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Reactions of Reduced Nicotinamide Adenine Dinucleotide in Acid: Studies by Reversed-Phase High-Pressure Liquid Chromatography†

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ABSTRACT: Reversed-phase high-pressure liquid chromatography was used to isolate acid breakdown products of reduced nicotinamide adenine dinucleotide (NADH) and products produced when NADH breakdown is catalyzed by glyceraldehyde-3-phosphate dehydrogenase (G-3-PD). Chromatographic and UV spectral data on these and related products support a mechanism for NADH acid degradation involving hydroxy addition at the nicotinamide C-6 followed by cyclization of the ring and the adjacent ribose moiety. G-3-PD is shown to catalyze a reaction in which two products are formed which are also intermediates in the acid degradation of NADH (α- and β-6-hydroxynicotinamide products). Formation of the major acid products fits a three-step, first-order mechanism curve, making it possible to calculate the rate constants k_2 and k_3 as well as the previously determined k_1.

Reactions of reduced nicotinamide adenine dinucleotide (NADH[1]) in acid are difficult to study because a complex mixture of compounds is formed. Although these reactions have been studied using ultraviolet (UV), fluorescence, circular dichroism (CD), optical rotatory dispersion (ORD), and nuclear magnetic resonance (NMR) spectroscopy, there remains conflicting opinion about the nature and number of products and the mechanism of NADH breakdown. The inability to isolate intermediates and transient species and to separate breakdown products from reactants has been a deterrant in understanding the acidic breakdown of NADH. The use of high-pressure liquid chromatography (HPLC) with microparticle packing has greatly improved the separation of NADH from its breakdown products (Margolis et al., 1976; Miksic and Brown, 1977a,b). With this technique, NADH acid product intermediates can be observed and measured.

The acid reactions of NADH center primarily around the labile nicotinamide ring. Until recently, it was believed that the primary acid product of NADH was formed from the addition of water across the 5,6 double bond of the nicotinamide ring, (6HTN)AD (1) (Anderson and Berkelhammer, 1958; Stock et al., 1961; Diekman et al., 1964; Kim and Chaykin, 1968; Choi and Alivisatos, 1968). The mechanism of the reaction was thought to involve two steps; the first involves a slow and probably reversible step in which H^+ is added at the C-5 position, and the second step involves electrophilic addition of the anion (OH^-) at position C-6 (Anderson and Berkelhammer, 1958; Kim and Chaykin, 1968). No intermediates for these mechanisms were observed, although Stock et al. (1961) have interpreted a 3-nm shift in the isobestic point as indicative that an anionic intermediate is formed.

Recently, Oppenheimer (1973), Oppenheimer and Kaplan (1974a), and Williams et al. (1976) have provided evidence based on NMR data that the structure of the primary acid product is a cyclic structure, in which the C-6 position of the nicotinamide ring and the 2′-hydroxy of the adjacent ribose moiety are bound. Oppenheimer refers to this product as cyclic tetrahydronicotinamide adenine dinucleotide (C-THN)AD. Miles et al. (1968) obtained the same CD spectra for the acid products of the two stereoisomers, α- and β-NADH. Since α- and β-NADH have different CD spectra, these data indicate that epimerization takes place between the α and β forms of NADH prior to formation of the acid product. Two mechanisms were suggested by Oppenheimer and Kaplan (1974a); one includes epimerization of β-NADH to α-NADH with subsequent protonation at the C-5 and cyclization between the 2′-hydroxy and the C-6. The other mechanism involves initial addition of water across the 5,6 double bond of the nicotinamide ring, epimerization, and then dehydration to form the cyclic product (Figure 1). Williams et al. (1976) have used the increased sensitivity of ^{13}C NMR to support formation of the cyclic product. In addition, they have identified a second conformational isomer of the acid product not observed by Oppenheimer and Kaplan (1974a). Johnson and Tuazon (1977) have studied the stability of NADH and its analogues over a wide range of pH and have also concluded the first step in the acid-catalyzed breakdown of NADH to be proton addition at the C-5 position.

A reaction product similar to the primary acid product results from catalysis of β-NADH in a neutral or acidic solution

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[1] Abbreviations used: NADH, reduced nicotinamide adenine dinucleotide, β form unless specifically stated; (C-THN)AD, cyclotetrahydronicotinamide adenine dinucleotide; (6HTN)AD, 6-hydroxytetrahydronicotinamide adenine dinucleotide; G-3-PD, glyceraldehyde-3-phosphate dehydrogenase; LDH, lactate dehydrogenase; NADHX, product formed from catalysis of NADH by G-3-PD.

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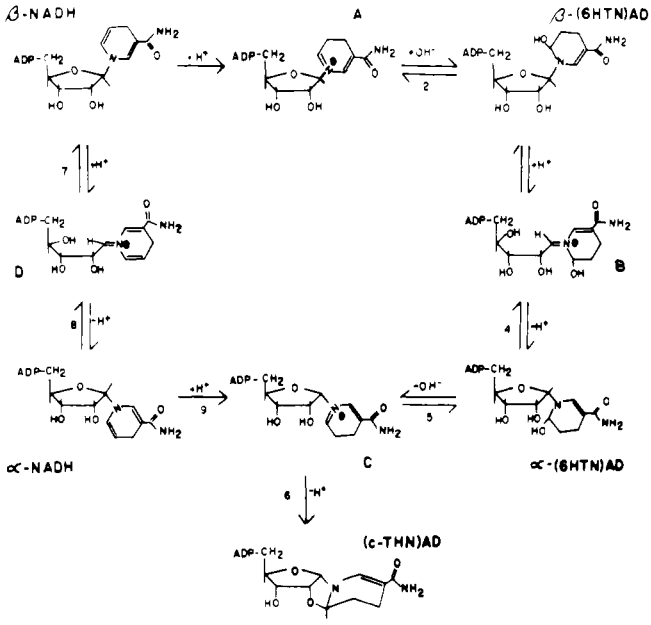

FIGURE 1: Reaction scheme for the breakdown of NADH in acid to the primary acid product. Redrawn from Oppenheimer and Kaplan (1974a).

of glyceraldehyde-3-phosphate dehydrogenase (G-3-PD). This product is referred to as NADHX. It has a UV-absorbance spectrum similar to that of the acid product; however, from its reaction with certain enzyme systems (Meinhart et al., 1956; Stock et al., 1961) it appears to be a different compound, although it is converted to the primary acid product with acidification. Oppenheimer and Kaplan (1974b) have assigned it the structure β-6-hydroxytetrahydronicotinamide adenine dinucleotide, (6HTN)AD, on the basis of NMR data.

In order to clarify the mechanism of NADH breakdown, we have used HPLC and UV spectral analysis to study the acid reaction intermediates of NADH breakdown. HPLC provided a sensitive means of observing intermediates and products which formed during the acid degradation of NADH. Integration of the UV signal gave a quantitative measurement of each product at the time it was chromatographed. For identification, the intermediates and products were collected for further analysis by other techniques. UV-absorbance ratios at two wavelengths were used both to characterize peaks and to determine the purity of the peak (Miksic and Brown, 1977a,b).

Experimental Procedure

Materials and Apparatus. A Waters Associates high-pressure liquid chromatograph equipped with a dual-wavelength detector (Model 440; 254 and 280 nm), a loop injector, and a gradient system (Model 660 programmer) was used for chromatographic work. Reversed-phase columns used were Whatman Inc. ODS/2 and Waters Associates Bondapak C-18. UV spectra were obtained on a Varian Associates Cary 15. Glass-distilled methanol and reagent-grade inorganic salts used for buffer solutions were filtered through 0.45-μm membrane filters. Nucleotide, nucleoside, and base standards were "Sigma grade"; G-3-PD from yeast in crystalline form (70% protein, 1.2 units/mg of protein) and lactate dehydrogenase (LDH) from rabbit muscle (L2500) were obtained from Sigma Chemical Co.

(c-THN)AD was made according to the method of Oppenheimer and Kaplan (1974a), except for their final chromatographic step. NADHX was made by adding 1.8 mg of G-3-PD (100 units) to 1 mL of a solution of 1 mM NADH in 0.05 M sodium pyrophosphate at pH 6.

Reversed-phase chromatographic conditions used were those described by Miksic and Brown (1977a,b).

Methods. NADH solutions (~1 mM) were prepared in sodium acetate (0.005–0.05 M), potassium phosphate (0.05 M), or potassium phthalate (0.05 M) solutions. The pH was adjusted by adding the acid or base of the salt used. All solutions were filtered through 0.25 μm membrane filters prior to preparation of the NADH solution. Solutions of NADH were stored dark in sterile containers at 27.0 °C. Aliquots were chromatographed at time intervals of about 40 min from the time of preparation. Peak height or peak area (electronic integration) was used as a quantitative measure of NADH and its acid products.

Breakdown products were isolated by collecting the appropriate fraction from the column. The UV spectra of these products were obtained in the effluent (0.02 M KH_2PO_4, pH 7.0, and trace MeOH). Peaks collected were acidified and rechromatographed to determine their stability. The peak corresponding to α- and β-NADH was collected and assessed for any α-NADH produced in solution. To assay for α-NADH, the following method was adapted from Jacobson et al. (1973). Fifty milliliters of a 10 mM solution of β-NADH was chromatographed. The "NADH peak" was collected, 2 mL of the effluent was placed in a cuvette, and 1.5 mL of sodium pyruvate (22.7 mM in 1 M phosphate, pH 7.5) was added. The UV absorbance was measured at 340 nm against a reference of equivalent salt concentration. Five milliliters of LDH was added to both cuvettes and the absorbance again measured. Since only β-NADH reacts with LDH, the final UV absorbance at 340 nm was due to α-NADH.

Results

Acid Breakdown of NADH. NADH was observed to form six major products as well as a number of minor products under acidic conditions (pH 4–6), in various buffer systems. When NADH broke down in acid, peaks 3, 4, and 5 were initially formed. Subsequently, peaks 7 and 8 were produced. Peak 1, formed from peak 7 and/or 8, was very stable in acid, although it slowly formed a number of smaller peaks, peak 2 and some peaks of longer retention time which are not in the chromatograms. From chromatograms of β-NADH in acidic solution the following breakdown sequence was observed (Figure 2).

NADH (5) → peaks 3 and 4

peaks 3 and 4 → peaks 7 and 8

peaks 7 and 8 → peak 1

Acidification and chromatography of collected peaks revealed no significant reversibility in any step of the process. In acid, product **3** (or **4**) was converted to products **7** and **8**, and then to product **1**. Peaks 7 and 8 formed and disappeared concurrently during acid breakdown of NADH. A consistent area ratio of 10:1 between peaks 7 and 8 developed in acidic conditions during their formation and disappearance. When isolated and acidified, peak 7 primarily degraded to peak 1. Trace amounts of product **3** were formed and peak 8 developed in the usual 10:1 ratio. Equilibrium between peaks 3 and 4 was obtained. Collection of either product **3** or **4** and chromatography after a few hours in neutral solution resulted in the appearance of approximately a 1:1 ratio of the two products (Figure 3).

UV Spectra of Products. UV spectra for peaks 7 and 8 were the same as the spectra reported by Stock et al. (1961) and

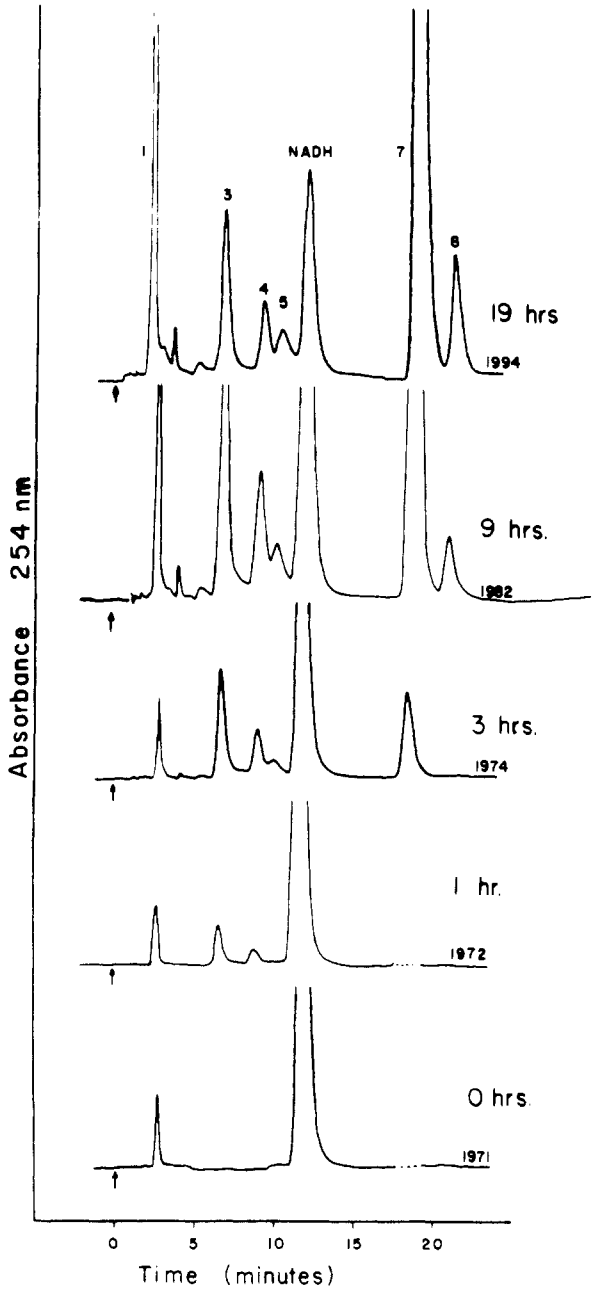

FIGURE 2: Reversed-phase chromatograms of an acidic solution of NADH (0.05 M NaAc, pH 5.4) stored at 27.0 °C from 0 to 19 h.

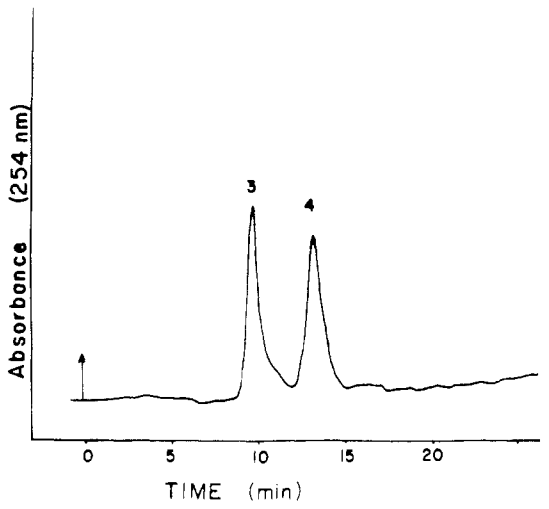

FIGURE 3: Chromatogram of an isolated sample of product **4** after standing 3 h in column effluent at room temperature.

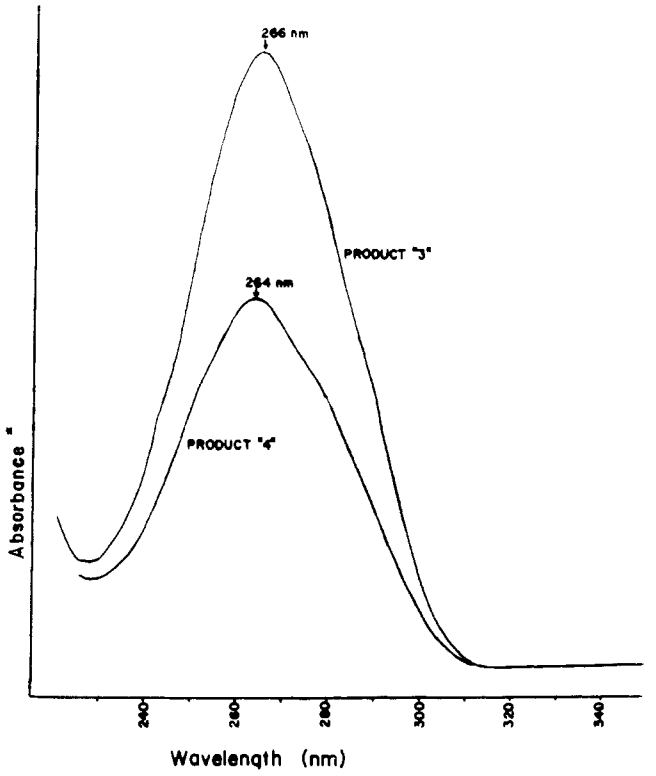

FIGURE 4: UV spectra of intermediate products **3** and **4**, produced by catalysis of NADH breakdown by G-3-PD. (*) Concentration undetermined for products **3** and **4**, therefore relative absorbance scale only.

Johnston et al. (1963) for the primary acid product. In addition, the retention times, peak-height ratios, and relative proportions of peaks 7 and 8 were the same as those of the products produced when (c-THN)AD was made according to the method of Oppenheimer and Kaplan (1974a).

For products **3** and **4**, the UV spectra were similar but not identical (UV maxima at 266 and 264 nm, respectively). Both products had a slight shoulder at higher wavelengths. The UV spectra obtained for these products formed when NADH breakdown was catalyzed by G-3-PD are shown in Figure 4. When the samples used to obtain spectra for products **3** and **4** were chromatographed, they showed some contamination from each other. Product **1** (secondary acid modification product) had a single sharp UV peak with a maximum at 260 nm, and peak 5 a maximum at 259 nm.

Catalysis by G-3-PD. When G-3-PD was used to catalyze the breakdown of NADH at pH 6.0, the principal products had the retention of acid intermediates **3** and **4** (Figure 5). They also had the same UV spectra, 254/280 nm absorbance ratios, and acid and base reactivity as products **3** and **4** of the NADH acidic systems. However, under conditions of G-3-PD catalysis, products **3** and **4** were fairly stable and did not readily decompose to products **7** and **8** as they did under other pyrophosphate and acidic breakdown conditions.

α-NADH. α-NADH (0.005 M NaAC, pH 5.2) broke down in a pattern similar to β-NADH, with the exception of an additional small peak between NADH and the primary acid product (peak 7). Peak-height ratios (280/254 nm) from α-NADH breakdown were equivalent to the corresponding peaks from β-NADH breakdown. When β-NADH was acidified to pH 5.5, the "NADH peak" was collected and assessed for α-NADH. It was found that approximately one-tenth of the

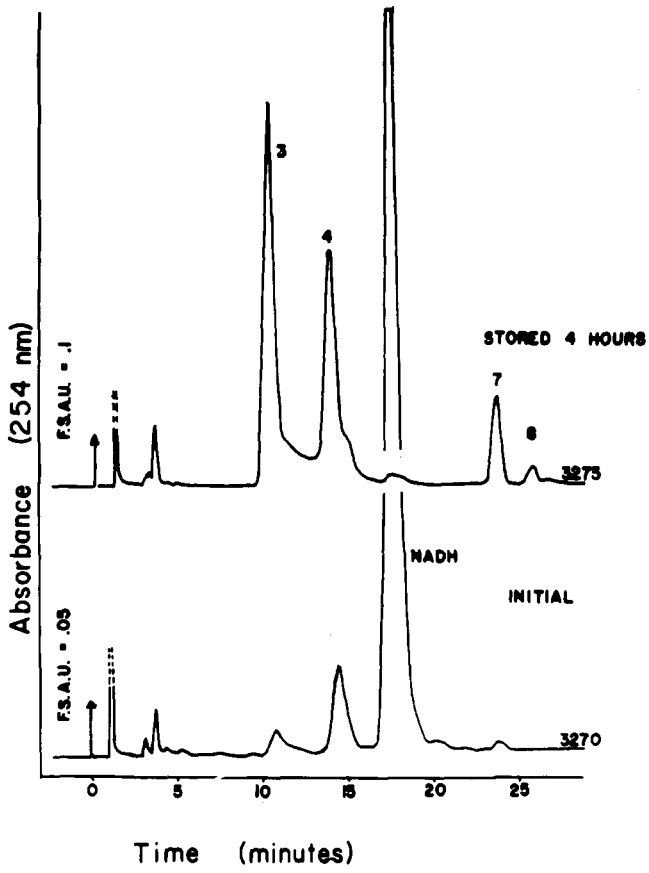

FIGURE 5: Reversed-phase chromatograms of solutions of NADH in the presence of G-3-PD, 0 and 4 h. Chromatographic conditions given by Miksic and Brown (1977a,b).

"NADH peak" had been converted to α-NADH in acid.

Kinetics. Acidic solutions of NADH between pH 5 and 6 were kept at 27.0 °C and chromatographed at time intervals of about 40 min. Peak area measurements of NADH and breakdown products **3**, **7**, and **1** were made. A graph of time of NADH in solution vs. peak area of each peak is shown in Figure 6. Rate constants (k') were found assuming first-order consecutive reactions and by fitting equations for these reactions to curves obtained chromatographically.

The initial breakdown of NADH to product **3** was found to be first order in H^+ concentration over the range examined. The value of k_1' (rate/$[H^+]$) was found to be 450 ± 10 min^{-1}.[2] The rate constant, k_2, for the reaction in which product **7** was formed from product **3** was found to be linearly dependent on H^+ concentration (Figure 7) and the value of $k_2/[H^+]$ was calculated to be 680 ± 75 min^{-1} by curve fitting. The rate constant, k_3, for the reaction in which product **1** was formed from product **7** was found using k_1, k_2, and theoretical curve fitting; thus, the error was increased significantly. The rate constant, k_3, also appears to be dependent on H^+ concentration and the value of $k_3/[H^+]$, found to be approximately 106 min^{-1}.

In order to adjust the measured peak areas of different products to a common concentration scale to fit theoretical curves, the absorbance ratios between products at 254 nm were calculated. The absorbance ratio of peak 3 to NADH was 0.68, and of peak 7 to NADH was 1.2.

[2] Lowry et al. (1961) give a value of 380 (H^+) min^{-1} at 23 °C. Johnson et al. (1963) found the rate to be 462 (H^+) min^{-1} at 25 °C.

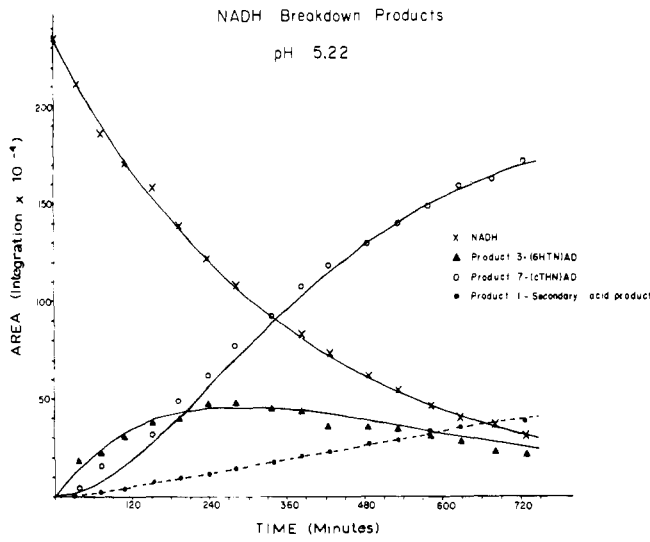

FIGURE 6: Formation of three major acidic breakdown products of NADH; 0.05 M NaAc, pH 5.22 ± 02: Solid lines represent calculated curves for 3-step, consecutive, first-order reactions in which k_1 = 0.00276, k_2 = 0.0042, and k_3 = 0.0007, and NADH intercept = 235 units.

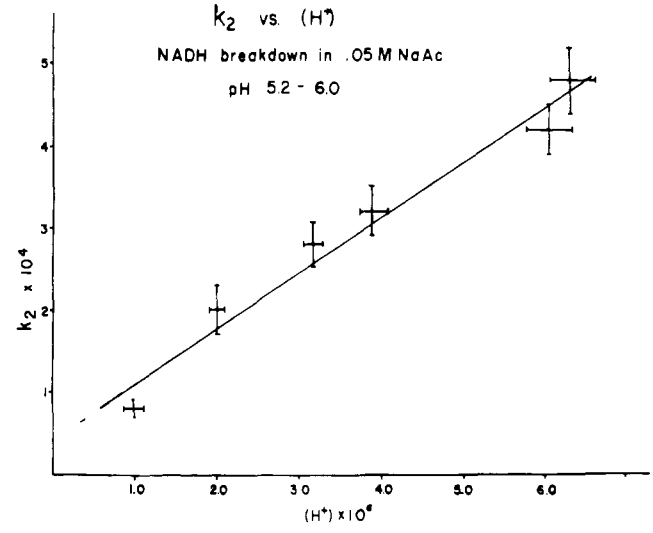

FIGURE 7: Dependence of k_2 on H^+ concentration between pH values 5 and 6 (NADH breakdown in 0.05 M NaAc buffered solutions).

Conclusions

Products **3** and **4**, which have not been isolated by any other method, were identified as reaction intermediates in the breakdown of NADH to the primary acid product. UV chromatographic and chemical data indicate that these products are also produced in the reactions of β-NADH catalyzed by G-3-PD. Furthermore, **3** and **4** do not appear to be anionic addition products of the buffer medium, since changing the anion had no effect on peak-height ratios or retention times of these peaks. The two products produced in the primary acid reaction (**7** and **8**) are thought to be the conformational isomers of (c-THN)AD as described by Williams et al. (1976). No intermediate products between the primary and secondary acid product were observed. It was demonstrated that α-NADH forms in acidic solutions of β-NADH and has a breakdown sequence paralleling that of β-NADH.

The data obtained by HPLC support the mechanism proposed by Oppenheimer and Kaplan (1974a) (Figure 2) and indicate that the primary mechanism of NADH breakdown

takes place through an addition process (clockwise). Products **3** and **4** fit the proposed mechanism as α- and β-(6HTN)AD. Apparently, intermediates in the addition and epimerization process are very short-lived and they could not be observed by HPLC. α-NADH degrades in acid primarily by the same clockwise mechanism, first epimerizing to β-NADH and forming the 6-hydroxy addition products, as predicted by Johnson and Tuazon (1977).

UV and kinetic data are consistent with the mechanism shown. The UV spectra of the (6HTN)AD products (**3** and **4**) are very similar and the shoulder at 280 nm indicates a double bond at the 2,3 position of the ring (Stock et al., 1961). Since products **7** and **8** have indistinguishable spectra, it is proposed that they are the conformational isomers. The excellent fit of the products to first-order curves indicates that the reactions occur primarily by a single mechanism. Rate constants calculated for the breakdown of NADH to the primary acid product (k_1 and k_2) would not be affected greatly by the equilibrium reaction between the isomers of (6HTN)AD, although k_1 is described more accurately by the reaction between NADH and intermediate A, and k_2 by the reaction between α-(6HTN)AD and intermediate C.

The breakdown of (c-THN)AD to a stable acid product, peak 1, does not appear to involve any intermediates. The retention time indicates that a highly polar compound is formed; presumably, breaking of the cyclization as well as the nicotinamide ring structure occurs. A value for the rate constant k_3 is calculated, although it is approximate, since it incorporates the accumulated errors for k_1 and k_2.

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