Evidence for an Acid-Catalyzed α,β Epimerization in Pyridine Nucleotides*

D. W. Miles, D. W. Urry, and H. Eyring

ABSTRACT: Circular dichroism and optical rotatory dispersion measurements on four dihydropyridine nucleotides indicate that an α,β epimerization at the nicotinamide–riboside linkage accompanies an addition reaction across the 5,6 double bond of the dihydropyridine moiety when the reduced pyridine nucleotides are in the presence of acids or polybasic anions. Reduced 1,4-dihydronicotinamide ribonucleotide (NMNH), reduced α-nicotinamide–adenine dinucleotide (α-DPNH), β-DPNH, and β-deamino-DPNH show quite different optical rotatory dispersion and circular dichroism properties at neutral pH, but at low pH or at neutral pH in the presence of polybasic anions all four compounds give almost identical 280-mμ Cotton effects. The acid-catalyzed epimerization appears to favor closure in the α configuration after hydration of the 5,6 double bond of the nicotinamide ring. The effect of $HgCl_2$ on the dihydropyridine nucleotide gives additional evidence in support of the epimerization reaction.

The instability of DPNH[1] in acid has been known for some time (Warburg and Christian, 1934; von Euler *et al.*, 1938; Haas, 1936), and is characterized by the disappearance of fluorescence, the loss of absorption at 340 mμ, and the appearance of a new band at 280–290 mμ. The investigation of the mechanism of this reaction utilizing model dihydropyridine derivatives has resulted in several conflicting proposals regarding the probable mechanism and the nature of the intermediates and products (Wallenfels *et al.*, 1959; Anderson and Berkelhammer, 1958; Stock *et al.*, 1961; Alivisatos *et al.*, 1964). Moreover it has been found that at neutral pH several other systems can cause similar changes in the absorption spectra of DPNH such as certain polybasic anions (Alivisatos *et al.*, 1965) (orthophosphate, for example), certain metal ions (Stock *et al.*, 1961), and the enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPD) (Rafter *et al.*, 1953; Meinhart and Chaykin, 1955). The product formed from DPNH in the presence of GAPD has been called DPNH-X (Chaykin *et al.*, 1956). DPNH-X may be reconverted to DPNH (as can the product formed in the early stages of the DPNH orthophosphate reaction (Alivisatos *et al.*, 1965) whereas the acid product or products cannot. A knowledge of the nature of these reactions and the products is of considerable interest from the standpoint of the chemistry of hydropyridine compounds.

The present communication utilizes the sensitivity of optical rotatory dispersion and circular dichroism to gain information on the molecular structure of the products of these reactions. The study includes both anomers of DPNH (Kaplan *et al.*, 1955) involving the nicotinamide–ribosidic linkage. The active form with yeast alcohol dehydrogenase has a β configuration (in which case the nicotinamide is on the same side of the ribose ring as the 5′-carbon) and the inactive form is the α derivative. In the α configuration the nicotinamide is on the same side of the ribose ring as the 2′- and 3′-hydroxyls.

Both the α and β forms of DPNH contain a β-adenylic acid moiety. Also included in the study is β-NMNH and β-deamino-DPNH which contains the hypoxanthine chromophore in place of the adenine chromophore. The results indicate that an α,β epimerization at the nicotinamide–riboside linkage accompanies an addition reaction, *i.e*, hydration, across the 5,6 double bond of the dihydropyridine moiety when the reduced pyridine nucleotides are in the presence of acids or polybasic anions. The acid-catalyzed epimerization appears to favor closure in the α configuration after addition to the 5,6 double bond of the nicotinamide ring.

Experimental Section

Circular dichroism and optical rotatory dispersion measurements were made on a Cary 60 spectropolarimeter equipped with the Model 6001 circular dichroism attachment. Scan speeds, pen period, and time constants were chosen to maintain sufficient response time to allow adequate signal-to-noise ratios. The circular dichroism unit was calibrated by using the Cary Model 1401 circular dichroism attachment for the Model 14. The standard used was an aqueous solution of *d*-10-camphor-

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: DPNH, reduced nicotinamide–adenine dinucleotide; NMNH, 1,4-dihydronicotinamide ribonucleotide; deamino DPNH, dihydronicotinamide–hypoxanthine dinucleotide; GAPD, glyceraldehyde 3-phosphate dehydrogenase.

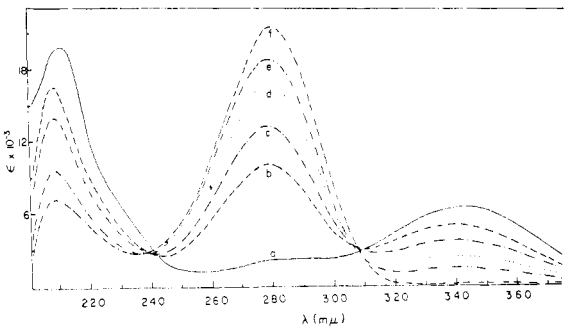


FIGURE 1: Spectral changes of β-NMNH in 1.5 M potassium orthophosphate (pH 6.6) at 25° as a function of time. Curve at zero time was run at 6.4×10^{-4} M in a 1-mm light-path cell without orthophosphate. All other curves were run at 3.2×10^{-4} M in a 2-mm cell. Appropriate blanks were placed in the reference beam. Time (in hours): a = 0, b = 4, c = 19, d = 34, e = 64, and f = 270.

sulfonic acid (J. T. Baker, lot no. 9-361) with an $\epsilon_1 - \epsilon_T$ of 2.20 ± 0.05 at 290 mμ. Sample temperatures were maintained with the Haake KT-62 Kryothermat and were monitored with a YSI Model 42SC telethermometer while spectra were being run. The sample was introduced by means of a syringe and Teflon tubing through a hole in the sample compartment so as to eliminate base-line anomalies arising from the manipulation of the cell compartment carriage. The base line was checked before and after all measurements. Absorption curves were determined on the Cary Model 14 spectrophotometer.

The biochemicals were purchased from Sigma Chemical Co. Assay data were supplied with each compound purchased. The purities of the biochemicals were all listed at 95% or better except for α-diphosphopyridine nucleotide (α-DPNH). The concentration of α-DPNH was established by using ϵ_{346} 5400 (Suzuki *et al.*, 1967). The superposition of the optical rotatory dispersion and circular dichroism curves of the α-DPNH and β-DPNH and acid product and phosphate product (Figures 5–7) verifies the use of the above extinction coefficient.

The circular dichroism and optical rotatory dispersion of the acid product and the mercury product were measured when the 340-mμ band had completely disappeared. Before performing the measurements on the acid product the pH was adjusted to 7. The circular dichroism of the phosphate product was measured immediately after the last curves in Figure 1–4.

Results

Figure 1 shows the effects of 1.5 M orthophosphate at pH 6.6 on the absorption spectra of β-NMNH as a function of time. The 340-mμ absorption band is observed to decrease progressively with the simultaneous appearance of a new band at 280 mμ. In addition a reduction in absorption at 210 mμ is also found to occur as the orthophosphate–NMNH reaction progresses. The time-dependent changes in the absorption spectra of α- and β-DPNH and β-deamino-DPNH in 1.5 M orthophosphate are given in Figures 2–4, respectively, and are quantitatively similar to the changes occurring in the β-NMNH once the contributions of the adenine or hypoxanthine chromophores of these compounds are removed from the absorption curve. The reaction of orthophosphate with either β-NMNH or α-DPNH proceeds twice as fast as with β-DPNH or β-deamino-DPNH. Alivisatos *et al.* (1965) has previously observed that

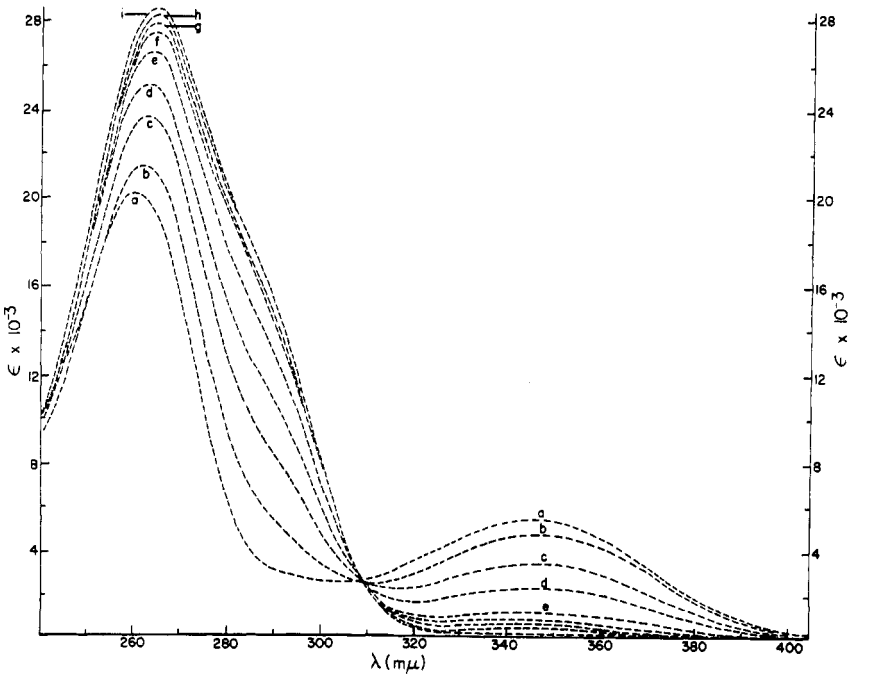


FIGURE 2: Spectral changes of α-DPNH in 1.5 M potassium orthophosphate (pH 6.6) at 25° as a function of time under conditions similar to those described under Figure 1. Time (in hours): a = 0, b = 3, c = 14, d = 33, e = 60, f = 91, g = 120, h = 210, and i = 360.

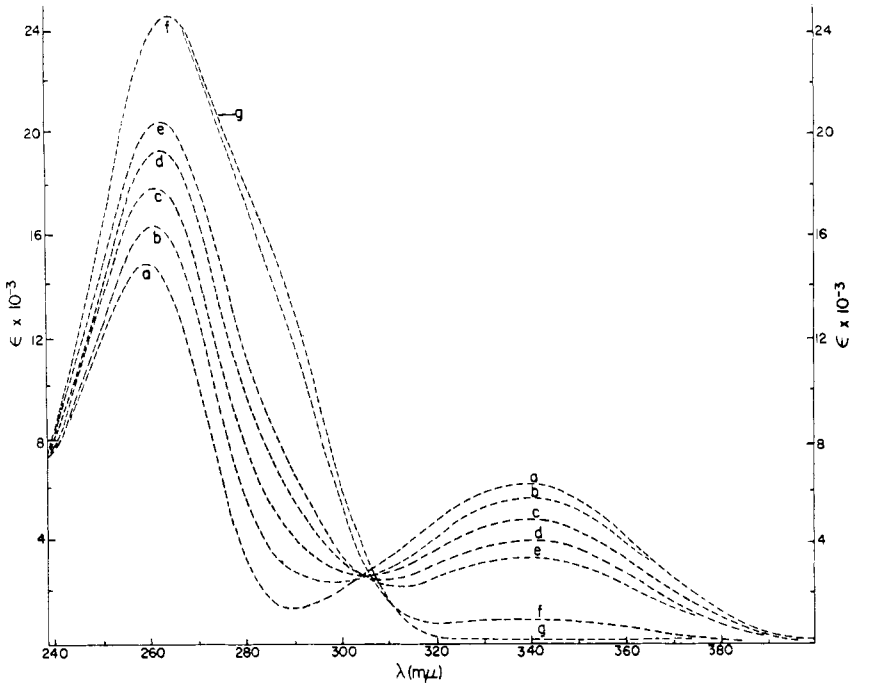


FIGURE 3: Spectral changes of β-DPNH in 1.5 M potassium orthophosphate (pH 6.6) at 25° as a function of time. Conidtions were similar to those described under Figure 1. Time (in hours): a = 0, b = 17, c = 45, d = 75, e = 110, f = 360, g = 900.

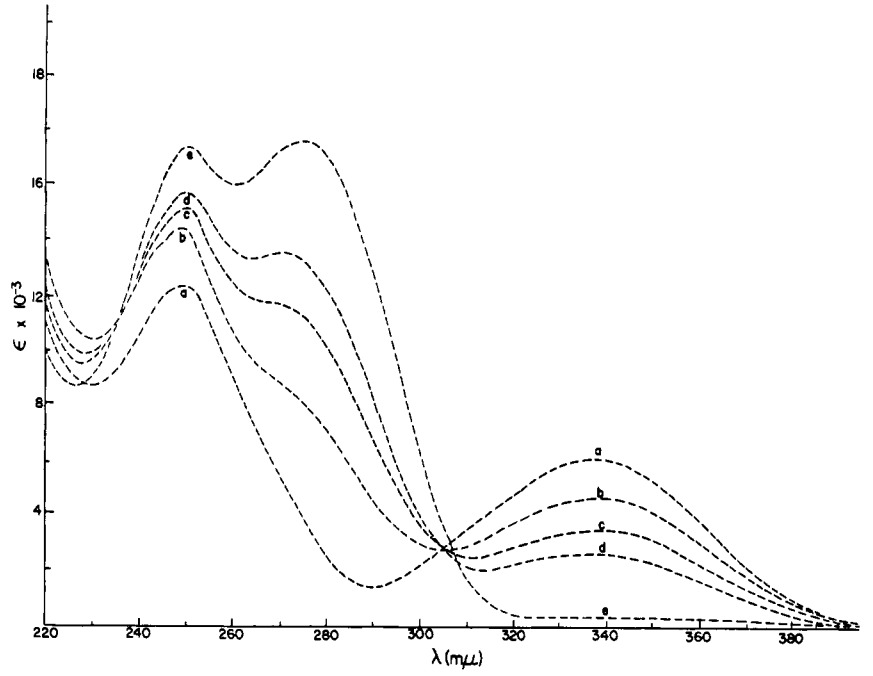


FIGURE 4: Spectral changes of β-deamino-DPNH in 1.5 M potassium orthophosphate, pH 6.6 at 25° as a function of time. Conditions were similar to those described under Figure 1. Time (in hours): a = 0, b = 60, c = 120, d = 180, and e = 900.

NMNH reacts much faster than β-DPNH with orthophosphate and related the difference in rates to the folded conformation of β-DPNH in solution.

Figure 5 contains the optical rotatory dispersion curves of α-DPNH and β-DPNH at neutral and acidic pH values. At neutral pH both the α-DPNH and β-DPNH have negative Cotton effects corresponding to the characteristic 340-mμ and 260-mμ absorption maximum. The β anomer displays a slight Cotton effect corresponding to the characteristic 340-mμ absorption maximum, while α-DPNH has a much more pronounced Cotton effect at this position. The situation is reversed for the Cotton effects centered around 260 mμ. These differences will be discussed in a following publication. In this communication we wish to emphasize the equilibrium curves at acidic pH. The acid product curves for both anomers are seen to be nearly identical. Furthermore the Cotton effects at 340 and 260 mμ have

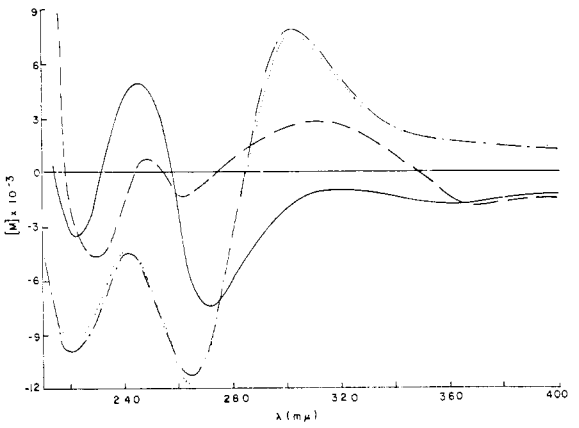


FIGURE 5: The optical rotatory dispersion of α-DPNH and β-DPNH at pH 7.6 and 3.0. The low pH curves were measured after 1-hr incubation time. Measurements on samples with or without 0.1 M phosphate buffer gave similar results. (——) β-DPNH, pH 7.6; (— — —) α-DPNH, pH 7.6; (— ·) β-DPNH, pH 3.3; (· · · ·) α-DPNH, pH 3.0.

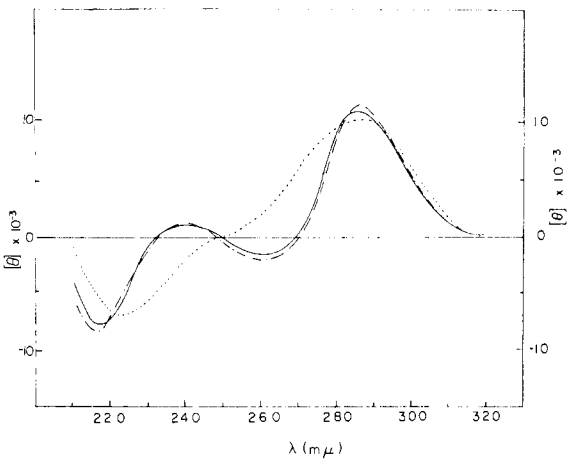


FIGURE 7: The circular dichroism of α-DPNH, β-DPNH, and β-NMNH after incubating in 1.5 M potassium orthophosphate (pH 6.6) at 25°. Curves were run immediately following the last absorption curve of Figures 1-3. (——) α-DPNH; (— · —) β-DPNH; (· · · · ·) β-NMNH.

disappeared and a relatively large positive Cotton effect centered at about 280 mμ now dominates the curves. This characteristic curve of the acid product of either α or β anomers of DPNH is not altered when the pH is adjusted to neutral pH.

The circular dichroism curves of α- and β-DPNH, β-NMNH, and β-deamino-DPNH at acidic pH values and in the presence of 1.5 M orthophosphates at neutral pH are shown in Figures 6 and 7, respectively. All four compounds give large, positive circular dichroism bands with maximum ellipticities of very nearly equal magnitudes at about 280 mμ. Note that in both figures the circular dichroism curves for α- and β-DPNH are almost superimposable. This supports the value reported by Suzuki *et al.* (1967) for the 346-mμ extinction coefficient of α-DPNH. The differences in the other curves of Figures 6 and 7 below 280 mμ may be ascribed to the Cotton effect arising from transitions in the adenine or hypoxanthine rings. What we wish to emphasize is that, while these compounds show quite different optical rotatory dispersion and circular dichroism properties at neutral pH, in acid environment or at neutral pH in the presence of polybasic anions all four compounds give almost identical 280-mμ Cotton effects despite differences in anomeric configuration or the presence or absence of the adenine ring and its ribophosphate moiety. The most logical interpretation of this striking similarity in circular dichroism behavior is the occurrence of an α,β epimerization involving the nicotinamide–ribosidic linkage (see Discussion section).

Figure 8 shows the effect of divalent mercury ions on the optical rotatory dispersion of the α- and β-DPNH. It is noted that both curves are greatly altered, but of special importance is the similarity of the 285-mμ Cotton effect of the α-DPNH–Hg system with that of the acid product optical rotatory dispersion curve given in Figure 3. Figure 9 shows that the effect of $HgCl_2$ on

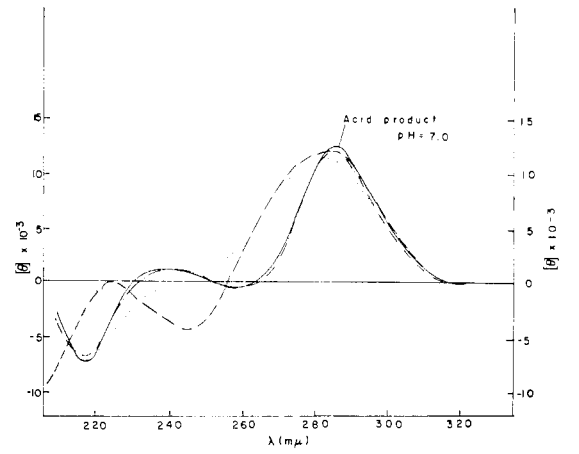


FIGURE 6: The circular dichroism of the acid product of α-DPNH, β-DPNH, β-NMNH, and β-deamino-DPNH. Curves were run after 1-hr incubation time at pH 3. Before making the circular dichroism measurements the pH was adjusted back to 7. Measurements were made on 6.5×10^{-4} M solutions in a 1-mm cell. (——) α-DPNH; (— · —) β-DPNH; (· · · · ·) β-NMNH; (– – –) β-deamino-DPNH.

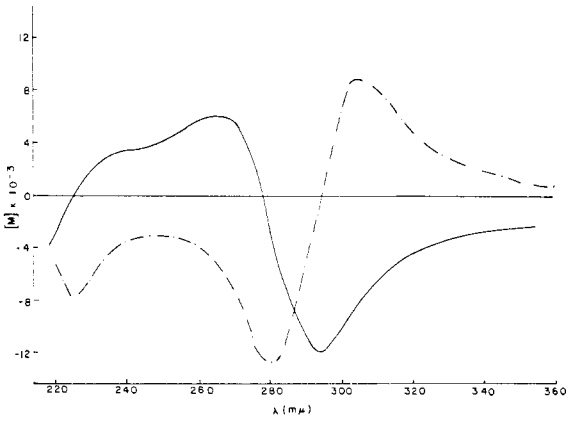


FIGURE 8: The optical rotatory dispersion of α-DPNH and β-DPNH in solutions containing equimolar concentrations of $HgCl_2$ at pH 7 in 0.1 M phosphate buffer. Measurements were made 1 hr after addition of $HgCl_2$. (——) β-DPNH–Hg^{2+} and (— · —) α-DPNH–Mg^{2+}.

NMNH is to produce a large negative Cotton effect at about 280 mμ. An absorption study on this system shows that addition of mercuric chloride in equimolar amounts quickly eliminates the 340-mμ absorption band of NMNH and produces a large 280-mμ absorption band in a manner similar to the effects of acid and phosphate. The reaction of mercury and other metal ions with these systems has been studied extensively by Stock *et al.* (1961). However the significance of the circular dichroism measurements depicted in Figure 9 is that the mercury product with NMNH gives a large negative Cotton effect at 280 mμ. Upon adding acid to this system an almost exact mirror image curve is produced. The results of Figures 8 and 9 will be discussed as additional evidence of an acid-catalyzed epimerization reaction in which the integrity of the ring system is maintained (Alivisatos *et al.*, 1965).

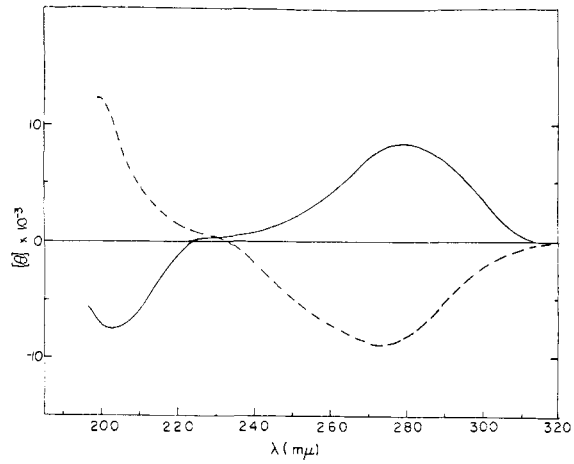
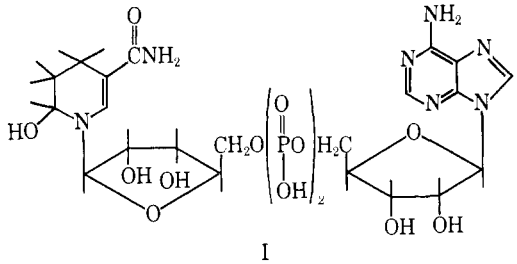


FIGURE 9: The circular dichroism of β-NMNH in a solution containing equimolar concentrations of $HgCl_2$ in 0.1 M phosphate buffer. The dashed line represents the measurement after 1-hr incubation time. The solid line represents the measurement after the pH was adjusted to 3 for 0.5 hr and then readjusted to pH 6.8. (- - - -) β-NMNH-Hg^{2+}, pH 6.8; (——) β-NMNH-Hg^{2+}, pH 6.8.

Discussion

An unambiguous feature of the reduced diphosphopyridine nucleotide curve (Figure 5) occurs when the pH is lowered to approximately three. Both α- and β-DPNH give optical rotatory dispersion curves of the same form, indicating the occurrence of an α,β epimerization (an inversion of the configuration at C-1'). Furthermore the amplitude of this 280-mμ Cotton effect provides strong evidence that the integrity of the ring system is maintained. A ruptured ring has been suggested for the acid product (Burton and Kaplan, 1961). Opening of the nicotinamide ring would not be expected to result in such a strong Cotton effect due to allowed rotations about the N–C_1 glycosidic bond and the N_1–C_2 bond of the former ring system of the chromophore (Kauzmann–Eyring (1941) rule). Furthermore raising the temperature to 70° causes no more than a 30% reduction in amplitude of the band. The optical rotatory dispersion results are more in accord with the structure of an acid product of a nicotinamide analog reported by Anderson and Berkelhammer (1958) where water had been added across the 5,6 double bond of the reduced nicotinamide ring as in structure I. Since both α- and

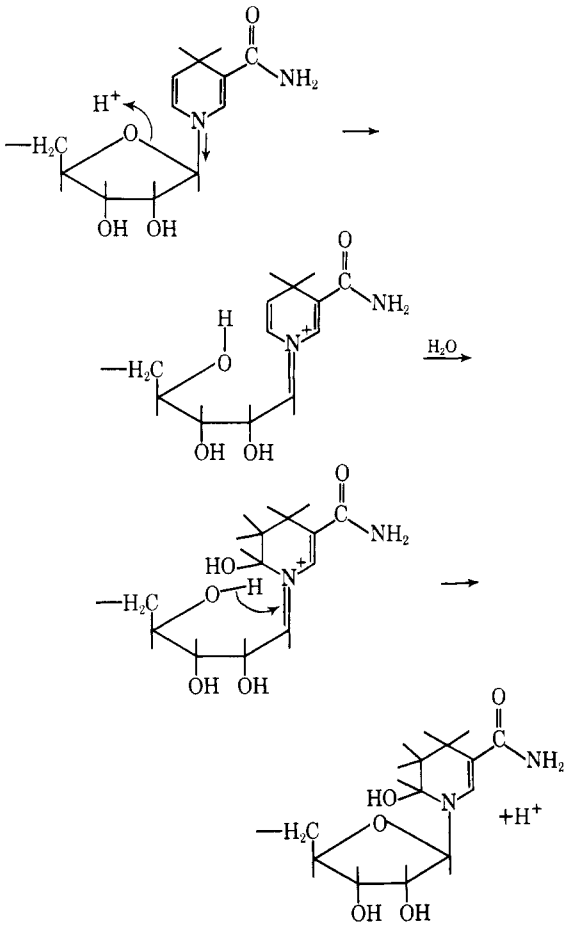


I

β-DPNH give the same acid product in which the ring system is maintained, we suggest that Scheme I be considered.

It may be seen that the 280-mμ Cotton effect is large when compared with the 340-mμ Cotton effect of α- and β-DPNH. This could be due to a sterically preferred closing of the ribose ring resulting in attachment of the modified nicotinamide ring and/or could be due to restricted rotation of the more completely saturated ring. Part of the increase, however, merely reflects the larger dipole strength of the 280-mμ band as compared to the 340-mμ band since the rotational strengths of strongly allowed electric dipole transitions arise mainly by the Kirkwood (1937) mechanism where in the dipole–dipole approximation each sugar–nicotinamide interaction is proportional to the dipole strength of the transition in question. The hydroxyl group was placed at the 6 position in analogy to the Anderson and Berkelhammer analog but does not represent an interpretation of the optical rotation data. The remarkable similarity of the 280-mμ Cotton effect for the product of the acid reaction or the reaction in orthophosphate for NMNH, α- and β-DPNH, and β-deamino-DPNH suggest the following: (1) that the rotational strength of this band arises mainly from ribophosphate–nicotinamide interactions, otherwise similar Cotton effects for β-NMNH and β-DPNH or β-deamino-DPNH would be quite fortuitous in that this would require some combination of ribophosphate–nicotinamide and adenine- (or hypoxanthine) β-nicotinamide interactions to give the same rotational strength as the ribophosphate–nicotinamide interactions in β-NMNH; (2) that an α,β epimerization reaction has occurred in addition to the reaction that modifies the reduced nicotinamide chromophore. Similar 280-mμ Cotton effects for α- and β-DPNH lead to the following considerations. In the α anomer the 2'-OH is *cis* relative to the chromophore and molecular models indicate that the hydroxyl group restricts the rotation about the C_1'–N bond leading to an increase in optical activity (the Kauzmann–Eyring rule). If the α,β configurations were maintained, it is difficult to conceive of a way in which the more freely rotating β anomer could match the α anomer in sign and magnitude of its 280-mμ Cotton effect. The large rotational strength of this band is evidence in favor of an α closure, *i.e.*, clo-

SCHEME I



sure leading to α configuration, in the epimerization reaction.

The effect of mercury on the reduced pyridine nucleotides provides additional information on the nature of the acid or polybasic anion product. As both curves for α- and β-DPNH are greatly altered by the presence of mercury it is apparent that it has effected either a structural or conformational change in these nucleotides. More specifically it may be noted in Figure 8 that there is a striking resemblance between the long-wavelength Cotton effect of the α-DPNH–Hg system and that of the acid product. This supports the view that the acid product involves a sterically preferred α closure of the ribose ring. It has been suggested on spectral grounds that mercury adds across the 5,6 double bond in a characteristic olefin addition (Stock *et al.*, 1961).

The effect of mercuric ions on the circular dichroism pattern of NMNH (Figure 9) at neutral pH is to produce a large negative 280-mμ Cotton effect. It seems reasonable to expect that the addition of mercury to the 5,6 double bond would not effect the configuration at the C-1′ of the ribose moiety. If acid is subsequently added to the Hg–NMNH system, the large positive circular dichroism band at 280 mμ is now generated. This effect is most simply and logically explained in terms of the aforementioned epimerization reaction induced by acid and involving a sterically preferred α closure of the ribose ring.

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