kaplan, N. O. *Biochemistry* **1974**, *13*, 4675–4685. Johnson, S. L.; Tuazon, P. T. *Biochemistry* **1977**, *16*, 1175–1183.

(5) NAD is now isolated from yeast. One pound of yeast (~$1) yields approximately 1 g of NAD: Kornberg, A. *Methods Enzymol.* **1957**, *3*, 876–879.

(6) For previous syntheses of NAD, see: Kornberg, A. *J. Biol. Chem.* **1950**, *182*, 779–793. Hughes, N. A.; Kenner, G. W.; Todd, A. R. *Ibid.* **1950**, *182*, 3733–3738. Traub, A.; Kaufman, E.; Teitz, Y. *Anal. Biochem.* **1969**, *28*, 469–473.

(7) Previous syntheses of NMN have used protecting groups to hold the ribose moiety in the furanose form and to allow selective phosphorylation of the primary hydroxyl group: Haynes, L. J.; Hughes, N. A.; Kenner, G. W.; Todd, A. R., *J. Chem. Soc.* (*London*) **1957**, 3727–3732. Atkinson, M. R.; Morton, R. K.; Naylor, R. *Ibid* **1965**, 610-15. Mellinikova, L. M.; Beresovskii, V. M. *J. Gen. Chem. USSR* (*Engl. Transl.*) **1967**, *37*, 1429–1432. Beresovskii, V. M.; Mellinikova, L. M.; Eremenko, T. V. *Ibid.* **1967**, *37*, 1433–1436. Jeck, R.; Heik, P.; Woenckhaus, C. *FEBS Lett.* **1974**, *42*, 161.

(8) The method used was adopted from Sokatch, J. R. *Biochem. Prep.* **1968**, *12*, 1–5. AMP was obtained from Kyowa Hakko Kogyo.

(9) Tipson, R. S. *J. Org. Chem.* **1961**, *26*, 2462-2464.

(10) Lettré, H.; Haede, W.; Ruhbaum, E. *Ann.* **1953**, *579*, 123–132.

(11) ATP was generated from AMP and acetyl phosphate as described previously: Baughn, R. L.; Adalsteinsson, O.; Whitesides, G. M. *J. Am. Chem. Soc.* **1978**, *100*, 304–306. Leuchs, H. J.; Lewis, J.; Rios-Mercadillo, V. M.; Whitesides, G. M. *Ibid.* **1979**, *101*, 5829–5830.

(12) NADPP was from Brewer's yeast: Kornberg, A. *J. Biol. Chem.* **1950**, *182*, 779–793.

(13) Pollak, A.; Blumenfled, H.; Wax, M.; Baughn, R. L.; Whitesides, G. M. *J. Am. Chem. Soc.* **1980**, *102*, 6324–6336.

(14) Grassl, M.; Möllering, H. In "Methods of Enzymatic Analysis", 2nd English ed.; Bergmeyer, H. U., Ed.; Academic Press: New York, 1974; Vol. 4, pp 2073–2077.

(15) Other materials present in significant quantities were unreacted r-5-P, α-NMN, and bis(5-phosphoribosyl)amine.

(16) NADPP catalyzes the hydrolysis of ATP to ADP.[14] It is, therefore, necessary to regenerate ATP continuously during the reaction which forms NAD.

0002-7863/80/1502-7805$01.00/0

to a volume of 2 L with distilled water, and the pH was adjusted to 7.2. Magnesium chloride (50 mmol) and 1,3-dimercapto-2-propanol (20 mmol, protein antioxidant)[17] were added, and the reaction was blanketed with argon. Diammonium acetyl phosphate solution[18] (AcP, 1 M, pH 7.0, stored at 0 °C) was added with stirring by peristaltic pump to maintain an ATP concentration above K_m for NADPP (0.5 mM). Additional NMN (20 mmol) and AMP (25 mmol) were added over 10 days. At the conclusion of the reaction, 100 mmol of AcP had been added and 39 mmol of NAD produced (97% based on NMN). The enzyme-containing gel was allowed to settle, and the reaction mixture was decanted. A repetition of the reaction on the same scale and using the same enzymes consumed 110 mmol of AcP and generated 37 mmol of NAD (91% based on NMN).

The solutions containing NAD could be used directly, without further purification, to provide NAD (or NADH) for cofactor-requiring enzymatic synthesis.[19] Treatment of this crude NAD-containing solution with NAD kinase (EC 2.7.1.23) and ATP (using the ATP regeneration system) also generated NADP uneventfully. Thus, whatever the impurities present in the unpurified NAD may be, they do not appear to inhibit or inactivate other enzymes. If desired, however, solid NAD can be obtained in >50% purity by acidifying the solution with Dowex 50 (H^+ form), precipitating impurities with $Ba(OH)_2$, and precipitating NAD^+ with ethanol.

This work has several interesting features. First, this synthesis of NAD from readily available starting materials involves only one isolation (of r-5-P; this isolation is required only to dry the r-5-P and is straightforward). For all other steps, unpurified reaction mixtures are used directly, and enzymatic selectivity is used to direct reactants efficiently to products. Isolations and separations of nucleotides are laborious: a synthesis which requires only one simple separation has an advantage in convenience. Second, the NAD produced appears to be suitable for use in cofactor recycling procedures *without further purification*. Thus, although the NAD produced here is only ~15-20% pure (without purification), its simple synthesis and its demonstrated utility in cofactor recycling should make it useful in enzyme-catalyzed organic synthesis. Third, all of the enzymes required for the synthesis are easily immobilized and very stable: the manipulation of the enzymatic catalysts is thus straightforward. Finally, we note that the facile synthesis of rA-5-P should find application in other areas of nucleotide chemistry, that the use of r-5-P as starting material avoids many of the problems encountered in more extensively developed synthetic routes to nucleotides, by avoiding the protecting groups often required to generate a product having the furanose configuration, and that preliminary studies suggest that NADPP has sufficiently broad specificity to catalyze the coupling of NMN and ATP moieties bearing at least some structural modifications.

(17) Szajewski, R. P.; Whitesides, G. M. *J. Am. Chem. Soc.* **1980**, *102*, 2011-2026.

(18) Lewis, J. M.; Haynie, S. L.; Whitesides, G. M. *J. Org. Chem.* **1979**, *44*, 864-865.

(19) For example, a turnover number of 1000 was obtained for NAD(H) in the preparation of D-lactate from pyruvate. The reaction mixture (20 mL) contained 0.05 mM NAD (0.34 mL of the solution prepared as described), glucose 6-phosphate (50 m*M*), pyruvate (50 m*M*), glucose-6-phosphate dehydrogenase (50 U) and D-lactate dehydrogenase (50 U). Reaction was complete in 24 h and generated D-lactate quantitatively. Indistinguishable results were obtained by using pure NAD (Sigma). Similar results have been obtained with lipoamide dehydrogenase and horse liver alcohol dehydrogenase. Impurities also do not seem to inhibit the enzymes used to make and assay NAD and NADP.

(20) CONACYT-MEXICO Predoctoral Fellow.

David R. Walt, Victor M. Rios-Mercadillo[20]
Jacques Augé, George M. Whitesides*

Department of Chemistry
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

Received September 15, 1980

Stereoselective Generation of *Z*-Enolates of Thioamides: Its Application to Diastereoselective Aldol Condensations and Thio-Claisen Rearrangements

Sir:

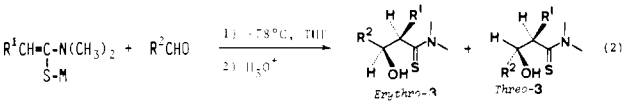
Compared with ordinary carbonyl compounds (ketones, esters, amides, etc.) and thiocarbonyl compounds (dithioesters,[1] thioketones,[2] etc.), thioamides are exceptionally reluctant to undergo carbophilic[3] or thiophilic addition of organometallics. This characteristic behavior in conjunction with our work on Michael addition reactions of a wide range of organometallics to α,β-unsaturated thioamides[4] suggests that thioamides are most likely to undergo proton abstraction α to the thiocarbonyl group to generate thioenolates in the reaction with organometallics.[5] Indeed, this proved to be the case and the enolates with Na^+ (**2b,c**) Li^+ (**2a-c**), and Mg^{2+} (**2a-c**) gegenions were generated very conveniently simply by exposure of thioamides to NaH (THF-Me_2SO, room temperature), *n*-BuLi (THF, -78 °C), and *i*-PrMgBr (THF, room temperature or 65 °C), respectively (eq 1).

Here we report diastereoselective aldol condensations and thio-Claisen rearrangements which take advantage of the ready availability of the thioamide enolates **2** in high geometrical purity (vide infra).

$$R^1CH_2C(=S)NMe_2 \;(\mathbf{1}) + MX \longrightarrow R^1CH{=}C(S\text{-}M)\text{-}NMe_2 \;(\mathbf{2}) \qquad (1)$$

a, $R^1 = CH_3$
b, $R^1 = Ph$
c, $R^1 = SPh$

Mx = *n*-BuLi, *i*-PrMgBr, or NaH



(2)

It is well documented that the appearance of kinetic aldol selection is attributed to the six-membered chairlike transition state with the R^2 group of the aldehyde in an equatorial position (Scheme I).[6] On this ground, the *E*-enolates of thioamides, as suggested by Brandsma et al.,[7] are expected to give rise to the *threo*-β-hydroxythioamides (*threo*-**3**) selectively. However, as

(1) (a) Meyers, A. I.; Tait, T. A.; Comins, D. L. *Tetrahedron Lett.* **1978**, 4657. (b) Burgot, J.-L.; Masson, J.; Vialle, J. *Ibid.* **1976**, 4775. (c) Gosselin, P.; Masson, S.; Thuillier, A. *J. Org. Chem.* **1979**, *44*, 2807.

(2) (a) Dagnonneau, M.; Vialle, J. *Tetrahedron* **1974**, *30*, 415. (b) Metzner, P.; Vialle, J.; Viet, A. *Tetrahedron Lett.* **1976**, 4295. (c) Burgot, J.-L.; Masson, J.; Metzner, P.; Vialle, J.; *Ibid.* **1976**, 4297. (d) Gassman, P. G.; Mullins, M. J. *Ibid.* **1979**, 4457; **1980**, *21*, 2219.

(3) (a) Beak, P.; Yamamoto, J.; Upton, C. J. *J. Org. Chem.* **1975**, *40*, 3052. (b) Walter, W.; Lüke, H.-W. *Angew. Chem.* **1977**, *89*, 550.

(4) (a) Tamaru, Y.; Harada, T.; Iwamoto, H.; Yoshida, Z. *J. Am. Chem. Soc.* **1978**, *100*, 5221. (b) Tamaru, Y.; Harada, T.; Yoshida, Z. *Ibid.* **1979**, *101*, 1316.

(5) For the metalation of the *N*-CH_3 of *N,N*-dimethylthiopivalamide with *sec*-BuLi, see: Seebach, D.; Lubosch, W. *Angew. Chem., Int. Ed. Engl.* **1976**, *15*, 313.

(6) (a) Evans, D. A.; Vogel, E.; Nelson, J. V. *J. Am. Chem. Soc.* **1979**, *101*, 6120. For diastereoselective aldol condensations of ketone enolates, see: (b) House, H. O.; Crumrine, D. S.; Teranishi, A. Y.; Olmstead, H. D. *Ibid.* **1973**, *95*, 3310. (c) Klebshick, W. A.; Buse, C. T.; Heathcock, C. H. *Ibid.* **1977**, *99*, 247. (d) Buse, C. T.; Heathcock, C. H. *Ibid.* **1977**, *99*, 8109. (e) Heathcock, C. H.; White, C. T. *Ibid.* **1979**, *101*, 7076. (f) Heathcock, C. H.; Pirrung, M. C.; Buse, C. T.; Hagen, J. P.; Young, S. D.; Sohn, J. E. *Ibid.* **1979**, *101*, 7077.

(7) *E*-Ketene *S,N*-acetals were obtained selectively by Shuijl et al. (Shuijl, P. J. W.; Bos, H. J. T.; Brandsma, L. *Recl. Trav. Chim. Pays-Bas* **1966**, *85*, 1263) by the alkylation of enolates generated by treatment of thioamides with $NaNH_2$ in liquid NH_3. The geometry of the ketene *S,N*-acetals was determined on the basis of the ^1H NMR chemical shifts of the vinyl protons. Similar selectivity was also observed by us when we prepared ketene *S,N*-acetals by alkylation of enolates generated by treatment of thioamides with *sec*-BuLi in THF: Tamaru, Y.; Harada, T.; Yoshida, Z. *J. Am. Chem. Soc.* **1978**, *100*, 1923.

0002-7863/80/1502-7806$01.00/0