Mechanisms of the Primary Acid Modification Reaction of Reduced Diphosphopyridine Nucleotide Models*

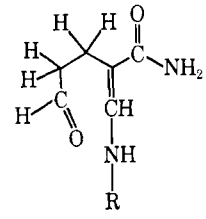
Chung Sul Youn Kim† and Sterling Chaykin

ABSTRACT: The primary acid modification reaction of a reduced diphosphopyridine nucleotide (DPNH) model compound has been studied. 1-(2,6-Dichlorobenzyl)-1,4-dihydronicotinamide (S) underwent reaction at pH 5 in tetrahydrofuran–water (40:60) to give a crystalline acid product (S–H_2O) in a good yield. S–H_2O was identified as 1-(2,6-dichlorobenzyl)-6-hydroxy-1,4,5,6-tetrahydronicotinamide by X-ray crystallography. An independent analysis of the nuclear magnetic resonance spectrum of S–H_2O led to the same conclusion. Kinetic analysis of the reaction mechanism was carried out using both spectrophotometric and isotopic rate effect techniques. The reaction proceeded in two steps, with initial protonation at the C-5 position of the nicotinamide ring and subsequent hydroxylation at the C-6 position. The reaction was second order (rate = $k(H^+)(S)$), indicating that only one of the steps was rate controlling. Under aqueous conditions the reaction showed a strong isotope effect when tritium or deuterium was used (k_H/k_T = 10 at 37° and 17 at 11°), indicating that protonation was the rate-determining step. However, under substantially anhydrous conditions (tetrahydrofuran or dioxane containing about 1% H_2O), the reaction showed no isotope effect, indicating that hydroxylation was the rate-determining step. The first step was shown to be reversible by a spectrophotometric method and by determination of the degree of tritium incorporation in the recovered reactant. The over-all reversibility of the reaction was demonstrated by subjecting S–H_2O to an anhydrous organic medium and observing the appearance of the characteristic absorption band of S (λ_{max} 350 mμ) and the formation of water (by nuclear magnetic resonance spectroscopy).

The instability of the reduced pyridine nucleotides, DPNH and TPNH, in acidic medium has been known for many years (Warburg and Christian, 1934; von Euler *et al.*, 1938; Haas, 1936; Karrer and Warburg, 1935). The reaction, which takes place in the nicotinamide moieties of these molecules is characterized by a shift of the ultraviolet absorption band with a maximum at 340 mμ downward to the 290-mμ region. The 290-mμ-absorbing compound, which has been referred to as the "primary acid modification compound" (Rafter *et al.*, 1953), is also unstable in acid. Its absorption at 290 mμ disappears in a so-called "secondary acid reaction."

The enzyme, glyceraldehyde 3-phosphate dehydrogenase, catalyzes a reaction of DPNH, which is characterized by a change in the ultraviolet spectrum similar to that which accompanies the primary acid reaction (Rafter *et al.*, 1953; Meinhart *et al.*, 1956). The product of the enzymic reaction is referred to as DPNH-X. Although "the primary acid product" is not identical with enzymatically produced DPNH-X (the former cannot be converted to DPNH by an enzyme system from yeast as DPNH-X can) it is thought that their chemical structures are very similar.

The chemistry of 1-substituted dihydronicotinamide compounds has been under continued investigation through the years (Burton and Kaplan, 1963; Segel and Stein, 1960; Stock *et al.*, 1961; Anderson and Berkelhammer, 1958; Diekmann *et al.*, 1964). These investigations, which have been carried out using the nucleotides themselves and various model compounds as well, have led to a number of different and conflicting mechanistic descriptions of the primary acid reaction. Burton and Kaplan (1963) proposed a reaction mechanism, which proceeds with initial protonation at the ring nitrogen of nicotinamide, ring opening, and the formation of an aldehydic function at C-6 (I). Segel



I

and Stein (1960) proposed the structure of the acid product to be a water adduct with the OH group on the C-5 position (II). This structure was based on their conclusion that the primary acid products formed from 1,4-dihydro (III) and 1,6-dihydro (IV) 1-substituted nicotinamides were identical.

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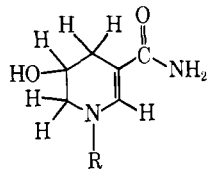
† Present address: Aerojet-General Corp., Sacramento, Calif.



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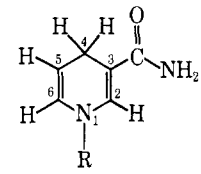

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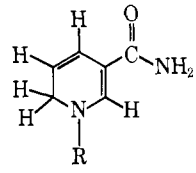
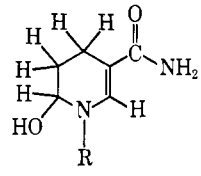


IV

Most of the other workers (Stock *et al.*, 1961; Anderson and Berkelhammer, 1958; Diekmann *et al.* 1964), however, favor a third reaction sequence in which a water adduct is formed with the hydroxy group on the C-6 position (V).



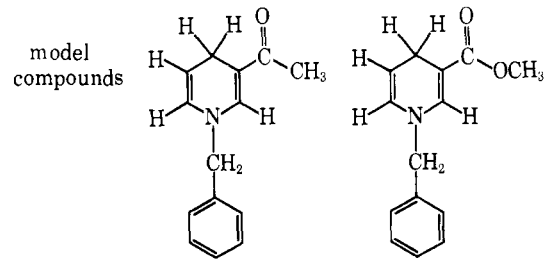
V

Anderson and Berkelhammer (1958) studied the kinetics of the acid reaction of various 1,4-dihydropyridine compounds in 50% ethanol–water solution. They found that all of the compounds they studied (VIa–e) underwent acid reactions characterized by second-order kinetics (rate = $K(H^+)$(reactant)). The rate of the reaction was directly related to the basicities of the dihydropyridine rings. Their extensive efforts to isolate crystalline acid products absorbing in the region near 290 mμ from these model compounds met with success only with VIa.

Although fraught with low yields and difficulties in the purification of intermediates, their direct chemical method to identify the "primary acid product" indicated that the structure (VII) which they assigned to the crystalline product was correct. However, the possibility remained that a different product might have predominated in the acid modification of the dihydropyridine compounds substituted with an amide group at C-3.

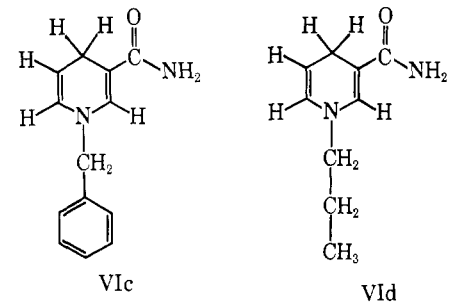
Diekmann *et al.* (1964) recently reported the isolation of a crystalline compound in a low yield (22%) (mp 161–162° dec) by treating VIII with diacetyl for a day, and believed that the crystalline compound was the primary acid product (IX). This assignment was primarily based on elemental analysis.

In summary, previous work reported in the literature indicates that the primary acid reaction of 1,4-dihydronicotinamide derivatives is second order, and that the product is a 1 mole of water adduct. The work of Anderson and Berkelhammer on the acetyl analogs suggested that, in the nicotinamide series, protonation at



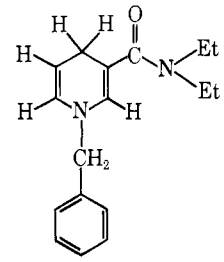
VIa VIb

relative rate 1 14



VIc VId

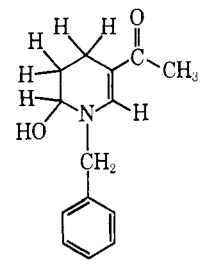
36 136



VIe

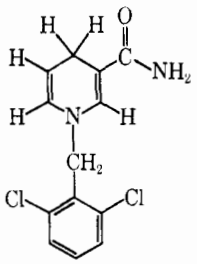
164

C-5 and hydroxylation at C-6 would also prove to be the rule; however, the conclusions of Segel and Stein (1960) have brought this extrapolation into question. The purpose of the present investigation was to clarify the mechanism of the primary acid reaction. Since current thinking on the mechanism by which inorganic

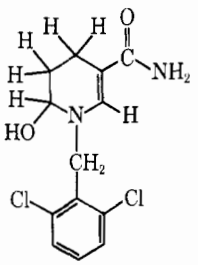


VII

phosphate might be fixed into a high-energy form during the mitochondrial oxidation of DPNH revolves about this question, it seemed worthwhile to firmly establish both the structures of the primary acid products of 1-substituted dihydronicotinamide compounds and the mechanisms by which they are formed.



VIII



IX

Experimental Procedure

Preparation of 1-Benzylnicotinamide Chloride. A solution of 61 g (0.5 mole) of nicotinamide (Sigma) and 107 g (0.85 mole) of benzyl chloride (Eastman) in 300 ml of methanol was refluxed over a steam bath for 3 hr.

Upon partial evaporation of the solvent and cooling of the reaction mixture, white colorless prisms formed, 118 g (95%). Recrystallization of the crude product from methanol yielded 94 g (75%) of colorless prisms: mp 241°, lit. (Anderson and Berkelhammer, 1958) 235–236° (with discoloration).

Preparation of 1-(2,6-Dichlorobenzyl)nicotinamide Chloride. A solution of 49 g (0.4 mole) of nicotinamide and 100 g (0.51 mole) of α-chloro-2,6-dichlorotoluene (K & K Laboratories, Hollywood, Calif.) in 200 ml of methanol was refluxed over a steam bath for 4 hr. The methanol was evaporated under vacuum, and the residual salt was washed with benzene to yield the crude product (109 g, 86%). Recrystallization of the crude product from methanol–water yielded 90 g (71%) of colorless prisms, mp 239–242°.

Preparation of 1-(2,6-Dichlorobenzyl)-1,4-dihydronicotinamide (S).[1] A solution of 12.8 g of 1-(2,6-dichlorobenzyl)nicotinamide chloride in 100 ml of water was added slowly at 45–50° to 600 ml of water in which 25.8 g of sodium dithionite (Mallinckrodt) and 13.8 g of anhydrous sodium carbonate had been dissolved. The reaction mixture was shaken for approximately 10 min and allowed to stand in the cold for 2 hr. The amorphous yellow precipitate which formed was ground to a fine powder and washed three times with water.[2] Recrystallization of the crude product from methanol–water yielded 10.8 g of yellow needles (95%): λ_{max} 350 mμ (ε 8 × 10³); mp 161°, lit. (Wallenfels and Schuly, 1957) 163–173° dec. 4-Monodeuterio-1-(2,6-dichlorobenzyl)-1,4-dihydronicotinamide was synthesized by the same procedure used above with the exception that D_2O was substituted for H_2O in the reduction mixture. Deuterium analysis indicated the incorporation of 0.99 atom of deuterium/mole.

Preparation of 1-Benzyl-1,4-dihydronicotinamide. The procedure was the same as that used in the preparation of S. The product was recrystallized from ethanol–water with a 75% yield: mp 110–112°, lit. (Anderson and Berkelhammer, 1958) mp 110–114°.

Primary Acid Reaction of S under Aqueous Conditions. Because of the insolubility of S in water, a mixed solvent system was used. Tetrahydrofuran (refluxed over sodium and distilled) was used as the first component in most experiments; dioxane and acetone were also found to be satisfactory. Acetate buffer (0.2 M, pH 4.0) was the second component. The buffer (600 ml) was flushed with nitrogen and added to a freshly prepared solution of 2.0 g of S in 400 ml of tetrahydrofuran. The reaction container, a brown bottle, was briefly flushed with nitrogen, sealed, and allowed to stand overnight at room temperature. After 18–20 hr, the apparent pH (as registered on a pH meter) of the reaction mixture was adjusted from pH 5 to 7. The optical density ratio, $\lambda_{290\ m\mu}/\lambda_{350\ m\mu}$, of the reaction mixture was approximately 20.

The solvent was removed from the reaction mixture by flash evaporation at room temperature. Small, pale yellow, starlike crystals slowly appeared in the residue. The crude product, after washing with water, weighed 1.7 g (85%). Most of the yellow material in the crude product was eliminated by washing with a small amount of tetrahydrofuran. The remaining powder was dissolved in tetrahydrofuran–water, treated with 0.5 g of Norit, and recrystallized to give 1.2 g of colorless diamond-shaped crystals of the acid product (S–H_2O) (60%): mp 185–190°; λ_{max} 287 mμ (ε 2.1 × 10⁴). *Anal.* (elemental composition based on a 1 mole of H_2O adduct) Calcd: C, 51.85; H, 4.68; Cl, 23.55; N, 9.30; O, 10.62. Found: C, 51.85; H, 4.65; Cl, 23.62; N, 9.30; O, 10.58 (by difference). Deuterated S–H_2O was synthesized by the same procedure used in the preparation of S–H_2O with the exception that D_2O was substituted for H_2O during the treatment with acid.

Primary Acid Reaction of 1-Benzyl-1,4-dihydronicotinamide. The reaction conditions used were the same as those used for the 1-(2,6-dichlorobenzyl)-nicotinamide derivative. The formation of the acid product was accompanied by an optical density ratio, $+\Delta_{290\ m\mu}/-\Delta_{350\ m\mu}$, equal to 3. This ratio is comparable to that observed in the formation of S–H_2O. The white amorphous product was used without recrystallization.

Reaction of S with Acid in Methanol. Freshly prepared S (0.5 g,) was dissolved in 200 ml of absolute methanol (dried over sodium sulfate); the yellow solution was placed in a brown bottle and flushed with nitrogen. The reaction was initiated by the addition of 20 μl of concentrated HCl and the reaction container was sealed. After 18 hr the reaction mixture was very pale yellow, and its optical density ratio, $\lambda_{290\ m\mu}/\lambda_{350\ m\mu}$, was 10–11. It was neutralized, dried with anhydrous sodium carbonate, filtered to remove the drying agent, and concentrated to 20 ml by flash evaporation. The concentrate was diluted with 50 ml of benzene and was again concentrated to about 20 ml. The crude product (pale yellow, 0.3 g) was precipitated out of the benzene solution by the addition of petroleum ether (bp 30–60°). It was dissolved in hot benzene, treated with about 0.5 g of Norit, and recrystallized from benzene–petroleum ether to give 0.16 g of colorless needles: mp

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: S, 1-(2,6-dichlorobenzyl)-1,4-dihydronicotinamide; S-MeOH, 1-(2,6-dichlorobenzyl)-6-methoxy-1,4,5,6-tetrahydronicotinamide.

[2] The product tends to incorporate sulfur, and careful washing is required to eliminate all traces of sulfur. The final crystalline compound gave a negative sulfur test.

162–164° (with slight discoloration); λ_{max} 287 mμ (ϵ 2.3 × 10^4). *Anal.* (elemental composition based on 1 mole of methanol adduct) Calcd: C, 53.35; H, 5.12; Cl, 22.50; N, 8.89; O, 10.15. Found: C, 53.42; H, 5.24; Cl, 22.62; N, 8.93; O, 9.79 (by difference).

The structure assigned to the product on the basis of its elemental analysis and nuclear magnetic resonance spectrum (Figure 1) was 1-(2,6-dichlorobenzyl)-6-methoxy-1,4,5,6-tetrahydronicotinamide (S-MeOH).

Tritium Isotope Effect in the Acid Reaction. A. IN 34:50 TETRAHYDROFURAN–WATER. Two different reaction temperatures were used.

Reaction at 37°. Acetate buffer (0.2 M, pH 4.0) (25 ml) was mixed with 100 μl of T_2O (specific activity approximately 1 Ci/ml) and placed in a brown bottle. The aqueous buffer was flushed with nitrogen before a fresh solution of 0.1 g of S in 17 ml of tetrahydrofuran was added. The mixture had an apparent pH of 5.1 (pH meter). The reaction bottle was flushed with nitrogen again, sealed, and placed in a 37° water bath for 4 hr. The reaction mixture was then chilled in an ice bath and the apparent pH was adjusted to 7 with 1 M NaOH. The optical density ratio, $\lambda_{290\ m\mu}/\lambda_{350\ m\mu}$, was between 13 and 14. The product, S–H_2O, was isolated by the method described above. The purity of the S–H_2O was verified by comparing its ultraviolet absorption spectrum with that of the S–H_2O prepared under the conditions specified above. The amount of tritium incorporated was determined by dissolving 2 mg of the dry sample (vacuum dried over P_2O_5 at room temperature for 2 hr) in 5 ml of Bray's scintillation solution[3] and counting in a Packard Tri-Carb scintillation counter. A second recrystallization of the sample from tetrahydrofuran–H_2O improved the purity (based on ϵ_{max}) from 80% to over 95%, but did not change the specific activity (after correction for purity on the basis of ϵ_{max}). The 2-mg sample did not give significant chemical quenching of an internal standard. The apparent isotope effect, k_H/k_T, was determined by dividing the specific activity expected if 1 g-atom of tritium had been incorporated per mole of acid product by the experimentally determined specific activity.

Reaction at 11°. The reaction conditions, except for temperature and reaction time, were the same as those used in the experiment carried out at 37°. S was allowed to react at 11° for 26 hr. The reaction mixture, after neutralization, gave an optical density ratio, $\lambda_{290\ m\mu}/\lambda_{350\ m\mu}$, of 8.5–9.0. The product, S–H_2O, was purified in the same manner as above, and the apparent isotope effect was determined.

B. IN SUBSTANTIALLY ANHYDROUS DIOXANE. A freshly prepared solution of 0.1419 g of S in 600 ml of anhydrous dioxane (spectroquality grade dioxane dried over sodium overnight) was placed in a brown bottle with a solution which consisted of 0.6 ml of 1.0 M HCl, 100 μl of T_2O (approximately 1 Ci/ml), and 10 ml of deionized water. The bottle was flushed with nitrogen, sealed, and allowed to stand at room temperature for 3 hr. The reaction mixture was then neutralized with 10^{-3} M NaOH. The optical density ratio, $\lambda_{290\ m\mu}/\lambda_{350\ m\mu}$, was approximately 6 before and after the neutralization. It was necessary to terminate the reaction at the end of 3 hr because of the many side reactions which appeared to become dominant at that time. (The optical density ratio, $+\Delta_{290\ m\mu}/-\Delta_{350\ m\mu}$, deviated from 3.) Dioxane was removed from the neutralized reaction mixture by flash evaporation. The precipitate which formed in the process was recovered by filtration, washed with water, and dried. Most of the yellow color associated with the crude product was eliminated when it was washed with a small amount of tetrahydrofuran. The product, S–H_2O, was recrystallized from tetrahydrofuran–water. Repeated recrystallization did not significantly change the tritium content of the sample. The purity of the S–H_2O was over 90% based on the ultraviolet spectrum (ϵ_{max} 2 × 10^4 at 287 mμ, $\lambda_{290\ m\mu}/\lambda_{350\ m\mu}$ 53); the yield was approximately 10%.

Reversibility of the Protonation Step in the Acid Reaction of 1-Benzyl-1,4-dihydronicotinamide. Since 1-benzyl-1,4-dihydronicotinamide reacted much faster than S, the former was used for this experiment. A freshly prepared solution (0.4 ml) of 1-benzyl-1,4-dihydronicotinamide (8 mg/5 ml of dioxane, sodium dried) was added to a solution containing 20 μl of T_2O (approximately 1 Ci/ml), 20 μl of 1.0 M HCl, and 80 μl of H_2O. Absorbance changes at 350 mμ were monitored during the 10-min course of the reaction (10 min, A_{340} 0.256). The reaction was then reversed by the addition of 5 μl of 1.0 M KOH per 3 ml of reaction mixture. When the absorbance changes associated with the addition of base had ceased (final A_{340} 0.500), dioxane was removed from the reaction mixture by flash evaporation. The residue was dissolved in methylene chloride, and 0.016 g of fresh reactant was added for cocrystallization. The methylene chloride solution was clarified by filtration, extracted with water, and finally evaporated to dryness before recrystallization of 1-benzyl-1,4-dihydronicotinamide from methanol–water. A portion of the yellow needles obtained in the first crystallization was recrystallized. Both the first and second crystals were carefully dried before their specific activities were determined; first crystals, λ_{max} 354 mμ (ϵ 7.0 ×10^3); second crystals, λ_{max} 354 mμ (ϵ 7.1 × 10^3). The same method used in the reaction of S with acid in methanol was employed in the determination of the tritium content in the recovered reactant. However, a correction for chemical quenching of these samples had to be made since 1.5 mg of the sample gave 26% quenching of an internal standard.

Results and Discussion

Reaction Conditions. The acid reaction of 1-substituted 1,4-dihydronicotinamides was studied under various conditions. The formation and isolation of the primary acid modification product were found to be dependent on the pH of the reaction mixture, the chemical reactivity and purity of the solvent, the presence of

[3] 2,5-Bis[2-(5-butylbenzoxazolyl)]thiophene (8 g) (Scintillation Grade from Packard), 60 g of naphthalene, 20 ml of ethylene glycol, and 200 ml of absolute methanol were dissolved in sufficient dioxane to make a total volume of 2000 ml.

oxygen, the concentration and purity of reactant, and the substituent on the ring nitrogen. Maximum yields of crystalline product were obtained when the reaction conditions conformed with the following. (a) The reaction mixture was only slightly acidic; this was in order that further reaction of the primary acid product could be avoided. (b) The water–organic solvent system was one in which the organic component did not enter into the reaction; *e.g.*, methanol was found to be an inappropriate solvent because it gave a methanol adduct as a side product. (c) The composition of the reaction medium had been adjusted to control the acid reaction rate. This point will be amplified later in the discussion. (d) The reaction was run in a nitrogen atmosphere; the presence of traces of oxygen tended to initiate free-radical reactions. (e) The reaction was carried out in dilute solutions of dihydropyridinium compounds, in order to avoid dimerization (Anderson and Berkelhammer, 1958). (f) Carefully purified reactant was used; impure reactant tended to give a significantly lower yield of the desired product. (g) The rate of the acid reaction was controlled by the proper choice of the substituent on the ring nitrogen; thus, when carefully purified 1-(2,6-dichlorobenzyl)-1,4-dihydronicotinamide (VIII) was allowed to react overnight at approximately pH 5 in aqueous tetrahydrofuran (40% tetrahydrofuran) at room temperature, the primary acid product, S–H_2O, could be obtained in good yield. Similar results were obtained using (3,4-dichlorobenzyl)-1,4-dihydronicotinamide.

Properties and Identification of S–H_2O. S–H_2O was stable at temperatures up to 180°, but rapidly decomposed at 185–190°. S–H_2O had an absorption maximum at 287 mμ (ϵ 2.1 $\times$ 10^4) in 95% ethanol. Its elemental analysis, after drying over P_2O_5 under vacuum, corresponded to the empirical formula expected from the addition of 1 mole of water to the reactant.

S–H_2O had unusual solubility characteristics. It was completely insoluble in solvents such as methylene chloride, chloroform, benzene, and diethyl ether; it was only sparingly soluble in solvents such as dioxane, tetrahydrofuran, and acetone. Upon slight dissolution in the latter group of solvents the crystalline compound immediately became amorphous. It was most soluble in pyridine, dimethyl sulfoxide, methanol, and ethanol. Its solubility was significantly enhanced when these solvents contained 5–10% water, although it was insoluble in water itself.

The air-dried crystalline S–H_2O was stable indefinitely under atmospheric conditions. However, when stored under dehydrating conditions (vacuum over P_2O_5) for 1 week at -10 to $+25$°, the crystals became amorphous. The ultraviolet spectrum of the amorphous material did not differ appreciably from that of the crystalline acid product. Warming of S–H_2O in an anhydrous, inert solvent caused it to deteriorate; the solution became yellow and the intensity of its absorption band at 287 mμ decreased. Evidence was obtained that various reactions took place under these conditions, including the slow conversion of S–H_2O back into S (increase in 350-mμ absorption) and loss of the group substituted on the ring nitrogen (higher N and lower Cl contents by elemental analysis).

It is most likely that S–H_2O is a primary acid modification product rather than a secondary or tertiary product since the ratio of the molar extinction coefficient of the product at 287 mμ to that of the reactant at 350 mμ ($\sim$3) is about the same as the ratio of $+\Delta_{287\,m\mu}/-\Delta_{350\,m\mu}$ which was observed during the initial stages of the primary acid reaction. The values of these ratios, however, were not exactly the same since λ_{max} and ϵ_{max} of the reduced nicotinamide derivatives varied significantly in different solvent media (Table I). Compounds absorbing in the 290-mμ region (IX and XII) were less prone to solvent-dependent spectral changes.

TABLE I: Absorption Maxima of 1-Benzylnicotinamide Derivatives.

	1,4-Reduced[a]		Primary Acid Product[a]	
Solvent	λ_{max} (mμ)	A	λ_{max} (mμ)	A
Dioxane	328	3.12	289	4.08
48% EtOH–H_2O	352	3.86	293	5.06
10^{-3} M NaOH in 48% EtOH–H_2O	350	6.65	293	5.07
10^{-3} M HCl in 48% EtOH–H_2O	358		293	5.08

[a] At the same concentration in all solvents.

It appeared that a choice among the several possible structures for the primary acid product could be made on the basis of a nuclear magnetic resonance study. Such an investigation was carried out and the results were consistent with S–H_2O being 1-(2,6-dichlorobenzyl)-6-hydroxy-1,4,5,6-tetrahydronicotinamide (IX) (Figures 1–8). The spectra of S–H_2O shown in Figures 4–6 were recorded on samples of S–H_2O which had been recrystallized from tetrahydrofuran–D_2O in order to remove exchangeable hydrogen atoms. Thus in Figure 4 the usual peak representing hydrogen atoms of amide and hydroxy groups was absent. The complex resonance peaks of δ values in the range 1.6–3.2 are typical of the interaction of the hydrogen atoms of adjacent CH_2 groups in a six-membered ring (Figures 4 and 5). This observation is not in agreement with Segel and Stein's proposal of hydroxy substitution at C-5 but does not permit a choice between a C-4 or a C-6 hydroxy group. Burton and Kaplan's proposed structure (I) is also incompatible with the presence of cyclic CH_2–CH_2 hydrogen peaks in the spectrum of S–H_2O. The absence of an aldehydic proton (δ 9–11 ppm) gives additional reason for believing S–H_2O is not I.

The use of deuterated compounds was resorted to in

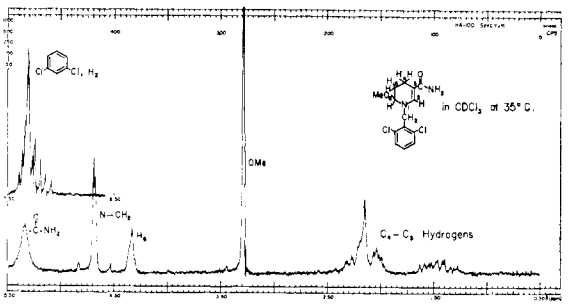


FIGURE 1: Nuclear magnetic resonance spectra of N-substituted dihydro- and tetrahydropyridine derivatives.

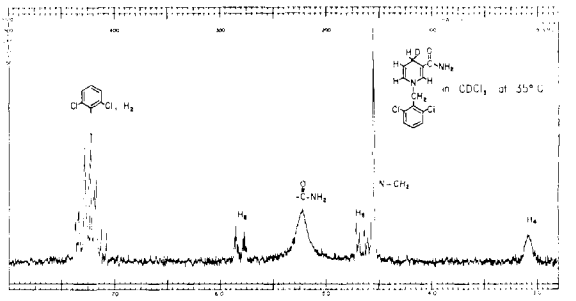
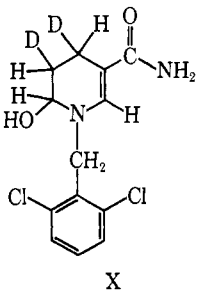
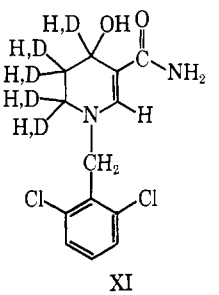


FIGURE 3: Nuclear magnetic resonance spectra of N-substituted dihydro- and tetrahydropyridine derivatives.

order to permit clear-cut peak assignments and to establish the pyridine ring position at which the hydroxy group was located; the spectrum of a dideuterio-S–H_2O is presented in the form of two half-spectra in Figures 7 and 8. The dideuterio-S–H_2O was prepared from 4-monodeuterio-S by reaction in acidic D_2O. The correctness of the assignment of the deuterium atom in the monodeuterio-S to C-4 can be verified by a comparison of the spectra in Figures 2 and 3. It is clear that the introduction of deuterium into S caused the area under the H_4 peak to fall to one-half of that in the nondeuterated S. The spectrum of dideuterio-S–H_2O (Figures 7 and 8) is consistent with a hydroxy group at C-6 (X) and not with a hydroxy group at C-4 (XI). The peaks at δ 2.45–5.02 are as expected for carbon atoms 4–6 each bearing a single hydrogen atom; a



X



XI

more complicated form would have been expected if dideuterio-S–H_2O was XI. Furthermore, in XI the peak corresponding to the CH α to the hydroxy group (δ 5.02) would be less than one-fourth of the area of the NCH_2 peak (δ 4.70), but the ratio of the peaks observed at δ 5.02 and δ 4.70 was 1:2. (More than 50% of the deuterium originally present in monodeuterio-S would have remained in XI if this hypothetical product had been formed in a reaction which involved an isotope effect.) A comparison of the NCH_2 peak of S–MeOH in Figure 1 with that of S–H_2O in Figures 7 and 8 also indicates the anion, the methoxy group, added at C-6 in that compound too. The NCH_2 peak of S–H_2O is a sharp singlet but that of S–MeOH is split; this is probably due to the steric effect of the methoxy group on C-6.

Although these peak assignments appeared to be correct it seemed desirable to obtain additional verification before attempting to use them in the determination of the structure of pyridine nucleotides, such as DPNH-X. X-Ray crystallography offered the means to obtain an unequivocal test of the preceding conclusions. To this end, Hope conducted an X-ray crystallographic study of S–H_2O, reported elsewhere (Hope, 1968), which proves the correct structure of S–H_2O is IX (Figure 9). Thus the crystallographic data provide the desired additional support for the conclusions of the nuclear magnetic resonance studies. The reason crystals of S–H_2O become amorphous immediately after dissolution in a substantially anhydrous organic solvent or upon exposure to a dehydrating atmosphere was also cleared up by the crystallographic study. The crystalline lattice of S–H_2O contains 2 moles of water/mole of S–H_2O. These water molecules, although crucial to the integrity of the crystal structure, are only held in the crystal lattice by weak hydrogen bonds. They can therefore be removed under anhydrous conditions, with the concomitant shattering of the crystal.

Reaction Mechanism. With the proposals of Segel and Stein (1960) and Burton and Kaplan (1963) clearly eliminated, the reaction sequence of Anderson and

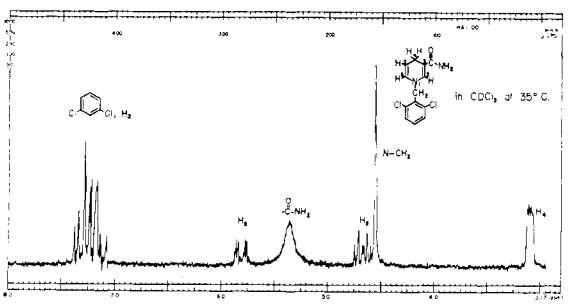


FIGURE 2: Nuclear magnetic resonance spectra of N-substituted dihydro- and tetrahydropyridine derivatives.

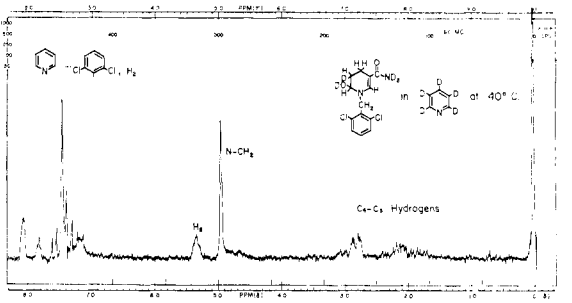
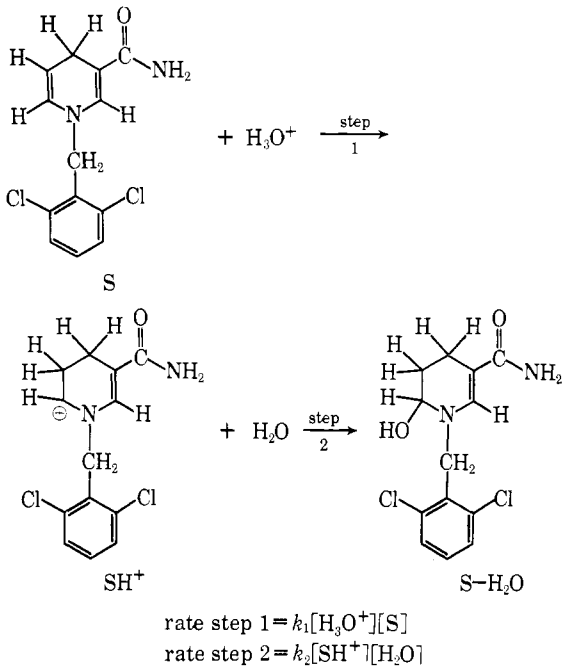


FIGURE 4: Nuclear magnetic resonance spectra of N-substituted dihydro- and tetrahydropyridine derivations.

SCHEME I

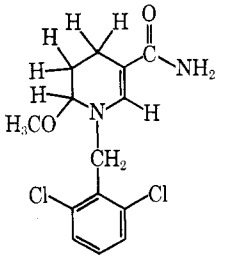


rate step 1 = $k_1[H_3O^+][S]$

rate step 2 = $k_2[SH^+][H_2O]$

Berkelhammer (1958) remained as the most reasonable description of the primary acid reaction (Scheme I).

This reaction sequence is analogous to that which has been assumed to be operative in other acid-catalyzed reactions of S. Wallenfels reported the addition of sulfurous acid (Diekmann *et al.*, 1964) and thiophenol (Wallenfels *et al.*, 1959) to 1,4-dihydropyridinium compounds. In this laboratory, S was shown to react with methanol under acidic conditions to give an addition product (XII). In short, all these reactions can be



XIII

described as proceeding by initial protonation and electrophilic reaction of the intermediate carbonium ion.

Kinetic studies by previous workers (Segel and Stein, 1960; Stock *et al.*, 1961; Anderson and Berkelhammer, 1958) as well as those performed in this laboratory show that the reaction is second order (rate = $k(H^+)(S)$) when the reaction is carried out in an aqueous medium. This indicates that either step 1 or step 2 is rate determining and that the rates in the two steps are not comparable. Anderson and Berkelhammer (1958) proposed a rapid equilibrium reaction in step 1 followed by a slow, essentially irreversible step 2. In order to clarify this aspect of the mechanism, the kinetics of the acid

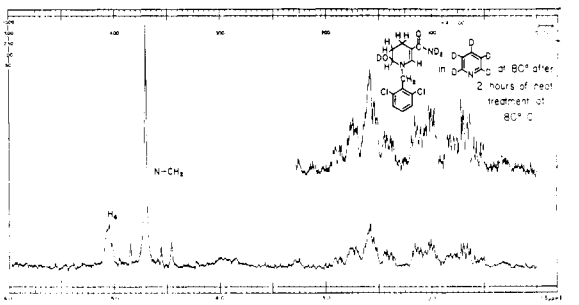


FIGURE 5: Nuclear magnetic resonance spectra of N-substituted dihydro- and tetrahydropyridine derivatives.

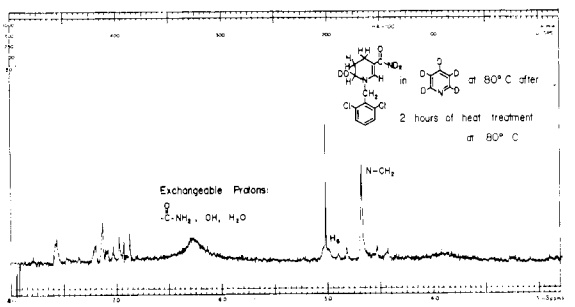


FIGURE 6: Nuclear magnetic resonance spectra of N-substituted dihydro- and tetrahydropyridine derivatives.

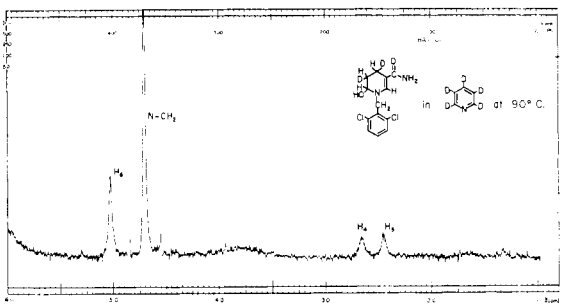


FIGURE 7: Nuclear magnetic resonance spectra of N-substituted dihydro- and tetrahydropyridine derivatives.

reaction were studied in reaction media containing T_2O or D_2O.

A. KINETICS IN AQUEOUS SYSTEMS. In the isotope studies which were carried out in mixtures of tetrahydrofuran and aqueous acetate buffer (pH 4.0), very strong isotope effects were found (Tables II and III). The incorporation of tritium into the acid product was significantly lower than would have been expected if there was no difference in the rates of reaction of tritium and hydrogen ions. It should be noted that the tritium atoms which were incorporated were nonexchangeable and that further purification of the acid product did not decrease the specific activity of the product (Table II). These data unequivocally demonstrate that the protonation step is accompanied by a significant isotopic rate effect, and that protonation is the rate-determining step in the over-all primary acid reaction. Tritium isotope effects have been shown to be temperature dependent, decreasing with increasing temperature (Wiberg, 1955). This was found to be true in the primary

TABLE II: Tritium Isotope Effect.[a]

Expt[b]	Reaction Temp (°C)	Wt Sample Counted (mg)	cpm/mg	cpm × 10^{-8}/mole[c]	App k_H/k_T	ϵ_{max} at 287 mμ
1A	37	1.9	2440	6.95	9.4	1.7×10^4
		2.2	2140	6.45		
1B		2.0	2040	6.14	10.1	2.0×10^4
		1.75	2060	6.20		
2A	37	2.6	2060	6.20	9.8	1.7×10^4
		1.6	2200	6.62		
2B		1.2	2240	6.75	9.6	1.9×10^4
		2.35	2090	6.30		
3A	11	2.1	1181	3.56	17.8	2.3×10^4
		2.3	1150	3.46		
4B	11	1.4	1250	3.75	17.1	
		2.5	1195	3.60		

[a] Acid reaction of 1-(2,6-dichlorobenzyl)-1,4-dihydronicotinamide in tetrahydrofuran–0.2 M acetate buffer (34:50) (pH 4.0), pH meter reading 5.1. [b] Samples of recovered reactant A were once recrystallized and samples B were twice recrystallized from tetrahydrofuran–H_2O. [c] Expected specific activity if one atom of hydrogen from water was picked up per mole of reactant = 6.25×10^9 cpm.

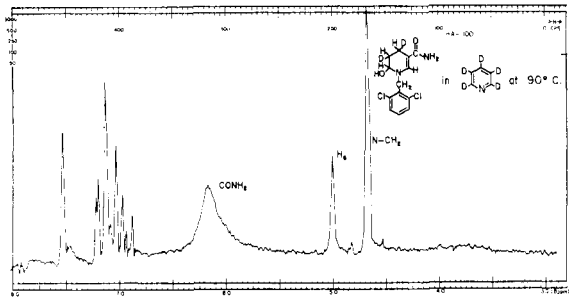
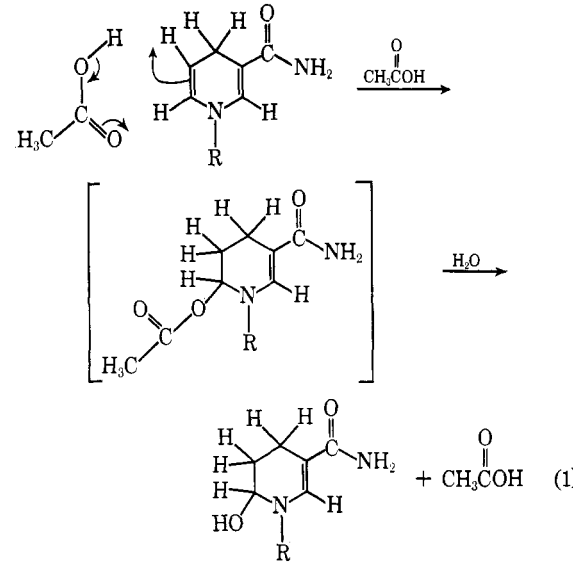


FIGURE 8: Nuclear magnetic resonance spectra of N-substituted dihydro- and tetrahydropyridine derivatives.

acid reaction system (Table II). The fact that the protonation step is the rate-determining step was given additional support by a more direct rate study. The relative rates of the acid modification reaction in H_2O and D_2O, as measured by the optical density ratios at 290 and 350 mμ, clearly show that the reaction is strongly retarded by deuterium (Table III). These findings are consistent with the general study of the mechanism of the hydration of enamines conducted by Stamhuis and Maas (1965) and Maas *et al.* (1967).

The strong isotope effect in the acid reaction led us to suspect that acetic acid might play an important role in the reaction system by competing with hydronium ion in the protonation step, possibly as shown in reaction 1. The kinetic experiments shown in Figures 10 and 11,[4] however, eliminate the direct reaction of acetic



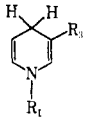
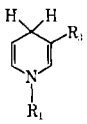
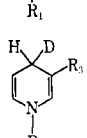
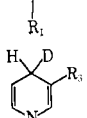
(1)

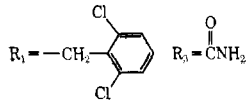
acid with the substrate as a major path for the synthesis of S–H_2O. Reaction systems B and C had the same free hydronium ion concentration, no greater than 10^{-3} M,[5] and system B contained 0.1 M acetic acid. The concentration of undissociated acetic acid was at least 100 times the hydronium ion concentration, yet the rates in systems B and C were about the same. Direct reaction with acetic acid was, therefore, negligible under these

[4] The differences in initial optical densities in the 350 mμ region were due to the differences in λ_{max} and ϵ_{max} of S in the different media; see Table I.

[5] Although hydronium ion concentrations determined with a pH meter are not accurate for solutions containing organic solvent, it was assumed that identical readings for systems having the same solvent mixture were indicative of the same hydronium ion concentrations.

TABLE III: Deuterium Isotope Effect.[a]

Expt	Reactant[b]	Solvent for Acetate Buffer	A at 290 mμ:A at 350 mμ after 20 hr
1		H_2O	15
2		D_2O	1.1
3		H_2O	17
4		D_2O	1.1

[a] Acid reaction in tetrahydrofuran–acetate buffer (40:60) (0.2 M, pH 4.0) at room temperature, pH meter reading 5.1. [b] 

reaction conditions. Further evidence in support of this argument was provided by the lack of reaction in a reaction mixture which contained acetic acid in substantially anhydrous tetrahydrofuran (system D in Figures 10 and 11). If the cyclic mechanism involving acetic acid did operate, the polarity of the solvent would not be expected to greatly affect the rate since charged intermediates are not involved. However, it is important to bear in mind that the primary acid reaction does occur by general acid catalysis although under the conditions used in these experiments the contribution by general acids was small compared to that of free hydronium ion. This small effect can be seen in Figures 10 and 11. The reaction mixture, which contained 0.1 M acetic acid in addition to hydronium ion, produced a somewhat greater rate (compare curves B and C). Near neutrality one would expect that the general acid catalysis would become relatively more important because of the very low concentration of free hydronium ion. Such effects have already been documented by Johnston and Alivisatos and their coworkers (Johnston *et al.*, 1963; Alivisatos *et al.*, 1965).

B. KINETICS IN SUBSTANTIALLY ANHYDROUS CONDITIONS. The rate of the acid reaction of the dihydronicotinamide derivatives in the presence of a strong acid was highly dependent on the nature of the reaction

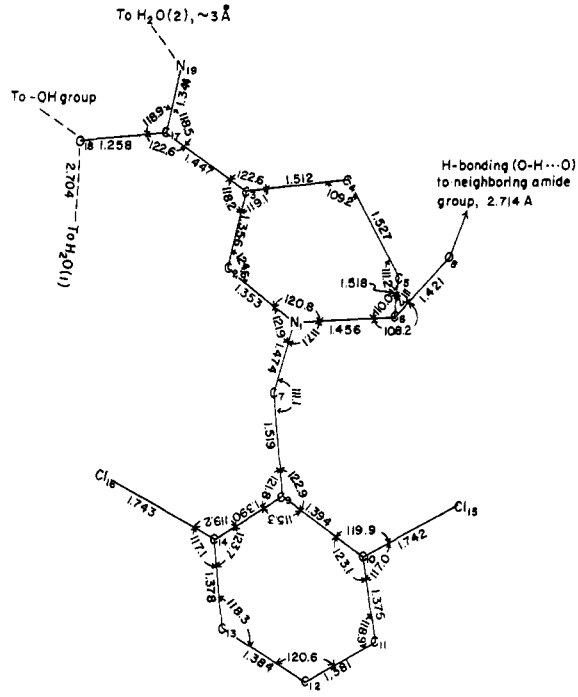


FIGURE 9: Distances and angles in S-$H_2O \cdot 2H_2O$. (1) Uncorrected for anisotropic motion; (2) hydrogen atoms omitted; and (3) atoms 1–4, 6, 7, and 17–19 are coplaner.

medium. Thus, absorption in the 350-mμ region fell more rapidly in substantially anhydrous media (Figure 12).

The increased rate of the protonation step might be explained by a decrease in the stability of the hydronium ion and an increase in the stability of the activated complex (assumed to be closely related to the protonated reactant (SH^+)) in solvents containing lesser amounts of water. These same conditions would also be expected to bring about a considerable retardation of the hydroxylation step because of the dependence of that

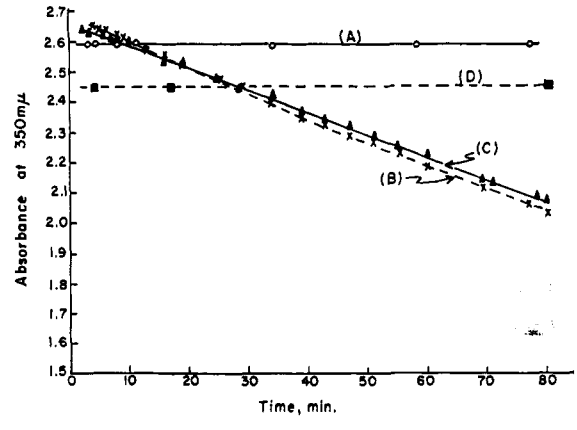


FIGURE 10: Decrease in absorbance at 350 mμ. Concentration of 1-(2,6-dichlorobenzyl)-1,4-dihydronicotinamide was 0.0060 g/50 ml of solvent. (○——○) (A) Control: H_2O–tetrahydrofuran (50:50), pH 7.0. (×——×) (B) 0.2 M acetic acid in H_2O–tetrahydrofuran (50:50), pH 3.65. (△——△) (C) H_2O–tetrahydrofuran (50:50), pH reading adjusted to 3.65 with HCl. (⊡——⊡) (D) 100% tetrahydrofuran containing 0.1 M acetic acid.

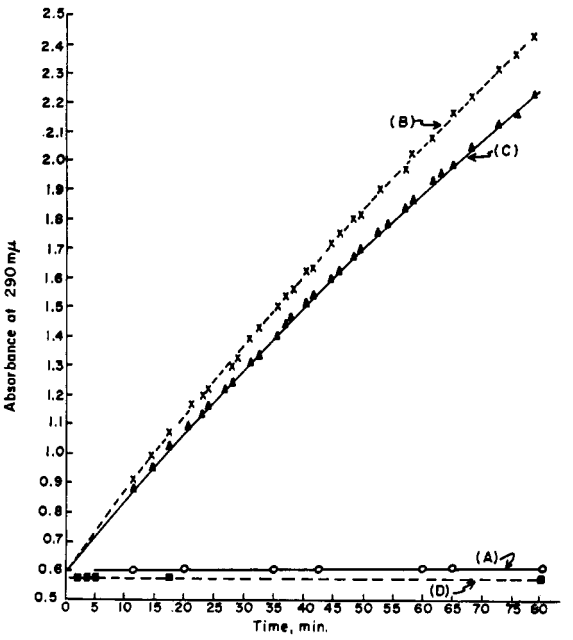


FIGURE 11: Increase in absorbance at 290 mμ. The same conditions as in Figure 10.

step on the water concentration. These two effects could and apparently do cause the hydroxylation step to become rate determining when the acid reaction is carried out in substantially anhydrous media. It is clear that the protonation reaction ceases to be rate limiting under such conditions since the isotope effect on the acid reaction of S disappeared when 0.97 M water in dioxane was used as solvent (apparent $k_H/k_T = 0.9$).

It has been reported (Meinhart and Hines, 1957) that the enzymatic formation of DPNH-X showed little isotope effect. It is possible in light of the previous discussion that the enzymatic reaction occurs in an environment which is substantially anhydrous; however, it is also possible that the orientation of DPNH on the enzyme prior to its protonation or a conformational change in the enzyme might be the rate-determining step in the enzymatic reaction.

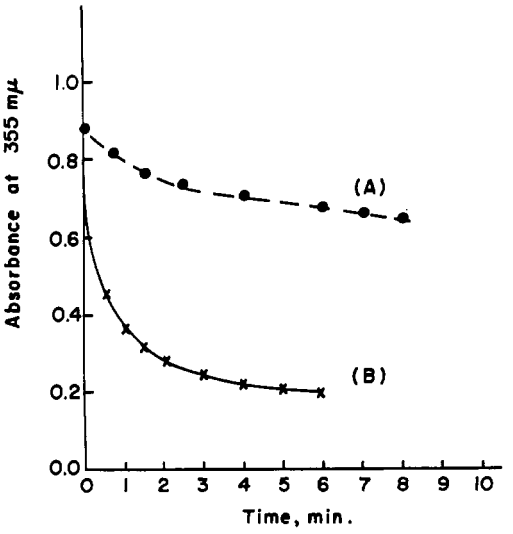


FIGURE 12: Relative rate of acid reaction. The reaction of 1.44×10^{-3} M 1-benzyl-1,4-dihydronicotinamide with 1×10^{-4} M HCl in (A) 48% ethanol–H_2O and (B) dioxane containing approximately 0.5% H_2O.

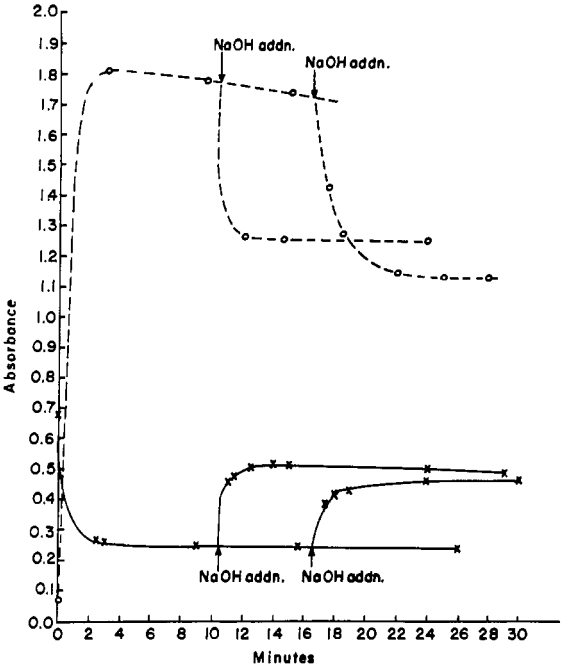


FIGURE 13: Reversibility of the protonation step. Absorption changes at 350 and 290 mμ which accompany the dissolution of 1-benzyl-1,4-dihydronicotinamide in dioxane containing 1.1% H_2O; 1.5×10^{-4} M reactant; 1.0×10^{-3} M HCl. (A) (O——O) 290 mμ; (B) (×——×) 350 mμ.

C. REVERSIBILITY OF THE ACID REACTION. It is not clear from a survey of the literature as to whether or not the acid reaction is reversible. Since no conclusive evidence has been presented for either alternative, an evaluation of this question was made as part of this investigation.

The reversibility of the protonation reaction was examined under anhydrous conditions. During the early phases of this reaction, SH^+ would be expected to be at its maximum concentration, since S is rapidly converted into SH^+ and SH^+ is only slowly converted to S–H_2O under anhydrous conditions. It was reasoned that if the protonation step was reversible the addition of base during the initial phases of the reaction should bring about the maximum observable reversal, *i.e.*, reappearance of absorption at 350 mμ. It was assumed in the design of such an experiment that the characteristic shift in absorption maximum from 350 to 290 mμ which accompanies the over-all reaction is associated with the protonation rather than the hydroxylation step. This was not considered unreasonable since protonation alone is sufficient to destroy one of the double bonds which make up the chromophore absorbing at 350 mμ. It was also important that only increases in 350-mμ absorption which were accompanied by decreases in 290-mμ absorption be considered as evidence for reversal. This was necessary because the 350-mμ absorption of any residual S would be expected to increase due to the greater extinction coefficient of S

at high pH values (Table I). Evidence for the reversal of the protonation reaction, which fits all of the criteria just outlined, is presented in Figure 13. The reaction rate and the degree of reversibility were found to be greatly affected by the concentration of water in the reaction mixture. For example, the protonation step in the forward reaction was 1.5 times faster in dioxane containing 1.1% water than in that containing 0.1% water. When S–H_2O was subjected to the same reaction conditions used in Figure 13 no evidence of reversibility was observed. Thus the reversible reaction documented in Figure 13 most probably is the protonation step. These data are also indicative of the fact that protonation can cause the shift in the absorption maximum of S from 350 to 290 mμ.

Further evidence for the reversibility of the first step of the acid reaction was obtained by recovering the reactant from a reaction mixture which contained T_2O. Except for the presence of T_2O the reaction conditions and procedures were the same as those used in Figure 13. The reactant which was recovered contained 0.23 g-atom of hydrogen derived from the medium (based on specific activity) per mole of reactant (based on 100% of the original reactant in the unreacted form); the control (pH 7.0, all other conditions the same) had about 2% tritium content. Here, the numbers themselves have no absolute significance since possible involvement of isotope effects was not calculated and neither the forward nor the reverse reactions were quantitative. However, the higher tritium incorporation into the recovered reactant under the acid conditions as compared to the control is strong indication of the reversibility of the protonation step.

Finally, it was possible to detect the over-all reversibility of the acid reaction by ultraviolet and nuclear magnetic resonance methods. The ultraviolet method was an extension of the procedure used to demonstrate the reversibility of the protonation reaction. Dehydrating conditions were substituted for the almost anhydrous system used earlier. The data in Table IV show that when S–H_2O was dissolved in dimethyl sulfoxide and allowed to stand in the presence of sodium hydroxide pellets the intensity of the 290-mμ absorption band decreased and the characteristic band of S at 350 mμ appeared. Reversal was not the only reaction which occurred; the observation of optical density ratios ($-\Delta_{290\ m\mu}/+\Delta_{350\ m\mu}$ greater than three are evidence for the existence of other reactions. It could be estimated from the peak ratios shown in Table IV that 21% of S–H_2O was converted into S and 14% of the 1-benzyl acid product was converted to its corresponding S before side reactions became dominant and interpretation of the results was made impossible.

Additional evidence in support of the over-all reversibility can be derived from the nuclear magnetic resonance studies. The sample of S–H_2O used in recording the spectra shown in Figures 4 and 6 was recrystallized from tetrahydrofuran–D_2O and therefore had deuterated amide and hydroxy groups. This was confirmed by the absence of an exchangeable hydrogen peak in the nuclear magnetic resonance spectrum (Figure 4). However, after a heat treatment of the sample in deuterated pyridine for 2 hr at 80° in a sealed system a typical broad peak corresponding to the combination of amide and hydroxy hydrogens appeared in the spectrum (Figure 6). This is a strong indication of the formation of water from S–H_2O, since all the hydrogen atoms in the S–H_2O sample were initially nonexchangeable. The same heat treatment of deuterated pyridine alone did not produce exchangeable hydrogen atoms.

TABLE IV: Spectrophotometric Changes of Acid Products When Dissolved in Dimethyl Sulfoxide over NaOH Pellets (20°).

Time	Absorbance $\lambda_{290\ m\mu}$	Absorbance $\lambda_{350\ m\mu}$	$\lambda_{290}/\lambda_{350}$	$-\Delta\lambda_{290}/+\Delta\lambda_{350}$
1-Benzyl-6-hydroxy-1,4,5,6-tetrahydronicotinamide				
0	1.51	0.021	72	
20 min	1.42	0.033	43	7(?)
1 day	1.29	0.065	20	4
5 days	1.19	0.111	11	3
7 days	1.17	0.091	13	
1-(2,6-Dichlorobenzyl)-6-hydroxy-1,4,5,6-tetrahydronicotinamide				
0	1.99	0.010	199	
20 min	1.66	0.044	40	10
1 day	0.503	0.139	3.6	16
2 days	0.382	0.125		

Acknowledgments

The advice of Professor Lloyd Ingraham is greatly appreciated. Nuclear magnetic resonance spectra were run by Dr. Norman Bhacca of Varian Associates, Palo Alto, Calif. The authors are indebted to one of the reviewers of the manuscript for calling the work of Stamhuis and Maas (1965) and Maas *et al.* (1967) to their attention.

Addendum

A paper concerning the characterization of the acid reactions of N-substituted dihydropyridine compounds using nuclear magnetic resonance spectroscopy appeared in this journal after the completion of this investigation (Choi and Alivisatos, 1968).

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Isolation of Co-5′-Deoxyadenosylcobinamide Guanosine Diphosphate, α-(2-Methyladenyl)-Co-5′-deoxyadenosylcobamide, and Co-Methylcorrinoids from *Clostridium thermoaceticum**

Eckart Irion† and Lars Ljungdahl‡

ABSTRACT: Co-5′-Deoxyadenosylcobinamide guanosine diphosphate (DA-GDP-cobinamide) and α-(2-methyladenyl)-Co-5′-deoxyadenosylcobamide (DA-factor A) have been found in *Clostridium thermoaceticum*. It is likely that the biosynthesis of complete B_{12} factors occurs as the Co-5′-deoxyadenosyl derivatives of corrinoids. DA-GDP-cobinamide, which is formed in a reaction between DA-cobinamide phosphate and guanosine triphosphate, is probably one of the intermediates in the formation of complete B_{12} factors. The presence of DA-factor A shows that *C. thermoaceticum* synthesizes B_{12} factors with derivatives of purines as the base of the nucleotide moiety as well as of the more abundant benzimidazoles, which were reported previously. Co-Methyl-factor IIIm, Co-methylcobyric acid, Co-methylcobyrinic acid pentaamide, Co-methylcobyrinic acid tetraamide, Co-methylcobyrinic acid triamide, Co-methylcobinamide, and a Co-methyl derivative with an unknown corrinoid moiety are present in *C. thermoaceticum*.

Corrinoids play an important role in the metabolism of *Clostridium thermoaceticum*. It is likely that α-(5-methoxybenzimidazolyl)-Co-methylcobamide (methyl-factor IIIm)[1] and Co-methylcobyric acid are intermediates in the total synthesis of acetate from CO_2 as performed by this organism (Ljungdahl *et al.*, 1965). The high content (30–70 μmoles of corrinoids/100 g of wet cells) and the great variety of corrinoids in *C. thermoaceticum* provide evidence that this organism also performs the biosynthesis of B_{12} compounds. We have previously reported (Irion and Ljungdahl, 1965) the presence of the complete B_{12} factor;[2] 5-methoxybenzimidazolylcobamide (factor IIIm) and of the incomplete B_{12} factors; cobinamide phosphate, cobinamide, cobyric acid, and di- to pentaamides of cobyrinic acid in *C. thermoaceticum*. These corrinoids occur mostly as their Co-5′-deoxyadenosyl derivatives. The incomplete factors are likely intermediates in the bio-

* From the Department of Biochemistry, Case Western Reserve University, Cleveland, Ohio 44106. *Received February 5, 1968.* This work was supported by a U. S. Public Health Service grant (GM 11839) from the Division of General Medical Sciences and by Contract AT (30-1)-1320 from the Atomic Energy Commission.

† Present address: Farbenfabriken Bayer A. G, Wuppertal-Elberfeld, Germany.

‡ Present address: Department of Biochemistry, University of Georgia, Athens, Ga. 30601. Address correspondence regarding this paper to this author.

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: factor IIIm, 5-methoxybenzimidazolylcobamide; factor A, α-2-methyladenylcobamide.

[2] Complete B_{12} factors are defined as corrinoids with a heterocyclic base normally attached as ligand to the cobalt atom (*e.g.*, 5,6-dimethylbenzimidazole in vitamin B_{12}). All corrinoids lacking a base are incomplete.