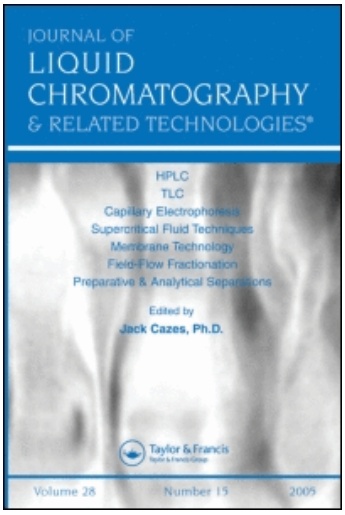

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Reverse-Phase Liquid Chromatographic Analysis of the Acid-Catalyzed Rearrangement of Nadh, Separation of New Products and an Analysis of the First Two Reactions of the Rearrangement

S. A. Margolis[a]; W. T. Yap[a]; B. Matthews[a]; R. Schaffer[a]
[a] National Measurement Laboratory Center for Analytical Chemistry National Bureau of Standards, Washington, D. C.

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REVERSE-PHASE LIQUID CHROMATOGRAPHIC ANALYSIS OF THE ACID-CATALYZED REARRANGEMENT OF NADH, SEPARATION OF NEW PRODUCTS AND AN ANALYSIS OF THE FIRST TWO REACTIONS OF THE REARRANGEMENT

S. A. Margolis, W. T. Yap, B. Matthews, and R. Schaffer (1)

National Measurement Laboratory
Center for Analytical Chemistry
National Bureau of Standards
Washington, D. C. 20234

ABSTRACT

The previously unresolved products of acid-catalyzed rearrangement of NADH have been separated into seven peaks by liquid chromatography on microparticle ODS. The peak with the second highest retention volume was identified as O^2 ,6-B-cyclotetrahydronicotinamide adenine dinucleotide. Based on the order of appearance and disappearance of the peaks, and on their production in the presence of glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12), four of the peaks have been assigned to specific products in the reaction sequence originally proposed by Oppenheimer and Kaplan [Biochemistry, 13, 4675, 1974]. The three remaining peaks (in addition to AMP) are previously unidentified products of the acid-catalyzed rearrangement of 6-B-cyclotetrahydronicotinamide adenine dinucleotide. The first step in the rearrangement of NADH to 6-B-cyclotetrahydronicotinamide adenine dinucleotide was shown to be dependent on the pH and ionic strength of the buffer, but neither the first product nor any other product incorporated the buffer into its structure.

INTRODUCTION

The modification of the dihydronicotinamide moiety of β NADH by acid was first described by Warburg et al. (3). This process was characterized by the loss of uv absorbance at 340 nm and a parallel increase at 280 nm (4). Two of the products are: NADHX (5) or (6HTN)AD (2,6) and the "primary acid product" (4) or (cTHN)AD (7). The structure of each of these compounds has been characterized by nmr (6-8). The existence of additional intermediates has been proposed (7-9) and there is spectrophotometric evidence for a third compound (9).

In the process of evaluating the purity of NADH by reverse-phase LC, we detected a number of peaks which absorb at 280 nm. These peaks did not correspond to any mono- or di-nucleotide we know of, but they were like the peaks we had found to be generated upon aging NADH in phosphate buffer at pH 7.0 (10).

Results of this study indicate that acid-catalyzed rearrangement of NADH produces seven compounds including (6HTN)AD and (cTHN)AD. By following the formation of these entities as a function of time, pH and ionic strength, the sequence and other aspects of the mechanism of their formation have been elucidated. The results are consistent with the scheme for the acid-catalyzed rearrangement of NADH (Figure 1) proposed by Oppenheimer and Kaplan (7). Furthermore, this study shows that LC is capable of rapidly resolving complex biochemical mixtures on microparticulate supports. It provides an ideal system for examining multistep chemical reactions containing transient intermediates.

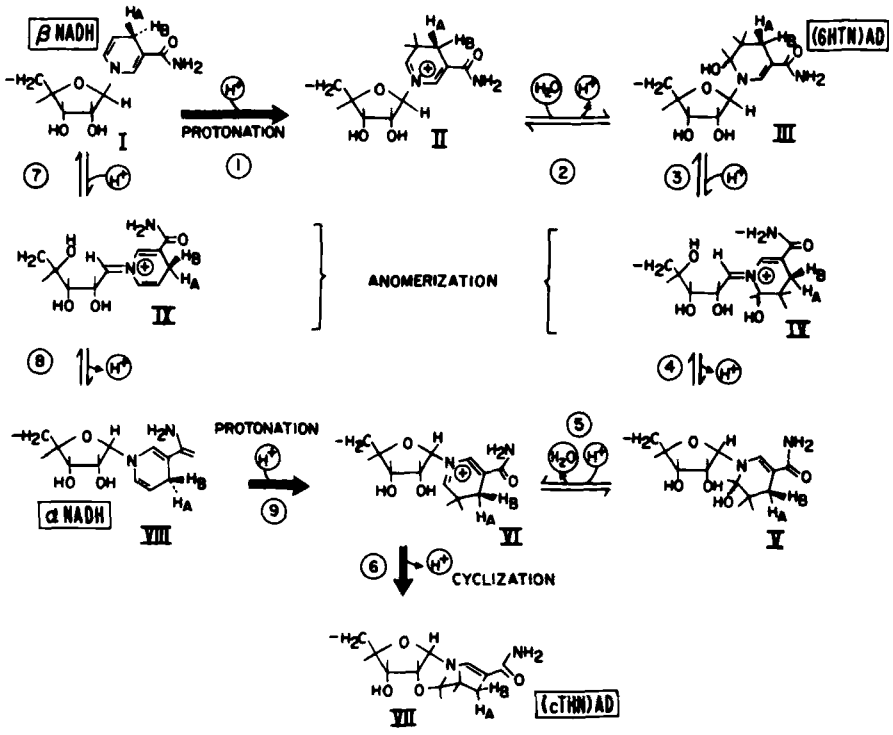


FIGURE 1

The proposed reaction sequence for acid-catalyzed rearrangement of NADH (5).

EXPERIMENTAL PROCEDURES

NADH was purchased from Sigma Chemical Company and Boehringer Mannheim Corporation. Yeast G3PD (EC 1.2.1.12) was purchased from Sigma Chemical Company. Sodium [2-^{14}C]-acetate was purchased from New England Nuclear Company. Difference spectra were measured with a Cary 14 spectrophotometer, which was periodically calibrated for wavelength accuracy against a holmium

oxide filter and the emission spectrum from a deuterium lamp, and periodically calibrated for absorbance accuracy with a set of NBS glass absorbance filters (SRM 930b). The difference spectra were measured with cuvettes in a thermostatted holder at 25 °C. The reference cuvette contained NADH in 0.1 mol/L ammonium bicarbonate buffer (pH 10.2) and the sample cuvette contained NADH at the same concentrations but in the experimental buffer.

The products of acid-catalyzed rearrangement of NADH were separated on μBondapak C_{18} by the LC method of Margolis et al. (10). The uv spectrum of substances in each chromatographic peak was recorded with a Schoeffel Model 770 scanning spectrophotometer which was connected to the μBondapak C_{18} column in series with two other detectors by a valve that permitted the effluent fractions to be diverted to and held in the spectrophotometer.

The pH was measured with a Corning model 12 research pH meter with a combination electrode.

(cTHN)AD was prepared by the method of Oppenheimer and Kaplan (7). NADHX (7,11) was formed by incubating 10 mmol of NADH in 0.5 ml of 50 mmol/L sodium pyrophosphate buffer (pH 6.0) containing 70 mmol/L NaCl and 10 mg/mL yeast G3PD for 2.5 hr at 22 °C. The reaction was terminated by the addition of 2 ml of 1.0 mol/L ammonium bicarbonate and the enzyme was removed by pressure dialysis through an Amicon PM-30 membrane. Instead of performing chromatography on DEAE-cellulose as the authors described originally, we analyzed the dialysate by reverse-phase LC. The progress of the reaction was followed by diluting 10 μL aliquots of the incubation mixture with 0.1 mol/L ammonium bicarbonate and measuring absorbance at 340 nm.

An attempt to synthesize an acetylated product from NADH was performed by incubating 1.4 mol/L NADH in 0.235 mL of 85 mmol/L sodium acetate buffer (pH 3.65) containing 5 μCi of sodium [2-^{14}C]-acetate (2 μCi/mmol) at 22 °C for 30 minutes. A 10 μL aliquot was fractionated on a μBondapak C_{18} column. Seventy-five one-min fractions were collected, dissolved in Aquasol II, and counted in a Packard Scintillation Counter.

RESULTS

Formation of Components of Acid-Catalyzed Rearrangement

The compounds that are obtained when NADH decomposes in an acid solution were separated by chromatography on μBondapak C_{18} (10), and the amount of each component formed in buffers of various pH and ionic strength was monitored at 254 nm and 279 nm as a function of the time of incubation.

The NADH initially showed very low levels of impurities (Figure 2); in ammonium bicarbonate buffer (pH 10.2) its decomposition was negligible during a 6-h period (10).

Ultraviolet difference spectra were also recorded. The differences between the spectra of NADH at pH 10.2 and those at either pH 5.70, 4.70, or 2.05 consist mainly of an increased absorbance at 279 nm and a decreased absorbance at 340 nm. The rate of these differential absorbance changes increased as the pH was decreased. As shown in Figure 3, at pH 2.05 the absorbance at 279 nm attained a maximum value within 3 min and then dropped to the starting value within 5 h while the absorbance at 340 nm reached a minimum within 3 min and then remained nearly constant. Similar results were observed at pH 4.70 and pH 5.70 but the progress of the reaction was much slower.

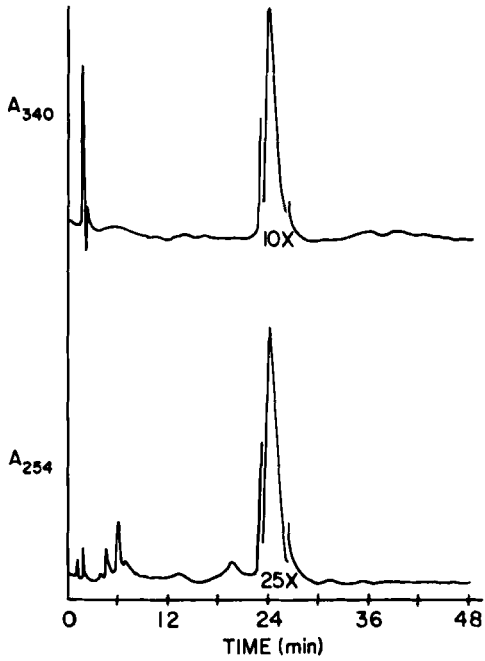


FIGURE 2

Chromatogram of pure NADH on μBondapak C_{18}. Ten μL of NADH 2 mmol in 30 mmol/L ammonium bicarbonate was applied to the column and eluted isocratically with 30 mmol/L potassium phosphate (pH 7.05). A. Absorbance tracing detected at 257 nm, absorbance scale 8 mA. B. Absorbance tracing detected at 340 nm, absorbance scale 10 mA. The numbers at the breaks in the curves (e.g., 10x) indicate the degree of peak attenuation.

Figure 4, a chromatogram on μBondapak C_{18} obtained after exposing NADH for 6 h to 0.1 mol/L sodium acetate buffer (pH 3.99), illustrates the separation of the seven peaks that formed by acid-catalyzed rearrangement. The peaks are numbered in order of elution. Peak 1 is AMP and was present in small amounts in the NADH originally but increased during the reaction.

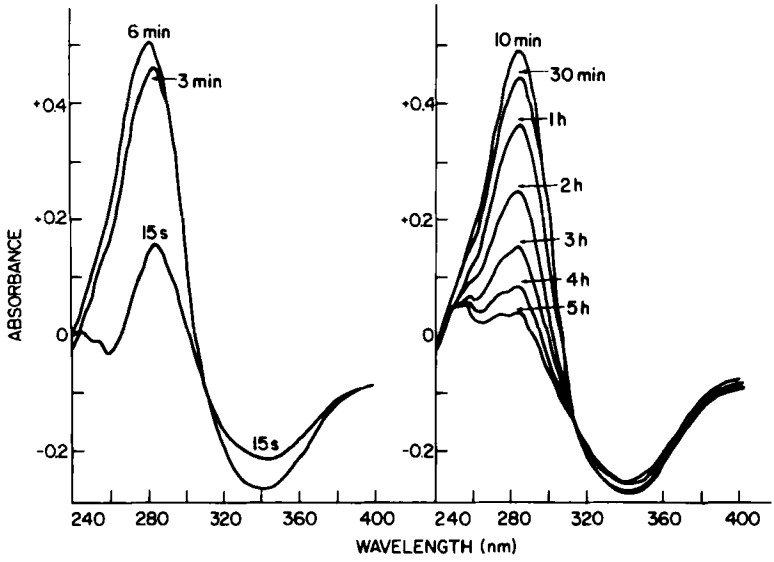


FIGURE 3

Difference spectra of NADH at pH 2.05 compared to NADH at pH 10.2. Spectra were recorded at the indicated time intervals by use of 1-cm light-path cuvettes, in a Cary 14 Spectrophotometer, at 25 °C. The cuvette in the reference beam contained 4.188×10^{-5} mol/L NADH in 0.1 mol/L ammonium bicarbonate (pH 10.2) and the cuvette in the sample beam 4.188×10^{-5} mol/L NADH in 0.1 mol/L potassium chloride-hydrochloric acid (pH 2.05) (5:1 v/v).

Peaks 1 and 2 overlap and peak 6 elutes just ahead of the NADH peak.

Progress curves for peaks 4, 5, and 7 are illustrated in Figure 5 for the rearrangement run at pH 5.70 in 0.1 mol/L potassium phosphate buffer. Peaks 4 and 5 were detected immediately by HPLC. Peak 7 was not evident until more than 3 h of reaction. Peaks 2, 3, 6, and 8 were not detected during the 6 h. The A_{254}/A_{279} absorbance ratios for peaks 4, 5, and 7 were approximately 5 times that for NADH (Table 1).

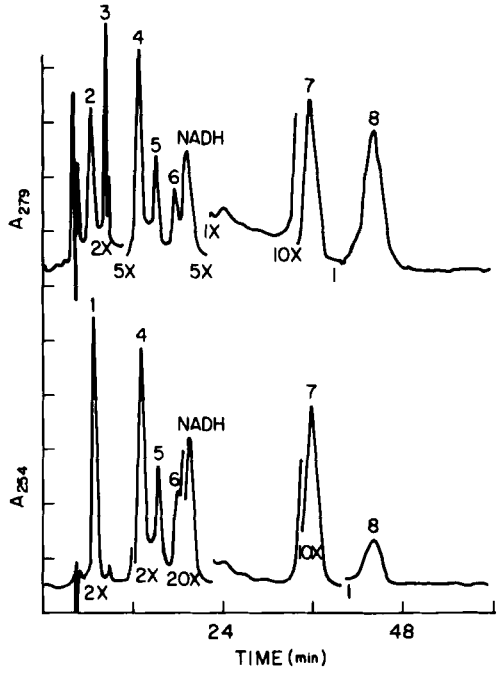
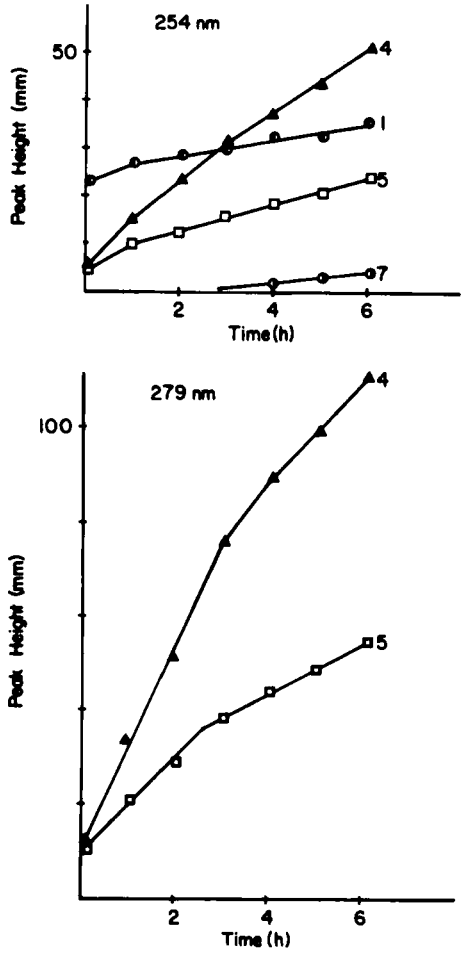


FIGURE 4

Chromatogram of the acid-catalyzed rearrangement products of NADH on μBondapak C_{18}. NADH 1.4 mmol/L in 0.1 mol/L sodium acetate (pH 3.99 was incubated at 4 °C for 6 hours. Ten μL of the solution was placed on the column and eluted isocratically with 30 mmol/L potassium phosphate (pH 7.05). A. Absorbance tracing detected at 254 nm, absorbance scale 8 mA. B. Absorbance tracing detected at 279 nm, absorbance scale 10 mA. The numbers at the breaks in the tracings (e.g., 20x) indicate the degree of peak attenuation. The peaks are numbered in order of elution.

However, in 0.1 mol/L sodium acetate at (pH 3.99) peaks 4, 5, and 7 were formed more rapidly. Also at this pH the formation of peaks 2, 3, and 6 became more evident (Figure 6). Peak 6 was detected with better resolution at 279 nm (Figure 4). Peak 1 contained two



Progress curves for the acid-catalyzed rearrangement products of NADH in 0.1 mol/L potassium phosphate buffer (pH 5.70) at 4 °C. Each chromatographic component was detected at both 254 nm and 279 nm the values represent the measured peak height times the attenuation factor. The curves are numbered to correspond with the peaks in Figure 4.

TABLE 1

Relative Retention Times and Absorbance Ratios of the Products of Acid-Catalyzed Rearrangement of NADH

Peak No.	Relative Retention Value[a]	A_{279}/A_{254}
1	0.35[b]	
2	0.36[c]	
3	0.46	2.76 ± 0.28
4	0.68	0.90 ± 0.01
5	0.79	0.99 ± 0.18
6	0.93	0.84 ± 0.04
NADH	1.00	0.18 ± 0.01
7	1.85	0.86 ± 0.03
8	2.27	1.44 ± 0.04

[a] Values for peaks 3-8 are calculated relative to NADH = 1.00, using A_{254} and A_{279}.
[b] Value calculated using A_{254}.
[c] Value calculated using A_{279}.

component compounds, one is AMP; the other absorbs at 279 nm (peak 2). The relative retention for peak 1 as measured at 254 nm was slightly less than that measured at 279 nm for peak 2 (Table 1). By 6 h the amounts of peaks 4 and 5 in the reaction mixture began to decrease, but the increase in the amount of peak 7 remained constant, confirming the observation made at higher pH that it is formed subsequent to peaks 4 and 5. Peaks 3 and 8 were detected at this lower pH, but they formed relatively slowly. At pH 4.8 in 0.1 mol/L sodium acetate buffer the same peaks formed more slowly.

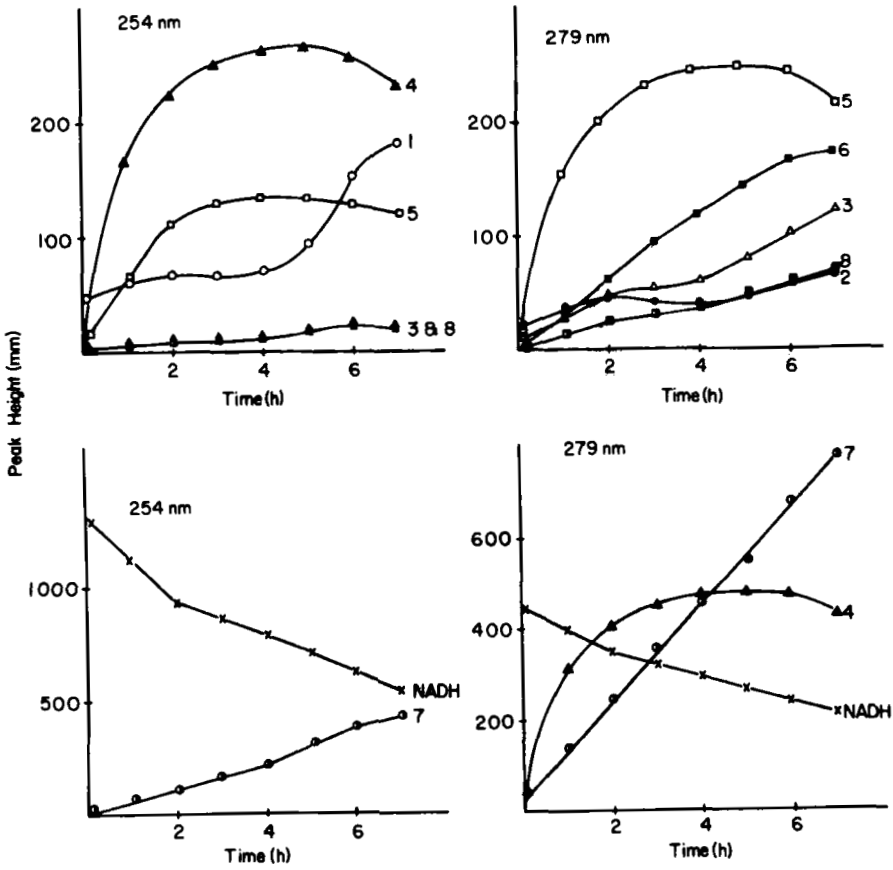


FIGURE 6

Progress curves for the acid-catalyzed rearrangement products of NADH in 0.1 mol/L sodium acetate buffer (pH 3.99) at 4 °C. Each chromatographic component was detected at both 254 nm and 279 nm. The values represent the measured peak height multiplied by the attenuation factor. The curves are numbered to correspond with the peaks in Figure 4.

However, the amount of peak 5 increased more rapidly than peak 4, indicating that peak 5 was formed before peak 4 (Table 2).

TABLE 2

Rearrangement of NADH in 0.1 mol/L Sodium Acetate (pH 4.8)

Time (min)	Peak Number 1	3	4	5	6	NADH	7	8
	Peak Height (mm)[a]							
2	34	--	9	18	--	1263	0	0
61	46	--	46	40	--	1233	5	0
125	55	11	78	52	8	1200	11	0
189	58	13	107	61	10	1146	16	2
253	62	14	140	68	11	1087	24	3

[a] Peak height equals the actual height at 254 nm times the attenuation factor.

In 0.1 mol/L KCl-HCl (pH 2.05), 90 percent of the NADH was destroyed within 15 min (Figure 7). No new peaks were formed. At 15 min, peaks 4 and 6 were also present in very small proportions compared to pH 3.99. (Figure 7 vs 6.) Peak 6 survived slightly longer than peaks 4 and 5; however, a comparison of the magnitude of peak 6 at pH 3.99 to that at 2.05 and an examination of the first 30 min of the reaction (Table 3) indicates that peak 6 also began to disappear at 15 min. Peak 7 reached a maximum at approximately 2 h whereas, peaks 1, 2, 3, and 8 continued to grow throughout the 5-h period. From these observations it appears that AMP, (peak 1) and peak 2 are final products and are preceded in order by peaks 3, 8, 7, and 6. All of these components were also observed at pH 5.70 after 72 h at 4 °C.

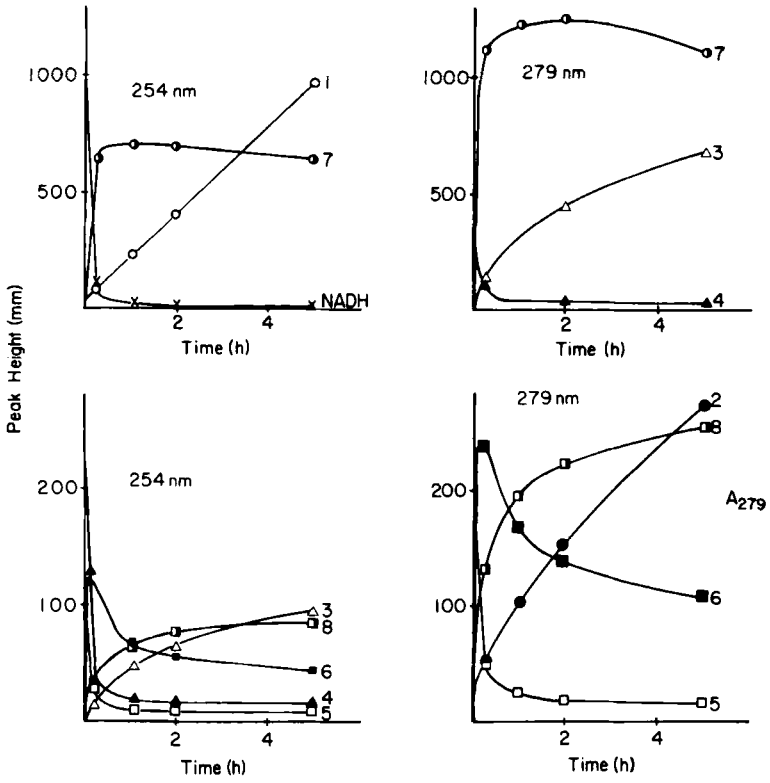


FIGURE 7

Progress curves for the acid-catalyzed rearrangement products of NADH in 0.1 mol/L potassium chloride-hydrochloric acid (5:1 v/v) (pH 2.05) at 4 °C. Each chromatographic component was detected at both 254 nm and 279 nm. The values represent the measured peak height multiplied by the attenuation factor. The curves are numbered to correspond to the peaks in Figure 4.

Identification of Compounds in Specific Peaks

In order to establish a relationship between the chromatographic peaks we found by reverse-phase LC and the structures in the acid-catalyzed rearrangement scheme (Figure 1), authentic specimens of the com-

TABLE 3

The Generation and Stability of (cTHN)AD and Other Compounds from NADH in 0.1 mol/L KCl-HCl (5:1 v/v) at pH 2.05

Time (min)	Peak Number 1	3	4	5	6	NADH	7	8
	Peak Height (mm)							
Experiment I[a]								
1	46	0	193	98	53	820	102	10
15	104	15	46	25	116	80	650	46
60	390	48	18	9	64	0	710	67
Experiment II[b]								
2	64	4	180	93	-	1600	43	3
5	137	8	305	160	-	1475	232	13
15	400	17	155	78	248	490	930	55
20	470	23	77	41	255	235	1070	69
20[c]	460	25	72	39	238	218	1030	62
30	630	26	47	26	227	95	1263	92

[a] Experiment I - LC analysis performed immediately after sample preparation.

[b] Experiment II - LC analysis performed on lyophilized samples.

[c] After solution for 4 h at 22 °C.

pounds in the scheme were needed. NADH was treated, as described by Oppenheimer and Kaplan (7), to produce (cTHN)AD. The compound formed at pH 2 within 15 minutes, and was the major product of the reaction (Figure 7 and Table 3, Experiment I). Its formation coincided with a 90 percent reduction in A_{340}. By reverse-phase LC the distribution of the products was unchanged when

examined after lyophilization and dissolution in 30 mmol/L ammonium bicarbonate (pH 10.2) (Table 3, Experiment II). The major peak had the same relative retention time as peak 7, indicating that peak 7 is (cTHN)AD.

(6HTN)AD was synthesized by use of G3PD (7,11). After 2.5 hours, when the A_{340} of the reaction mixture was reduced by 72 percent, the reaction mixture and appropriate controls were chromatographed on μBondapak C_{18}. The total integrated absorbance at 254 nm of the chromatographed products obtained in the presence or the absence of G3PD was the same. The data in Table 4 also indicate that during the incubation period 72 percent of the NADH was degraded in the presence of G3PD and that the major products (54% of the total area of all peaks) were peaks 4 and 5. Significant amounts of peaks 7 and 8 were also formed. Unexpectedly, in the absence of G3PD, the same products formed in the presence of pyrophosphate buffer but much more slowly. Furthermore, trace amounts of the same products were

TABLE 4

Percent Distribution of Rearrangement Products During Synthesis of (6HTN)AD

Peak Number	With G3PD	Without G3PD	Control[a]
1	5.5	5.0	5.0
4	32.6	6.6	1.3
5	21.7	3.9	0.9
NADH	27.7	82.3	92.2
7	9.7	1.9	0.2
8	2.3	0.2	0

[a] no G3PD and addition of NH_4HCO_3 to a final concentration of 0.8 mol/L.

detected in the pyrophosphate buffer in the absence of G3PD but with 0.8 mol/L ammonium bicarbonate present. The results also show that G3PD acts as a catalyst for this reaction, but is not essential. Since peaks 1 and 7 are known to be AMP and (cTHN)AD respectively, the product (6HTN)AD is either peak 4 or 5. The occurrence of 2 major peaks (peaks 4 and 5) in the incubation mixture which was reported to contain only (6HTN)AD indicates the presence of another compound which may possibly have been unstable and easily converted to (6HTN)AD or may have been lost in the reported isolation procedure (6).

Some authors have suggested that acids react with NADH in the initial step of the rearrangement (4,9,12). This possibility was tested by use of our LC method of analysis (10). NADH was incubated for 0.5 h in the presence of sodium [2-^{14}C]-acetate in which time 80 percent of the NADH was converted into products. No evidence was observed for the incorporation of [^{14}C]-acetate into any of the products. Despite the absence of evidence for an acetylated intermediate, acetate was found to accelerate the rate of acid-catalyzed rearrangement. The contribution of the various ionic and nonionic species to this acceleration was assessed by using the sodium acetate buffer in place of buffers of polyvalent anions (9). When the hydrogen ion concentration was held at pH 4.35 and the total acetate concentration was increased, the rate of rearrangement increased. When the hydrogen ion concentration was increased and the acetate was held constant, the rate of rearrangement also increased confirming the observations of other investigators (9,12,14). The rate of formation of each intermediate product was modified to the same degree for each set of experimental conditions in the presence of acetate buffer.

The late occurring peaks (1, 2, 3, and 8) are acid-catalyzed rearrangement products of NADH that have not been previously reported. Their uv spectra (Figure 8) indicate that they differ structually from their precursors. Peak 1 has the spectral characteristics of an adenine nucleotide, whereas peak 3 has an absorbance maximum at 280 nm. Since peaks 1 and 3 appear to be formed concurrently (Figures 6 and 7), they may be products of pyrophosphate cleavage of the dinucleotide. Spectra were not obtained for peak 2 and nor is any

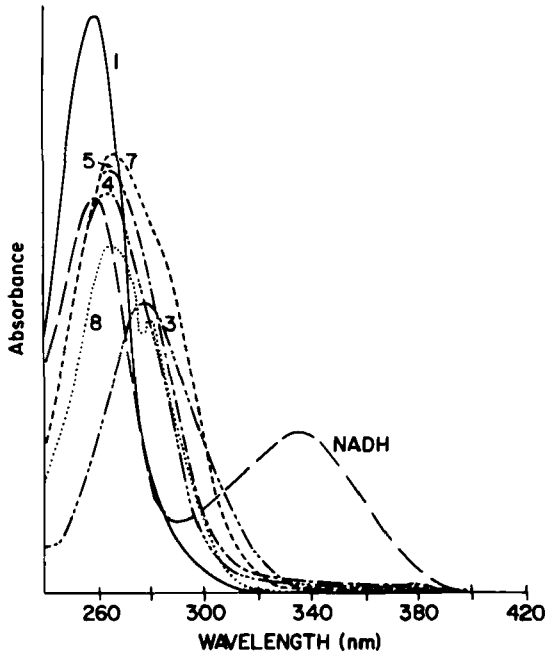


FIGURE 8

Absorption spectra of the materials separated by chromatography on μBondapak C_{18}. The curves are numbered to correspond to the peaks in Figure 4.

structural information available. Peak 8 shows λ_{max} at 267 nm and at 281 nm. The presence of λ_{max} at 267 nm and the more pronounced absorbance at 281 nm indicates a dinucleotide whose nicotinamide ring is modified beyond that of peak 7. Of the remaining compounds, peaks 4 and 5 have similar spectra with their λ_{max} shifted to 265 nm. A spectrum was not obtained for peak 6. The λ_{max} for peak 7 is at 268 nm with a distinct shoulder at 280 nm. This is consistent with the further modification of the nicotinamide structure.

DISCUSSION

Identification of Chromatographic Peaks

(cTHN)AD, the primary acid-catalyzed rearrangement product of NADH (3) and other acid-catalyzed rearrangement products referred to as NADHX (5) had been isolated and structually characterized by the use of nmr (6-8). We synthesized both compounds as previously described (6,7), and chromatographed them on μBondapak C_{18}. (cTHN)AD was found to correspond by this chromatography to our peak 7. It was the major product in the reaction mixture. It did not degrade after the reaction had been terminated by neutralization with ammonium bicarbonate nor after lyophilization followed by chromatography (Table 3). While synthetic NADHX had been identified as (6HTN)AD (6), our chromatography revealed two major products, with relative retention values that corresponded to chromatographic peaks 4 and 5. Furthermore, both 4 and 5 were found to be stable under lyophilization conditions (Table 3). Thus the prior identification of NADHX as solely (6HTN)AD appears to be in error.

The structure of NADHX is not certain. The addition of water across the 5-6 double bond has been demonstrated with model dihydropyridine compounds (13) and with NADH (6). Thus one of the two compounds may be the (6HTN)AD, but the nature of the other is not known. The later is not a buffer-anion derivative of (6HTN)AD since the acid-catalyzed rearrangement of NADH in [2-^{14}C]-acetate buffer did not result in the incorporation of radioactivity into the compounds in peaks 4 or 5 or any other peak. Since there is no evidence for a 2-hydroxy or a 2,6-dihydroxy structure in the pyridine ring of the products derived from the acid-catalyzed rearrangement of NADH (6-8), the most likely structure is the protonated NADH (II, Figure 1), proposed by Anderson and Berkelhammer (13), Oppenheimer and Kaplan (7) and Johnson and Tuazon (14).

The Chemical Basis for Chromatographic Separations on Microparticle ODS

The retention of the different nicotinamide adenine dinucleotides appears to be a function of both the dinucleotide structure and the nature of the nicotinamide ring. The less polar NADH and (cTHN)AD are better retained than the more polar NAD^+, (6HTN)AD, and protonated NADH. The absence of dinucleotide structure (AMP and NMN) markedly reduces the relative retention value to less than 0.36 (Table 1). We have also found a similar reduction in the relative retention value with more polar triphosphate compounds such as $NADP^+$ and NADPH. However, anomers are not separated by this system, e.g., α and β forms of NADH and NAD^+. Thus, the separation of the anomers of acid-catalyzed rearrangement products also might not occur.

Scheme of Acid-Catalyzed Rearrangement of NADH

The initial reactions for acid-catalyzed rearrangement have been studied previously with model dihydropyridine compounds (13,15) and with NADH and NADH analogs (4,6,7,9,12,16). Two rearrangement products of NADH, (cTHN)AD and (6HTN)AD, have been identified (6,7, 16). They exhibit enhanced absorbance at 270-280 nm compared to NADH. Although, Alivisatos et al. (9) on the basis of kinetic data, proposed that 3 compounds possessing this spectral behavior were produced in the acid-catalyzed rearrangement, we have detected seven by reverse-phase LC.

The sequence of formation of these peaks was uncovered by studying the reaction mixtures by reverse-phase LC after varying the pH and the duration of the reaction. The peaks are produced from NADH in the order 5, 4, 6, 7, 8, 3, 2. The number of peaks we detected correlates well (Table 5) with the structures proposed in the Oppenheimer and Kaplan scheme (7). Only the anomeric products that they proposed were not separated.

The multiplicity of NADH rearrangement products that have higher absorbance than NADH between 280 and 290 nm, validates the use of the $A_{260\ nm}/A_{280\ nm}$ or $A_{260\ nm}/A_{290\ nm}$ ratios as indicators of the extent of the acid rearrangement of NADH (10).

Several laboratories (9,12,13) have studied the kinetics of acid-catalyzed rearrangement of NADH assuming that the reaction yields only two or three compounds that absorb at 280 to 290 nm. This assumption is valid for reactions run at pH 5.7 in phosphate buffer at 4 °C for 6 h (Figure 5); however at low pH (Figures 6 and 7) or at higher temperatures, the compounds formed in peaks 2, 3, and 8 are present in

TABLE 5

Correlation of the LC Peaks with Their Precursors and Proposed Structures

LC Peak No. in Order of Appearance	Precursor LC Peak No.	Proposed Structure[a]
5	NADH	II and/or VI[b]
4	5	III and/or V[b]
6	4	IV
7	6	VII
8	7	dinucleotide; nicotinamide ring unknown
3	8	Lacks dinucleotide; nicotinamide ring unknown
2	3	unknown
1	8	AMP

[a] Roman numerals refer to structures in Figure 1.

[b] Anomers.

significant proportions and contribute significantly to the absorbance at 280 and 290 nm. Furthermore, at lower pH and higher temperatures only the rate of disappearance of absorbance at 340 nm is valid since none of the products of acid-catalyzed rearrangement absorb above 330 nm (Figure 8).

The Mechanism of the Initial Steps of Acid-Catalyzed Rearrangement of NADH

Johnson and Tuazon (14), after studying the stability of NADH in a large number of buffers in the pH range of 1 to 7, proposed that the first step in the acid-catalyzed rearrangement is a proton transfer from a general acid to the C_5 position of the NADH. At mod-

erate pH, they reported that the rate of proton transfer was dependent on the hydrogen ion concentration. A similiar first step in the rearrangement run in the presence of polybasic anions had been proposed earlier (9). It was again proposed, on the basis of the identification by nmr of (6HTN)AD and (cTHN)AD, (6,7).

The ability to separate rapidly the initial products of the rearrangement after they are formed in the presence of a monobasic buffer system afforded us the means for assessing the mechanism of the acid-catalyzed rearrangement. We found no evidence for the formation of any [2-^{14}C]-acetate intermediates using experimental conditions in which the intermediate products were demonstrably stable (Table 3). However, the rate of the rearrangement process was dependent on both the hydrogen ion concentration (at constant acetate concentration) and on the acetate concentration (at constant pH). Thus both the hydrogen ion and either the anion or the undissociated acid play an active role in a catalytic process, but it is not possible to define the precise role on the basis of these results. These observations are consistent with the proposed mechanism (7,9,12,14) and indicate that it is highly unlikely that a distinct chemical species is formed between the buffer and the nicotinamide ring.

REFERENCES

1. This research was partially supported by a research agreement with the National Institute of General Medical Sciences, NIH, and USPHS.
2. Abbreviations used: G3PD, glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12); (cTHN)AD, $O^{2'}$, 6-B-cyclotetrahydro-nicotinamide adenine

dinucleotide; (6HTN)AD, β-6-hydroxytetrahydronicotinamide adenine dinucleotide; LC; liquid chromatography.

3. Warburg, O., Christian W., and Griese, A., Biochem. Z., 282, 157, 1935.
4. Burton, R. M. and Kaplan, N. O., Arch. Biochem. Biophys., 101, 150, 1963.
5. Rafter, G. W., Chaykin, S., and Krebs, E. G., J. Biol. Chem., 208, 799, 1954.
6. Oppenheimer, N. J. and Kaplan, N. O., Biochemistry, 13, 4685, 1974b.
7. Oppenheimer, N. J. and Kaplan, N. O., Biochemistry, 13, 4675, 1974a.
8. Williams, T. J., Ellis, P. D., Bryson, T. A., Fisher, R. R., and Dunlap, R. B., Arch. Biochem. Biophys., 176, 275, 1976.
9. Alivisatos, S. G. A., Ungar, F., and Abraham, G. J., Biochemistry, 4, 2616, 1965.
10. Margolis, S. A., Howell, B. F., and Schaffer, R., Clin. Chem., 22, 1322, 1976.
11. Meinhart, J. O., Chaykin, S., and Krebs, E. G., J. Biol. Chem., 220, 821, 1956.
12. Stock, A., Sann, E., and Pfleiderer, G., Justus Liebigs Ann. Chem., 647, 188, 1961.
13. Anderson, A. G. and Berkelhammer, G., J. Amer. Chem. Soc., 80, 992, 1958.
14. Johnson, S. L. and Tuazon, P. T., Biochemistry, 16, 1175, 1977.
15. Wallenfels, K., Hofmann, D., and Schuly, H., Justus Liebigs Ann. Chem., 621, 188, 1959.
16. Chaykin, S., Meinhart, J. O., and Krebs, E. G., J. Biol. Chem., 220, 811, 1956.