THE STRUCTURE OF AN NAD-PYRUVATE COMPLEX*

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Summary

The structure of an NAD-pyruvate complex was determined by nuclear magnetic resonance, infrared and ultraviolet spectroscopic methods. Pyruvate in alkaline solution adds as an enolate anion specifically to the C-4 position of NAD^+ to yield an addition complex which has a maximum absorption at 340 mμ. This complex undergoes a slower cyclization reaction involving the carbonyl group of the pyruvate and the amino group of the nicotinamide. The cyclized product has an absorption maximum at 417 mμ. The complex is not active with respect to lactic dehydrogenase but has the same biological activity in stimulating ^{32}P-orthophosphate into lipids of rat liver homogenates as does a physical mixture of NADH and pyruvate.

The labelling of phosphatides with ^{32}Pi in a mammalian system was shown to be dependent upon Mg^{++}, NADH (NAD^+), and fructose diphosphate or pyruvate (Erbland, Marinetti, and Chatterjee, 1968). The possibility of an NAD-pyruvate complex (NAD-PYR) acting in the system led us to study its mechanism of formation and structure and to see if it had biological activity in the stimulation of lipid phosphorylation in rat liver systems.

The reaction of carbonyl compounds with NAD^+ has been studied extensively (Burton and Kaplan, 1954). The rate of addition of DHA to NAD^+ was shown to depend on pH. Burton and Kaplan found the relative rate of addition of pyruvate to NAD^+ at pH 9.8 was zero compared to the DHA addition under conditions where the concentration of NAD^+ was 0.167 mM and that of

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Abbreviations: DHA, dihydroxyacetone, GALD, glyceraldehyde, NAD-PYR, NAD-DHA, NAD-GALD, the products formed by reaction of DHA, pyruvic acid, and GALD with NAD, nmr, nuclear magnetic resonance, ^{32}Pi, radioactive orthophosphate, TSS, 3-(-trimethyl-silyl)-1-propanesulfonic acid (sodium salt).

the carbonyl compound was 33.3 mM. We found that by increasing the concentration of NAD^+ to 2.0 mM and maintaining the concentration of pyruvate at 4.0 mM, the reaction went essentially to completion at pH 10 after 90 minutes. The relative rate of formation of NAD-DHA to NAD-GALD and NAD-PYR under these conditions was 100:33.5:3.5.

Infrared and nmr studies of the reactants and products were used to characterize the product and to substantiate the reaction mechanism proposed by Burton and Kaplan.

The NAD-PYR complex was active in stimulating lipid phosphorylation in a rat liver homogenate, but its relative activity in comparison to a physical mixture of NADH and pyruvate varied with the lipid examined.

Materials and Methods

NAD^+ and NADH were obtained from P-L Laboratories Inc.; DL-glyceraldehyde and dihydroxyacetone from Calbiochem; sodium pyruvate from C. F. Boehringer and Sons (Mannheim, Germany); ^{32}Pi from Nuclear Science and Engineering Corp.; TSS from Eastman Organic Chemicals.

Solutions for ultraviolet spectroscopy were prepared in 0.1 M K_2HPO_4 buffer adjusted to various pH values with 1.0 N NaOH or 1.0 N HCl. The spectra were obtained with a Microtek (Unicam) SP-800 Spectrophotometer.

The barium salt of NAD-PYR was prepared by a procedure similar to that for the preparation of the barium salt of NAD-DHA (Burton, San Pietro, and Kaplan, 1957). Two tenths gram of NAD^+ and 0.3 g of sodium pyruvate were dissolved in 6.0 ml of water. Two milliliters of absolute ethanol were added followed by 0.24 ml of 3.5 N NaOH slowly with stirring. The solution was allowed to stand for 15 minutes. Two milliliters of 25% barium acetate were added and the mixture centrifuged. The supernatant fluid was removed and mixed with two volumes of cold absolute ethanol and placed in an ice bath for 20 minutes. The yellow precipitate which formed was centrifuged and washed with 12.0 ml of cold 80% ethanol-20% ethyl ether mixture followed by 12.0 ml of cold anhydrous ether. The precipitate was dried in vacuo over P_2O_5. Yield: 0.05 g of a

yellow powder. Considerable loss of product occurs during the precipitation and washing steps.

Sodium pyruvate, NAD^+, NADH, NAD^+ + sodium pyruvate, and the barium salt of NAD-PYR were prepared in KBr pellets at a concentration of one percent. The spectra were run on a Perkin Elmer 421 Grating Spectrophotometer.

One tenth molar solutions of NAD^+, NADH, sodium pyruvate, and NAD-PYR were prepared in D_2O. pH adjustments were made with either 0.1 N NaOH in D_2O or with an HCl solution made by bubbling anhydrous, gaseous HCl into D_2O. The nmr spectra were run by Mrs. Judy Lewis of the University of Rochester Chemistry Department using a high resolution 100 MC Japan Electron Optics Lab. Inc. NMR Instrument. The internal standard was TSS.

Results and Discussion

A mechanism for the reaction of NAD^+ and pyruvate is given in Figure 1. The mechanism involves a base catalyzed nucleophilic addition of the pyruvate enolate anion to the 4 position of the pyridine ring, followed by a slower cyclization reaction and dehydration. Product I has an absorption maximum at 340 mμ whereas product III because of increased resonance has an absorption maximum at 417 mμ.

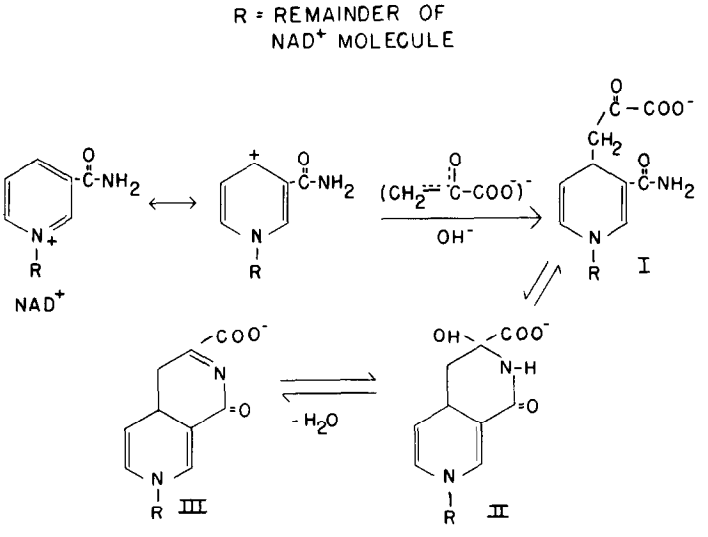

Figure 1. Postulated Mechanism for the Reaction of Pyruvate with NAD^+.

The increase in absorption at 340 mμ for a basic solution of NAD^+ and sodium pyruvate was linear for the first hour and the rate was directly proportional to the pH of the solution. The product gave a yellow color in solution which turned to a yellow-green flourescence upon standing at room temperature for several hours. The flourescence is associated with a new absorption maximum at 417 mμ. The 417 mμ peak began to form 6-8 hours after the start of the reaction and was accompanied by a decrease in the 340 mμ peak. After 24 hours the 417 mμ peak was the predominant one. NAD-DHA and NAD-GALD did not show the appearance of new peaks at 417 mμ upon standing.

Table I

Infrared Analysis of Sodium Pyruvate, a Physical Mixture of NAD^+ + Sodium Pyruvate and the NAD-PYR Complex

Assignment*	NaPyruvate cm^{-1}	Physical mixture NAD^+ + NaPyruvate cm^{-1}	NAD-PYR Complex cm^{-1}
CH_3=O wag	745	740	---
CH_3 rock	978	---	---
CH_3 rock	1015	---	---
C-CH_3 stretch	1182	1172	1174 (very weak)
Sym. CH_3 stretch	2935	---	---
Sym. CH_3 def.	1352	1345	1345 (very weak)
Sym. CO_2^- stretch	1400	1392	1390
Asym. CO_2^- stretch	1610	1612	1610
C = O stretch	1703	1690	1700 (weak)

* (Long and George, 1960).

Comparison of the infrared spectrum of sodium pyruvate, for which all the absorption frequencies have been assigned (Long and George, 1960), with the spectrum of a physical mixture of NAD and sodium pyruvate shows that most of the peaks attributable to CH_3-C=O deformations and C-CH_3 stretching in the pyruvate ion are still present in the physical mixture (Table I). However the infrared spectrum of NAD-PYR shows that all of the stretching vibrations connected with the methyl group of the pyruvate have essentially completely disappeared. This shows that the addition product was formed through the methyl group of the pyruvate. Furthermore, the pyruvate ketone carbonyl at 1703 cm^{-1} is shifted to 1690 cm^{-1} in the physical mixture of NAD^+ plus pyruvate and is markedly diminished in the NAD-PYR complex. The product isolated by barium precipitation is therefore a mixture of compounds I, III and possibly II (Figure 1).

The nmr spectra helped to elucidate the mechanism of pyruvate addition. Previous work has led to the assignment of chemical shifts for all the adenine and pyridine ring hydrogens of NAD and NADH (Jardetsky and Jardetsky, 1966, Lemieux and Lown, 1963, Meyer, et. al., 1962). The nmr spectrum of sodium pyruvate in D_2O at pH 6.7 showed only a single absorption peak due to the methyl group which disappeared when the pH was raised to 10.0. The exchanges between hydrogen and deuterium due to enolate anion formation would predict this. When the pH was brought back down to 6.7, the absorption peak of the -CH_3 group reappeared, but at a considerable decrease in intensity and sharpness because of dilution by CHD_2-, CH_2D-, and CD_3-species.

Table II gives the chemical shifts of the ring protons of NAD^+, NADH and NAD-PYR along with the absorptions of the C_1 hydrogens of ribose. The C_5 and C_6 hydrogens of the pyridine ring in NADH are shifted upfield to a position where they are obscured by the ribose C-H absorptions. Also included in the table is the chemical shift value for the methyl absorption of pyruvate. NAD-PYR was prepared by taking a solution of NAD^+ and sodium pyruvate in D_2O, bringing the pH to 10.0 for 30 minutes and then bringing it back down to 6.7. The spectrum

Table II

Nuclear Magnetic Resonance Analysis of Sodium Pyruvate, NADH, NAD^+, and the NAD-PYR Complex

	Chemical Shifts in CPS*								
	Adenine Hydrogens			Pyridine Hydrogens+					Pyruvate Methyl
Compound	C_8	C_2	C_1'	C_2	C_6	C_4	C_5	C_1'	C_1
Na Pyruvate									237
NADH	845	815	599	693	---	273	---	610	
NAD^+	840	808	601	936	916	885	822	611	
NAD-PYR	846	817	600	707	---	---	---	610	

* relative to TSS

\+ the pyridine C_5 and C_6 hydrogen in NADH have been shown by Meyer, Mahler and Baker, 1962 to be obscured possibly by an interaction of the pyridine ring with the adenine ring.

of NAD-PYR when compared to the spectrum of unreacted sodium pyruvate and NAD^+ shows (1) a complete disappearance of the methyl absorption (2) a disappearance of the C_4 pyridine absorption (3) a large shift of the C_2 pyridine absorption close to its corresponding value in NADH (4) a shift of the C_5 and C_6 pyridine absorptions to a region where they are obscured by the ribose absorptions and (5) the shift of the adenine absorptions very close to their corresponding positions in NADH. These results are consistent with pyruvate adding in its enolate form essentially exclusively at the C_4 position of the pyridine ring.

The NAD-DHA complex was found to be inactive as a coenzyme in a number of dehydrogenase systems (Burton and Kaplan, 1957). We found that the NAD-PYR complex could not replace NADH in a serum lactic dehydrogenase assay (Bergemeyer, Bernt, and Hess, 1965). The NAD-PYR complex was nearly as active as a physical

mixture of NADH and pyruvate in stimulating incorporation of ^{32}Pi into lipids of rat liver homogenates but was much less active in a microsomal-cytoplasm system.

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