CARBONYL ADDITION REACTIONS OF NICOTINAMIDE ADENINE DINUCLEOTIDE IN FROZEN ALKALINE SOLUTION

M. I. Dolin and K. Bruce Jacobson

Biology Division, Oak Ridge National Laboratory,* Oak Ridge, Tennessee

Received March 21, 1963

In a previous communication dealing with the H_2O_2 content of NADH solutions (Dolin, 1962) it was reported that storage of commercial NAD in frozen, weakly alkaline solution resulted in the formation of a highly fluorescent modification product. The product differed from that formed in 5 N KOH (Kaplan et al., 1951) and its synthesis was accompanied by the formation of H_2O_2. By chromatography on DEAE-cellulose, five modification products have now been isolated from solutions of commercial NAD frozen at -20°C at pH 11-11.9. These have been designated I-V in order of increasing mobility on DEAE-cellulose. One of the major products (V), isolated in pure form, is a dextrorotatory pyridine nucleotide in which a modified NMN moiety is combined in a pyrophosphate linkage with 5'-β AMP. The dinucleotide has the following characteristics (Dolin and Jacobson, in preparation). (a) $[M]_D^{22} = +27,500$. (b) Absorption peaks occur at the following positions (molar absorbance shown in parenthesis). At pH 7.2, 255 mμ (17.5×10^3) and 363 mμ (14.2×10^3). At pH 12, 252 mμ (19.6×10^3), 340 mμ (4.07×10^3) and 404 mμ (11.3×10^3). Both spectrophotometric titration and pH vs. fluorescence curves indicate a pK_a at 9.5. This is confirmed by paper electrophoresis.

On examination of the spectra of known pyridine nucleotide analogs, it became obvious that the spectrum of V closely resembled that of oxidized NAD-acetone (Burton et al., 1957). To substantiate this tentative identification, oxidized NAD-acetone was prepared

*Operated by Union Carbide Corporation for the U. S. Atomic Energy Commission

according to the method of Burton et al. (1957) and then purified on a DEAE-cellulose column. The following properties of this preparation were shown to be identical, within experimental error, to those of compound V. (a) Ultraviolet and visible spectra, at pH values of 7.2 and 12, (b) molar fluorescence, at pH values of 7.2 and 12, (c) molecular rotation, (d) mobility on DEAE-cellulose paper. Further, mass spectrometry (Biemann and McCloskey, 1962) showed that the pyridine ring of V has the molecular weight of the modified pyridine ring postulated by Burton et al. (1957). This compound (a bicyclic structure) is formed by condensation between the added acetone moiety and the carboxamide group, followed by a dehydration.

It then became apparent that compound IV was the reduced form of NAD-acetone since (a) the mobility of IV on DEAE-cellulose is identical to that of reduced NAD-acetone prepared according to Burton et al (1957), and (b) both IV and known reduced NAD-acetone are autoxidizable in frozen alkaline solution, the product being a compound with the R_f and spectral properties of oxidized NAD-acetone. The acetone necessary for the synthesis of the adduct is present in all of the commercial preparations of NAD which have been found to undergo the modification reaction in frozen solution (Table 1). The acetone, which the table shows is present also in commercial preparations of NADH, has been identified spectrophotometrically and chromatographically as the dinitrophenylhydrazone derivative (Meigh, 1952); quantitative estimates show the presence of approximately 0.25-0.3 mole of acetone per mole of NAD. This agrees well with the finding that at maximum, the sum of the oxidized and reduced NAD-acetone formed in frozen alkaline solutions of commercial NAD is approximately 0.2 mole per mole of initial NAD. With NAD preparations freed of acetone, the concentration of NAD-acetone formed in frozen alkaline solutions is nearly stoichiometric with the concentration of added acetone (Table 2), at least for the lowest acetone concentrations used. If the experiment of Table 2 is carried out at 25°C, approximately 100 μmole/ml of acetone is required to cause the formation of 1 μmole/ml of NAD-acetone.

Table 1. Acetone Content of Commercial Preparations of NAD and NADH

Sample	NAD	NADH	Acetone	Acetone / Pyridine Nucleotide
	μmole	μmole	μmole	
β NAD*	17.2		< 0.02	0
Sigma 112B-708	13.7		3.24	0.24
Pabst 330	10.5		3.57	0.34
Pabst 2208		14.7	4.61	0.31
Pabst 6501		15.6	5.95	0.38
Sigma 1-44+		13.3	< 0.02	0

*Prepared as in Table 2.

+This sample differed from the others in that it consisted of only 10 mg, furnished "preweighed" in a glass vial.

Acetone was determined as the 2,4-dinitrophenylhydrazone. Approximately 10 mg of sample was dissolved in 4 ml of water and mixed with 1 ml of a saturated solution of 2,4-dinitrophenylhydrazine in 6 N HCl. After 30 min at room temperature, the solution was extracted 6 times with 4 ml portions of heptane. Acetone standards were carried through the same procedure and the acetone content of the NAD samples was then estimated from the absorbance of the heptane extracts at 345 mμ. Chromatography according to Meigh (1952) revealed that the dinitrophenylhydrazones prepared from the NAD samples had the same mobility as acetone 2,4-dinitrophenylhydrazone. Spectral properties and melting points of the dinitrophenylhydrazones also indicated that acetone was the major or sole neutral carbonyl compound present.

In frozen alkaline solution, carbonyl addition products of NAD have also been prepared in the presence of the following compounds: acetaldehyde, acetoin, fructose, ribose-5-phosphate and adenosine diphosphoribose. In the absence of added carbonyl compounds, β NAD, prepared as in Table 2, gives rise, in frozen alkaline solution, to three orange compounds, all of lower R_f on DEAE-cellulose than either IV or V. The oxidized forms of the orange compounds have spectra that are very similar to those shown by the oxidized forms of all the carbonyl adducts so far examined (acetone, acetaldehyde, acetoin,

Table 2. Formation of NAD-Acetone in Frozen Alkaline Solution

Acetone Added (μmole/ml)	NAD-Acetone Formed (μmole/ml)		
	reduced form	oxidized form	total
0	0.14*	0	0.14*
1	0.27	0.55	0.82
2	0.64	0.84	1.48
4	1.10	1.06	2.16
6	1.58	0.67	2.25

*Unidentified compound, calculated as NAD·acetone.

Acetone-free β NAD was prepared by enzymic reduction of commercial NAD (Racker, 1950) to NADH. The latter, after isolation on a DEAE-cellulose column, was oxidized enzymically to NAD and rechromatographed on a column of DEAE-cellulose. Elution of NADH was carried out with 0.1 M LiCl and of NAD with 0.04 M LiCl. Excess LiCl was removed from the preparations by extraction with ethyl alcohol-diethyl ether 1:1. The compounds were then washed with diethyl ether and dried in vacuo. Reaction mixtures for the synthesis of the acetone adduct contained: NAD, 5 μmoles; acetone, as shown; KOH, 20 μmoles and water to 1 ml (final pH, 11.9). The mixtures were stored at -20°C for 3 days and then 0.2 ml aliquots were chromatographed on sheets of DEAE-cellulose (chloride form). Development was carried out with Tris-Cl, 0.02 M, pH 7.5, containing 0.1 M NaCl. The R_f values of the reduced and oxidized acetone adducts are almost identical with those of NADH and NAD respectively. The determination of oxidized and reduced NAD-acetone singly and in mixtures is described elsewhere (Dolin and Jacobson, in preparation).

---

fructose). With both groups of compounds, an absorption band in the region of 360 mμ-390 mμ is shifted approximately 40 mμ in the direction of longer wavelengths when the pH is raised from 1.5 to 12. (The fructose compound has a double peak at alkaline pH.) The compounds prepared by Burton et al. (1957) show similar behavior, as does the oxidized form of an inhibitor isolated from frozen NADH solutions (Fawcett et al., 1961; Fine et al., 1962).

References

Biemann, K. and J. A. McCloskey, J. Amer. Chem. Soc. 84, 2005 (1962).
Burton, R. M., A. San Pietro and N. O. Kaplan, Arch. Biochem. Biophys. 70, 87 (1957).

Dolin, M. I., Biochim. Biophys. Acta 63, 219 (1962).
Dolin, M. I. and K. B. Jacobson, in preparation.
Fawcett, C. P., M. M. Ciotti and N. O. Kaplan, Biochim. Biophys. Acta 54, 210 (1961).
Fine, I. H., C. P. Fawcett, J. Everse and N. O. Kaplan, Amer. Chem. Soc. Abstracts p. 55C (1962) (March).
Kaplan, N. O., S. P. Colowick and C. C. Barnes, J. Biol. Chem. 191, 461 (1951).
Meigh, D. F., Nature 169, 706 (1952).
Racker, E., J. Biol. Chem. 184, 313 (1950).