Spontaneous Reactions of 1,3-Substituted 1,4-Dihydropyridines with Acids in Water at Neutrality. I. Kinetic Analysis and Mechanism of the Reactions of Dihydronicotinamide–Adenine Dinucleotide with Orthophosphates*

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ABSTRACT: The spontaneous reactions of reduced nicotinamide–adenine dinucleotide (NADH, DPNH) with orthophosphates in neutral watery milieu were analyzed kinetically. They can be described by three consecutive second-order (pseudo-first-order) reactions. A graphical procedure, suitable for this kinetic analysis, was based on the observation that $k_1 \gg k_2 \gg k_3$. With the aid of this kinetic analysis it was shown that the reaction most probably involves three consecutive direct transfers of protons from the polybasic anions, which in this case act as proton donors, to the 1,3-substituted 1,4-dihydropyridine. Proton transfer depends on the dissociation constant of the acid (*i.e.*, the "polybasic anion") and other factors. When other conditions are kept constant, the rates of the reaction may serve as measures of the nucleophilicity of the enamine carbons in 1,3-substituted 1,4-dihydropyridines. With NADH as a reactant, initial protonation gives rise to a product which is very similar to the enzymatically prepared "DPNH-X." This indicates that the catalytic action of glyceraldehyde 3-phosphate dehydrogenase in promoting formation of "DPNH-X" is related to an acceleration of the direct transfer of protons from the "polybasic anions" to NADH.

We previously reported changes in the ultraviolet absorption spectra of nicotinamide–adenine dinucleotide (NAD)[1] in the presence of orthophosphates and certain other anions (Ungar and Alivisatos, 1961). In an extension of our observations, we recently studied the spontaneous reactions of NADH with dihydrogen phosphate and other "polybasic anions" (Stock *et al.*, 1961; Alivisatos *et al.*, 1964b). The products of these primary interactions react further with the acid anions, in a series of consecutive second-order reactions. A detailed kinetic analysis of these processes, involving graphical evaluation of spectrophotometric data, became necessary. A description of this graphical analysis and its application in the study of the probable mechanism of the reaction and the identity of intermediates and products are presented in this communication.

Materials and Methods

Inorganic monopotassium orthophosphate (Merck and Co.) was recrystallized four times from deionized water. Solutions of the recrystallized orthophosphate were adjusted to the desired pH values with 2 M potassium hydroxide free of carbonate. The absorbance of 1.5 M solutions of this material at pH 6.62 was ≤ 0.018 at 260 mμ, when readings were taken against water in 1-mm light path cuvets. The phosphorus content of the solutions was determined by the method of King (1932). Other chemicals were Fisher reagents with the exception of arsenic acid, which was Mallinckrodt Analytical Reagent. Nonbuffering salt solutions were kept at the desired pH with Tris–HCl, as described in the Results.

NAD and its various analogs mentioned in this paper, NADP, NMN, adenosine diphosphoribose, NADH, and NADPH, were purchased from the Pabst Laboratories. 1,4-Dihydropyridine analogs of NADH were prepared by incubation of 10 mg of each NAD analog in its oxidized form with 0.1 mg of crystalline alcohol dehydrogenase (Sigma Chemical Co.) in the presence of 50 μmoles of Tris–HCl at pH 9.0 and 0.05 ml of ethyl alcohol. The volume of incubation was 1.0 ml and the temperature was held at 37°. The time of incubation varied with the different analogs

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[1] Abbreviations: In accordance with accepted terminology, NAD analogs were abbreviated by the suffix AD (adenine dinucleotide) added to the common name of the substituted pyridine: *e.g.*, 3-acetylpyridine–adenine dinucleotide = 3-acetylpyridine-AD; N, nicotinamide, P, phosphate, H, reduced form (thus NADPH, reduced nicotinamide–adenine dinucleotide phosphate); NMNH, 1,4-dihydronicotinamide ribonucleotide.

(3-acetylpyridine-AD,[1] 15 min, 87% reduction; nicotinamide–hypoxanthine dinucleotide, 30 min, 75% reduction; 3-pyridinealdehyde-AD, 60 min, 50% reduction). At the end of the incubation the mixtures were immersed for 30 sec in boiling water and centrifuged in the cold (15,000 × *g*). The supernatant was used directly in our studies. NMNH was prepared by reduction of NMN with sodium dithionite (Liao *et al.*, 1962) with 77% yield. Reduction of NAD by sodium borohydride was performed according to Mathews (1948; Mathews and Conn, 1953) with a 58% yield. Nicotinamide 1-propochloride was prepared by the method of Holman and Wiegand (1948). It was reduced to 1-propyl-1,4-dihydronicotinamide following a procedure of Karrer (Karrer *et al.*, 1936) as described by Suelter and Metzler (1960).

An enzyme preparation from air-dried baker's yeast was prepared according to the first method described by the group of Krebs (Meinhart *et al.*, 1956). Mitochondrial preparations (Schneider and Hogeboom 1950) were exposed to 0.075 M sucrose for 10 min (Tapley and Cooper, 1956) in order to facilitate permeability of NADH or its products from the milieu. Hexokinase (Type IV) and glucose 6-phosphate dehydrogenase were products of the Sigma Chemical Co. Antimycin A was purchased from the California Corp. for Biochemical Research. Proteins were determined by the biuret method (Gornall *et al.*, 1949).

Spectrophotometric measurements were carried out in a PMQ II Zeiss spectrophotometer equipped with a servo-mechanism for automatic slit control. Differential spectrophotometry was performed in either a Beckman DK-1 or a Cary 14 recording spectrophotometer, using the "tandem cell" technique (Herskovits and Laskowski, 1961). All three instruments were equipped with thermoregulated cell compartments. Quartz cuvets of 1 mm and 10 mm light path were used. Nuclear magnetic resonance studies (in D_2O, to be described in detail in a following publication)[2] were performed with a Varian A-60 instrument located at the University of Illinois School of Pharmacy.

Ionophoretic studies and detection of ultraviolet-quenching spots on paper were as described previously (Alivisatos *et al.*, 1960). Detection of ^{32}P-labeled compounds on paper was accomplished autoradiographically (Alivisatos *et al.*, 1963). The radioactivity of eluted samples was determined in a D-47 gas flow counter equipped with a micromil window (150 $\mu g/cm^2$) supplied by the Nuclear Chicago Corp. (Liakopoulou and Alivisatos, 1965).

Results

As shown in Figure 1, the absorption spectrum of NADH at pH 6.62 changed considerably when the molarity of potassium orthophosphate in the milieu was increased from 0.05 to 1.5 M. Maximal differences

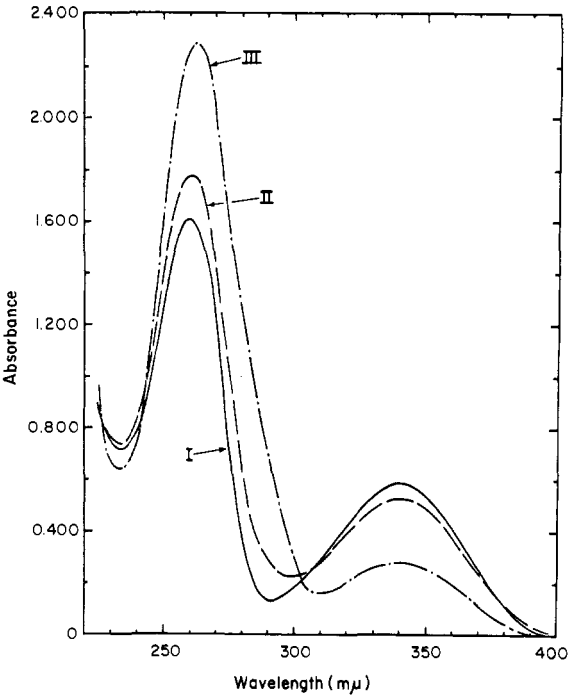


FIGURE 1: Spectral changes of NADH in potassium orthophosphate, pH 6.62, at 15.5°. Initial NADH concentration for all samples was 8.98×10^{-4} M. Readings in 1-mm light path cuvets, in a Cary Model 14 recording spectrophotometer. Absorbances above 1.8 were read in a PMQ II Zeiss spectrophotometer. Curve I, 0.05 M orthophosphate, zero time; curve II, 1.5 M orthophosphate, immediately after preparation of the sample; curve III, 1.5 M orthophosphate, after incubation for 19 hr at 15.5°. Blanks were appropriate orthophosphate solutions.

in absorbances were observed at 340 mμ and at about 280 mμ (Figure 2).

Kinetic Analysis. Changes occurring to NADH in the presence of orthophosphates at pH 6.62 could be written in a general form as follows:

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C \xrightarrow{k_3} D \xrightarrow{k_4} \text{.....etc.} \quad (1)$$

In this sequence, *A* stands for NADH and *B*, *C*, *D*, etc. are products of the consecutive reactions. Our studies indicate that under the conditions specified above three reactions occur during the experimental period shown in Figure 3 (see below). In the graphical kinetic treatment that follows, it is implicitly assumed that all reactions are irreversible and that the individual reaction rates are not influenced in any way by the accumulation of the reaction products of the immediate or the following reactions.

The time dependence of the transformation $A \rightarrow B$ was followed by the decrease in absorbance at 340 mμ. It is apparent from differences in the slopes of the lines in Figure 4A that the reaction is second order. How-

[2] C. L. Bell, F. Ungar, K. S. Choi, and S. G. A. Alivisatos: manuscript in preparation.

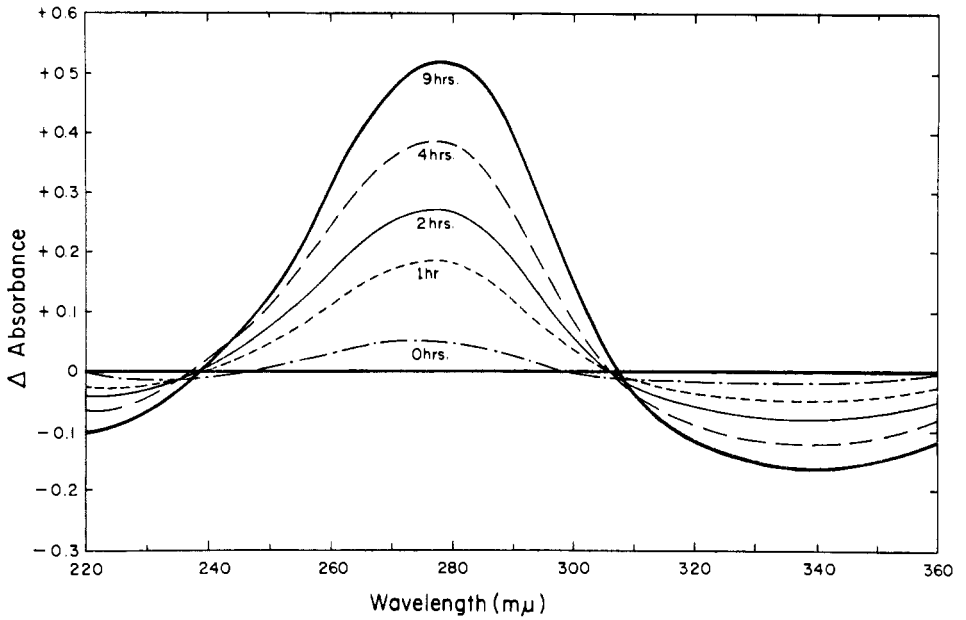


FIGURE 2: Difference spectra of NADH in potassium orthophosphate at pH 6.62. Readings in 1-mm light-path cuvets, in a Beckman DK-1 spectrophotometer, at 15.5°, in a tandem system. Cuvets A′ (toward the light source) and B′, both in the reference beam, contained 3.725×10^{-4} M NADH in 0.05 M orthophosphate and 1.5 M orthophosphate, respectively. Cuvets A (toward the light source) and B, both in the sample beam, contained 3.725×10^{-4} M NADH in 1.5 M orthophosphate and 0.05 M orthophosphate, respectively.

ever, considering the large excess of orthophosphate, as compared to NADH, the reaction may be described in terms of pseudo-first-order kinetics (Figure 4B). Thus, the equation

$$k_1(t_2 - t_1) = \frac{2.3}{d} \log \frac{a}{a - x} \qquad (2)$$

was found applicable (Alivisatos *et al.*, 1964b). In this equation, d is the total orthophosphate concentration in moles/liter (however, see following section); a and $a - x$ are the absorbances of NADH at 340 mμ at zero time (t_1) and at time t_2, respectively; and k_1, in M^{-1} min^{-1}, is the rate-constant for the first reaction.

Changes in absorbance at 280 mμ also depended on time and the concentration of the reactants (Figure 3). However, the kinetics of these changes were more complex. In general, it was expected that the integral equations

$$A + B + C + D = A_0 \qquad (3)$$

$$A\epsilon_{A(280)} + B\epsilon_{B(280)} + C\epsilon_{C(280)} + D\epsilon_{D(280)} = \text{absorbance}_{280} \qquad (4)$$

should apply for any time t during the experimental period (Figure 3). In these equations, A, B, C, and D are the same as in eq 1 and A_0 is the (known) initial concentration of A. It is evident that for a quantitative consideration of transformations following the formation of B it was necessary to determine the molar absorptivities of the individual components in this system.

The value of $\epsilon_{A(340)}$ was assumed to be known (Kornberg and Horecker, 1953) and $\epsilon_{A(280)}$ was determined from the absorbance of A_0 extrapolating to zero time the initial (linear) part of curves representing the over-all changes in absorbance[3] at 280 mμ (Figure 3). Thus the value of the expression $A\epsilon_{A(280)}$ was independently known for any time t from absorbance readings at 340 mμ. This permitted plotting of the difference

$$\text{absorbance}_{280} - A\epsilon_{A(280)} \qquad (5)$$

vs. time (curve 1, Figure 5).

For the determination of the molar absorbtivity of B at 280 mμ, it seemed reasonable to assume that at the onset of the reaction there was no appreciable accumulation of C or D (*i.e.*, $k_1 \gg k_2$) and that the absorbance at 280 mμ was solely due to A and B. Thus, *for this initial period:*

[3] Previously reported rapid initial ("zero time") changes in the absorbances of NADH solutions in orthophosphates at 340 mμ (decrease) and at 280 mμ (increase) (Alivisatos *et al.*, 1964a,b) were only in part due to reversible deformation of NADH of a nature similar to that previously described for NAD (Ungar and Alivisatos, 1961). The most important factor in these initial changes at both wavelengths was partial oxidation of NADH, which could be avoided by careful preparation of the samples. For simplicity, such initial changes (smaller than 6%) were neglected in the present work and a value of $\epsilon_{A(340)}$ (Kornberg and Horecker, 1953) was used throughout. Partial oxidation of NADH under similar conditions was also observed by A. G. Hilvers (personal communication).

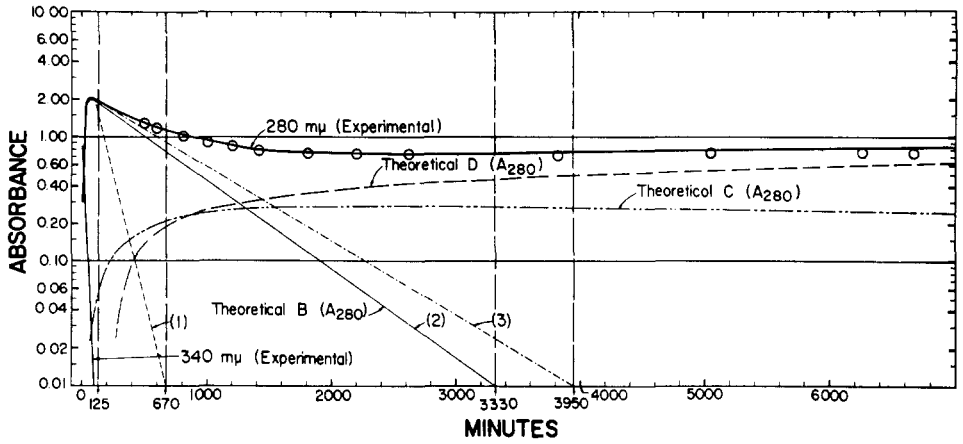


FIGURE 3: Absorbance changes in 340 and 280 mμ occurring during the spontaneous NADH–orthophosphate interaction at pH 6.62. The concentrations of NADH and of orthophosphate were 1.342×10^{-3} and 1.5 M, respectively. The temperature was 43.9°. Spectrophotometric readings in covered 1-mm light-path cuvets against 1.5 M orthophosphate, pH 6.62, in a Zeiss PMQ II spectrophotometer. The theoretical curves for the absorbance$_{280}$ of *B*, *C*, and *D* were calculated from the corresponding molar absorbtivities and rate constants. Curves 1, 2 (theoretical absorbance$_{280}$ of *B*), and 3 represent the (hypothetical) decrease in absorbance$_{280}$ due to *B*, assuming that $\epsilon_{C(280)}$ had the values: zero or 2700 or 11,000, respectively. Circles along the curve "280 mμ (Experimental)" correspond to resynthesis points (*i.e.*, addition of the calculated absorbancies at 280 mμ of the products *B*, *C*, and *D* at various times) and serve to demonstrate the close approximation to the experimental curve within the dimensions of the Figure.

$$B = A_0 - A \tag{6}$$

and the difference (6) could be set equal to $B\epsilon_{B(280)}$. In accordance with the above,[4] a mean value of $\epsilon_{B(280)}$ was obtained from the initial portion (45 min) of curve 1 in Figure 5 and from the corresponding *B*. This curve represents the conversion $A \rightarrow B$ under the assumption that *B* is the end product in sequence 1. Under this hypothetical condition, the limiting value $B_\infty = A_0$. However, when absorbance values obtained from (5) were plotted against time (curve 2 in Figure 5), it was observed that curves 1 and 2 deviated from each other. Evidently, *B* was slowly transformed to product(s) of lower molar absorbtivity (Figure 5).

In previous attempts to study the fate of *B* (Alivisatos *et al.*, 1964a,b) it was assumed for simplicity that the product *C* is stable. In the present study numerous systematic discrepancies between the experimental data and values computed from theory led to the conclusion that the transformation $B \rightarrow C$ was followed by a third transformation, $C \rightarrow D$, which ought to be considered. It was also evident that, for a relatively short period of time after practical completion of the transformation $A \rightarrow B$ (150–300 min in Figure 3), the only conversion occurring at a significant rate in this system was that of $B \rightarrow C$. Thus, during this period, a plot of the logarithm of the absorbance$_{280}$ *vs.* time resulted in a straight line (Figure 7). Consequently, during this interval, the relations

$$B + C = A_0 \tag{7}$$

$$B\epsilon_{B(280)} + C\epsilon_{C(280)} = \text{absorbance} \tag{8}$$

were considered valid. There are three unknowns in this system (*i.e.*, *B*, *C*, and $\epsilon_{C(280)}$). The obvious limitations to the infinite number of possible solutions for *B* are that $B \leq A$ and $\epsilon_{C(280)} \leq \epsilon_{B(280)}$ (see Figure 5). If *B* remained unaltered, then $\lim B(t \rightarrow \infty) = A_0$ and $C = 0$ (curve 1, Figure 5) [the same curve 1 (Figure 5) would be obtained if $\epsilon_{C(280)} = \epsilon_{B(280)}$]. For a given reaction rate, the difference between curves 1 and 2 (Figure 5) at any time *t* in the time interval specified above would increase as the ratio $\epsilon_{B(280)}/\epsilon_{C(280)}$ increased. A family of straight lines, intersecting at one point, was obtained by assigning arbitrarily to $\epsilon_{C(280)}$ values ranging from $\epsilon_{C(280)} = 0$ to $\epsilon_{C(280)} \leq \epsilon_{B(280)}$ and plotting the logarithms of the corresponding *B* values *vs.* time (Figure 7). It is obvious that, at the time defined by the point of intersection, the concentration of *B* is independent of the (arbitrary) ratio of the molar absorbtivities (92 min in Figures 5 and 7). Introduction of this value of *B* in the system of eq 8 and 9 would allow

[4] The "apparent" value of $\epsilon_{B(280)}$ is smaller at the beginning of the reaction, and it increases gradually to a constant value (Figure 6). This was attributed (see Discussion) to the initial formation of a product (*B'*) which is probably identical with the so-called "DPNH-X" (Chaykin *et al.*, 1956). *B'* has smaller molar absorbtivity$_{280}$ than *B*. The latter is probably identical with the "primary acid modification product" (Rafter *et al.*, 1954). As expected, the limiting value of $\epsilon_{B(280)}$ depends on the composition of the mixture (*i.e.*, $B' + B$), which varies within narrow limits with the experimental conditions (see Table I). Although these changes encompass the part of the reaction which is probably the most interesting from the biological viewpoint (see Discussion), in the kinetic approach depicted in the present communication the transition $B' \rightarrow B$ was not taken into consideration.

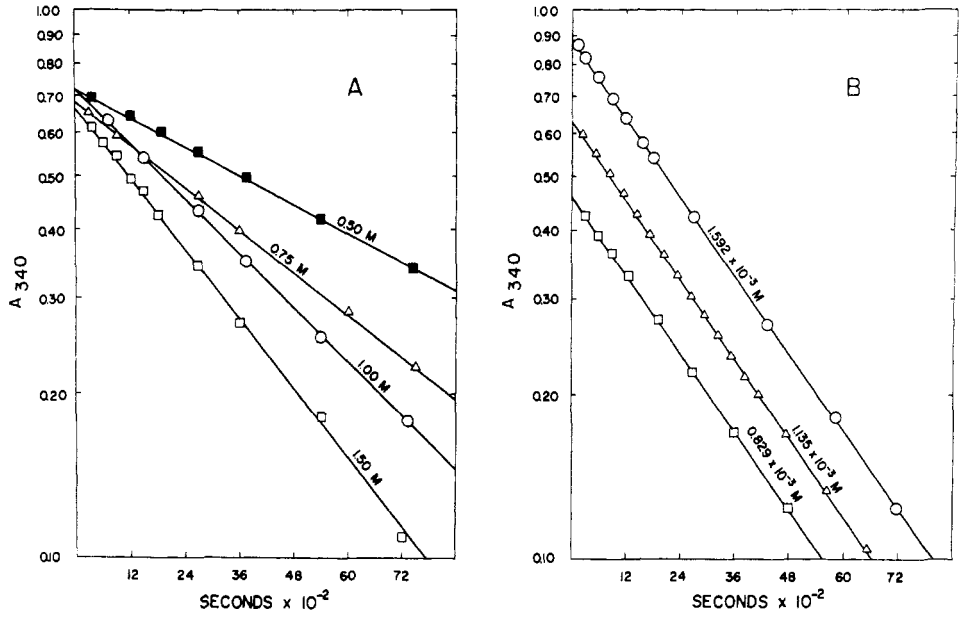


FIGURE 4: Time dependence of the absorbance changes of NADH in orthophosphate, pH 6.62, at 34.4°. (A) NADH (average 1.127 mM; range 1.122 mM to 1.138 mM) in various orthophosphate concentrations, as indicated in the figure. (B) 1.5 M orthophosphate and various NADH concentrations, as indicated in the figure. All readings in uncovered 1-mm light path cuvets against appropriate orthophosphate solutions in a Zeiss PMQ II spectrophotometer.

TABLE I: Molar Absorptivities and Rate Constants of Components and Reactions in the NADH–Orthophosphate System.

Component	Molar Absorbtivity 340 mμ	Molar Absorbtivity 280 mμ	Reaction No.	Rate Constant[e] (M^{-1} min^{-1})
A (NADH)	6220[a]	3,388 (SD ± 559)[b]	1	5.51×10^{-2}
B		18,327 (SD ± 1,067)[b]	2	2.05×10^{-3}
C		2,700[c]	3	1.45×10^{-5}
D		18,327[d]		

[a] Kornberg and Horrecker (1953). [b] Mean values from 12 experiments. [c] Pabst Laboratories (1956). [d] This value was taken as equal to $\epsilon_{B(280)}$ (see text). [e] Rate constants refer to the experiment shown in Figure 3. In this calculation the concentration of $H_2PO_4^-$ ions was taken as equal to 0.82 M (computed from the Hasselbach equation).

unequivocal estimation of the desired $\epsilon_{C(280)}$. However, the value of $C\epsilon_{C(280)}$ at this time is very small as compared to the total absorbance (see curves 2 and 3 in Figure 5). Consequently, values of $\epsilon_{C(280)}$ obtained in this manner are only approximations [$\epsilon_{C(280)} \simeq {}^1/_{10} \cdot \epsilon_{B(280)}$].

Alternatively, k_2 (hence $\epsilon_{C(280)}$) may be approximated at a time when the expression 5 reaches a maximum (first arrow at 72 min in Figure 5). At this time, it is permissible to neglect the absorbance due to D in this system. Justification of this statement is derived from the demonstration (see above) that at a later time (*i.e.*, second arrow at 92 min in Figure 5) accumulation of C is still very small. Thus, it may be assumed that the time of maximum accumulation of B coincides with the time of maximal value for expression 5 (see curve 2, Figure 5) and that the value of B at this time is known (*i.e.*, only B accounts for this value). Considering the pseudo-first-order character of these reactions (*i.e.*, $A \rightarrow B \rightarrow C$), it is expected (Frost and Pearson, 1961, p. 166) that the differential equation

$$dB/dt = k_1 A e^{-k_1 t} - k_2 B \qquad (9)$$

would be valid. Furthermore, at the time of maximal accumulation of B, eq 10 should be equal to zero. In eq 10 B_{max} was calculated from the maximal value of (5) divided by $\epsilon_{B(280)}$, and k_1 (4.52×10^{-2} min^{-1}) was obtained through multiplication of the value of k_1 in Table I (5.51×10^{-2} M^{-1} min^{-1}) with the molarity

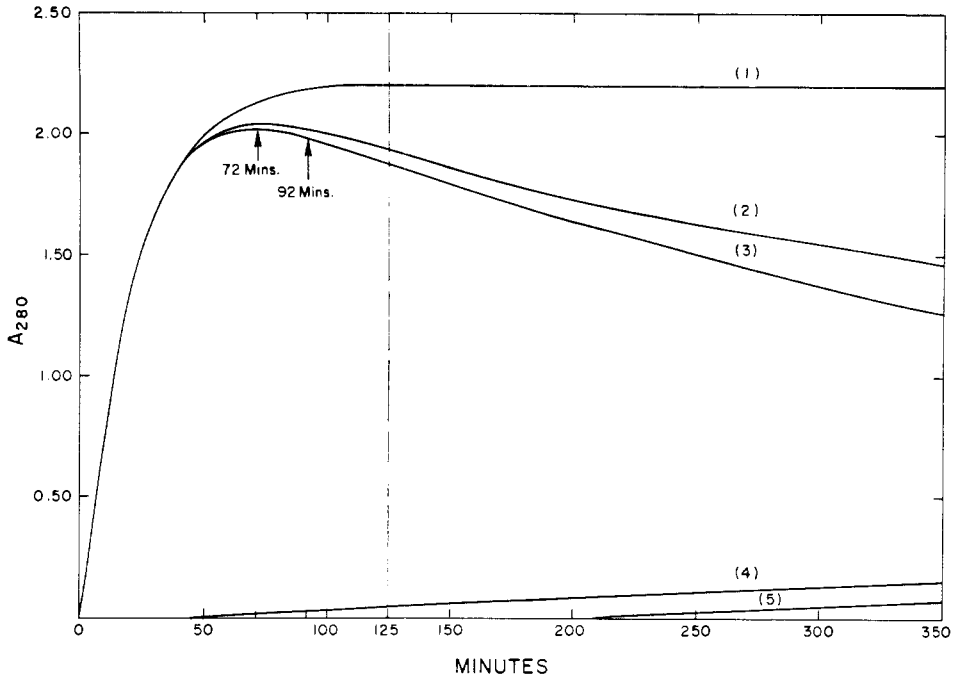


FIGURE 5: Analysis of the initial phase of absorbance changes in NADH–orthophosphate mixtures. Conditions as in Figure 3. Curve 1 corresponds to the transformation $A \rightarrow B$ (B regarded as stable). Curve 2 corresponds to [actual absorbance$_{280}$ $- A\epsilon_{A(280)}$]; it represents the combined absorbances of $B + C + D$. Curves 3, 4, and 5 correspond to the calculated absorbances of B, C, and D, respectively (see text). The arrow at 72 min indicates the point of maximum accumulation of B. The arrow at 92 min corresponds to the point of intersection of curves shown in Figure 7.

of the $H_2PO_4^-$ ions (0.82 M) (see effect of the pH in the following section). The k_2 (first order) computed in this manner was converted to a second-order constant ($k_2 = 2.31 \times 10^{-2}$ M^{-1} min^{-1}) in an analogous manner. The value of $\epsilon_{C(280)}$ corresponding to this k_2 was 3876 [calculated from an equation similar to (2) in the interval 92–236 min; see below] (Figure 7).

The low $\epsilon_{C(280)}$, as estimated from either of the above methods, was taken as an indication that the transformations of the dihydronicotinamide moiety of NADH led to a product which was not absorbing light at 280 mμ (see Discussion). Accordingly, a value $\epsilon_{C(280)} = 2700$ was assigned arbitrarily to C. This value represents the molar absorbtivity of the adenosine moiety of NADH (Pabst Laboratories, 1956). Assuming that the conversion $B \rightarrow C$ was also dependent on the concentration of the proton donor (see following section), the rate-constant k_2 was finally obtained from an equation similar to (2).

An understanding of the fate of C was achieved as follows. It was assumed that, from a time that no significant quantities of B were present in the system (see point of intercept of curve 2 with the abscissa in Figure 3), the only components in the system were C and D. This permitted utilization of a system of equations for C and D similar to eq 7 and 8, and the value of C was obtained from:

$$C = \frac{\text{absorbance}_{280} - \epsilon_{D(280)}A_0}{\epsilon_{C(280)} - \epsilon_{D(280)}} \qquad (10)$$

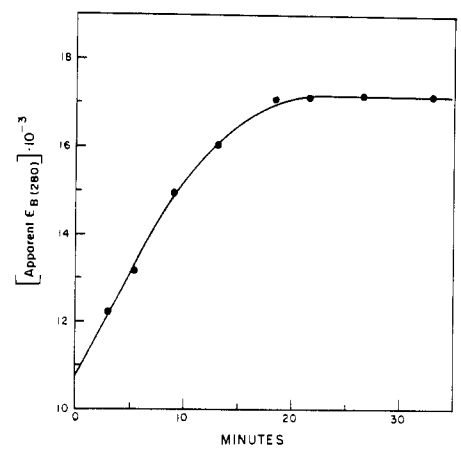


FIGURE 6: Changes of the apparent $\epsilon_{B(280)}$ at the initial stages of the NADH–orthophosphate reaction. Conditions and spectrophotometry as described in Figure 3, but with 1.25 M orthophosphate. The values of $\epsilon_{B(280)}$ for this particular experiment at various times were calculated from eq. 6, as described in the text.

Equation 10 was considered valid in the time interval following practical completion of the reaction $B \rightarrow C$. It was obvious that the molar absorbtivity of D should have a value, $\epsilon_{D(280)} > \epsilon_{C(280)}$ (see part of the curve extending beyond 3330 min in Figure 3). Through a procedure of trial and error it was found that $\epsilon_{D(280)} =$

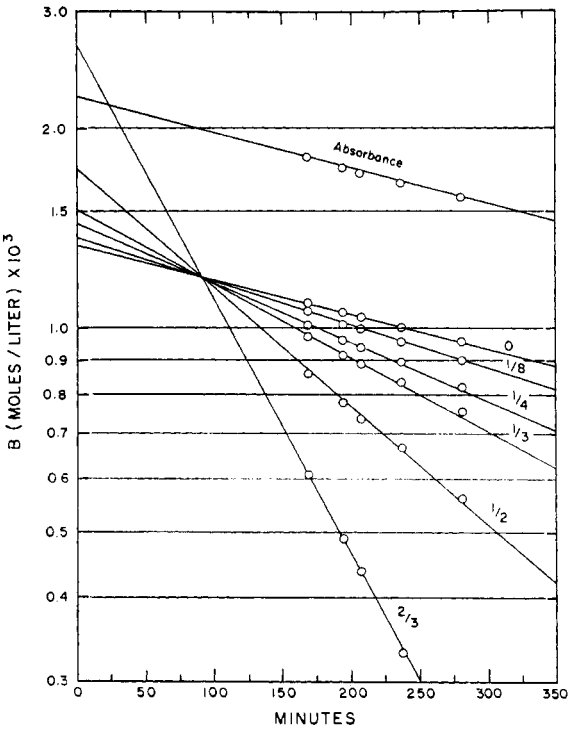


FIGURE 7: Graphical method for the determination of the composition (*A*, *B*, and *C*) of NADH–orthophosphate mixtures at a certain time of the initial phase of the reaction (see text). Experimental conditions as in Figure 3. The concentrations of *B* were obtained from the system of eq 7 and 8. The curve "absorbance" represents the over-all absorbance of the mixture at 280 mμ. Curve 0 corresponds to $\epsilon_{C(280)} = 0$. The other curves correspond to values of $\epsilon_{C(280)}$ equal to $^1/_8$, $^1/_4$, $^1/_3$, $^1/_2$, and $^2/_3$ of the $\epsilon_{B(280)}$, respectively. Note that at the point of intersection of the various lines (*i.e.*, at 93 min) the concentration of *B* is independent of the $\epsilon_{C(280)}$ used in the calculations.

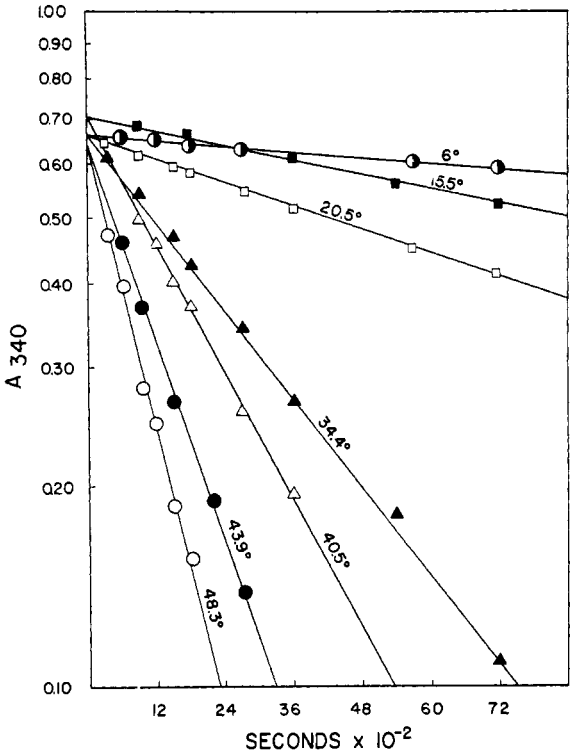


FIGURE 8: Temperature dependence of the rate of decrease of absorbance at 340 mμ of NADH (average 1.107 mM; range 1.063–1.140 mM) in 1.50 M orthophosphate, pH 6.62. Readings in uncovered 1-mm light path cuvets against appropriate orthophosphate solutions in a Zeiss PMQ II spectrophotometer.

$\epsilon_{B(280)}$ (Table I). The rate constant k_3 was obtained from an equation similar to (2). In the above derivation it was assumed that the conversion $C \rightarrow D$ also is phosphate dependent. A justification for this assumption will be provided in the following section.

Effects of Temperature and pH

The rates of these transformations depended on the temperature. For the transformation $A \rightarrow B$ this is shown in Figure 8. In Figure 9, the logarithms of the velocity constants, k_1, were plotted against the inverse of the absolute temperature of the reaction mixtures. Applying the Arrhenius equation, the energy of activation (E_a) was estimated in the vicinity of 16.000 cal/mole. The frequency factor of the Arrhenius equation was approximated to about 4.36×10^7 M^{-1} min^{-1} by extrapolation of the line in Figure 9 to infinite temperature. The dependence of k_2 on temperature was demonstrated by the constancy of the k_1/k_2 ratio, as obtained from mixtures studied at various temperatures (Table VI). The rate constant, k_3, also depended on temperature. However, considering that k_3 was three orders of magnitude smaller than k_1 (Table I), it was difficult to carry out measurements at lower temperatures within reasonable experimental periods.

Next, we studied the dependence of k_1 on the pH. Such studies revealed that within the range of two pH units (pH 6.0–8.0) k_1 depended on the concentration of the $H_2PO_4^-$ ions rather than on the total orthophosphate concentration (see previous section). This is demonstrated by the numerical values of k_1 in Table II. No attempt was made to correct for ionic strength, and the pH values recorded in this table are those obtained directly from a Beckman Zeromatic pH meter. This is one of the reasons for the observed small deviations of the k_1 values in the last column of Table II. Considering the wide range of variation in $H_2PO_4^-$ concentrations in this table, the agreement of k_1 values is remarkable. The dependence of k_2 on the pH was reflected in the constancy of the k_1/k_2 ratio observed in NADH–orthophosphate mixtures at various pH values (Table VI). The low value of k_3 prevented a study of the effect of the pH on this transformation (however, see following section).

TABLE II: Rate Constant, k_1, of the Time-Dependent Decrease in Absorbancy at 340 mμ of NADH Solutions in 1.5 M Potassium Orthophosphate and at Different pH Values.[a]

			$k_1 \times 10^2$ (M^{-1} min^{-1})	
No.	pH	$H_2PO_4^-$ (M)	From Total Concentration of Phosphate	From ($H_2PO_4^-$)
1	7.98	0.075	0.08	1.80
2	7.10	0.427	0.50	1.85
3	6.98	0.516	0.76	2.20
4	6.62	0.819	1.18	2.09
5	6.33	1.051	1.43	2.03

[a] Experimental conditions as in Figure 4A; the $H_2PO_4^-$ concentrations were calculated from the Hasselbach equation and a pK' value of 6.7. Measurements of the pH were carried out at 26° and were not corrected for the difference in temperature (34.4°). The mean value of k_1 determined from the $H_2PO_4^-$ concentration in 18 experiments (including the above) performed at various orthophosphate concentrations (0.25 to 1.5 M) and at various pH values (pH 7.98–6.22) was 0.0212 (SD ±0,0020).

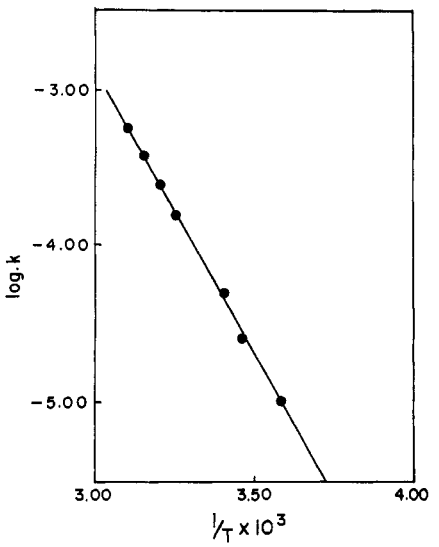


FIGURE 9: Arrhenius plot of the dependence of the pseudo-first-order velocity constant (k_1) of the decrease in absorbance at 340 mμ of NADH in orthophosphate on the temperature (absolute scale). Conditions as in Figure 8. k_1 values were determined in M^{-1} sec^{-1} from eq 2. In this equation d was set equal to the total orthophosphate concentration (*i.e.*, 1.5 M).

Effects of Variation of the Reactants

Variation of the cation accompanying the orthophosphate produced only small differences in k_1 (Table III). Thus, in the presence of potassium the decrease in absorbance at 340 mμ proceeded somewhat faster than in the presence of sodium, but these differences were not considered significant. Slower rates were observed in the presence of ammonium and imidazolium ions (experiments 7–11 in Table III). This may be attributed to a competition for protonation between imidazole (or ammonia) and the dihydropyridine moiety of NADH (see Discussion).

Similar differences in the reaction rates were encountered when $H_2PO_4^-$ was replaced by other anions. Thus, sulfite was most effective in promoting the rate of the reaction, but its mechanism of action is probably different from that of other anions.[5] The next fastest rate was observed with arsenate, followed by phosphite and phosphate (Table III). Compared to the above, the reaction rate was manyfold times slower in sulfate and chloride (Table III; see also Discussion). Replacing phosphate with either 5′-AMP, or ATP, or NADP was attempted. When these substances were incubated at equimolar concentrations (0.5 mM) with NADH at pH 6.62, there was no measurable reaction rate. This failure may be attributed to the low concentration of the proton donors (*i.e.*, the 5′-AMP, etc.). The high absorbance of these substances in the 260–280 mμ region prohibited their utilization at higher concentrations.

Variation of the anion (*i.e.*, the proton donor) had a similar effect on k_2. This is shown by the constancy of the k_1/k_2 ratio observed in experiments with arsenates or phosphites instead of orthophosphates (Table VI). It became evident from prolonged experiments with arsenate that k_3 is also affected by the nature of the proton donor (*i.e.*, faster rates of the transformation $C \rightarrow D$). It is also probable that in arsenate D is further transformed to a product of lower ϵ_{280}. In phosphate this transformation either does not occur or it proceeds at such low rates that it cannot be observed within reasonable experimental periods (*i.e.*, up to 10^4 min). In arsenate a change becomes evident at later stages of the over-all reaction, manifesting itself in a new slow wave of decrease of the absorbance at 280 mμ. However, this phenomenon was not analyzed kinetically in the present study.

Further insight on the mechanism of the transformation from A to B was achieved by the use of various analogs of NADH. The use of simpler model compounds, like 1-propyl-1 4-dihydronicotinamide, was neither convenient nor helpful in the present studies, due to the fast rate of their transformations at relatively

[5] Sulfite reacts with 1,3-substituted 1,4-dihydropyridines differently, according to conditions, *e.g.*, the nature of solvents, the H^+ concentration, the presence of catalysts, etc. (Wallenfels *et al.*, 1959; Stock *et al.*, 1961; Kosower, 1962, p. 210).

TABLE III: Rate Constants of the Time-Dependent Decrease in Absorbancy at 340 mμ of NADH Solutions in the Presence of Various Ions.[a]

No.	Anion	Cation	pH	Estmd Concn of Acid Anion[b] (M)	$k_1 \times 10^2$ (M^{-1} min^{-1}) From Total Anion Concn	$k_1 \times 10^2$ (M^{-1} min^{-1}) From Concn of Acid Anion
1	Sulfite[c]	Na^+	6.96	0.085	1.68	3.94
2	Sulfite[c]	Na^+	6.65	0.121	2.47	4.09
3	Sulfite[c]	Na^+	6.20	0.162	2.82	3.48
4	Arsenate[c]	K^+	6.62	0.819	1.66	3.04
5	Phosphite[d]	Na^+	6.98	0.373	0.50	2.01
6	Phosphite[d]	Na^+	6.30	0.920	1.07	2.13
7	Phosphate	K^+	6.62	0.819	0.95	1.73
8	Phosphate	Na^+	6.62	0.819	0.69	1.26
9	Plus chloride[e]	Na^+	6.62	0.819	0.68	1.24
10	Phosphate	NH_4^+	6.62	0.819	0.52	0.95
11	Phosphate	Imidazole[f]	6.62	0.819	0.45	0.83
12	Sulfate	NH_4^+	6.62	1.500	0.09	0.09
13	Chloride	Na^+	6.62	1.500	0.02	0.02

[a] The reaction mixtures contained 1.11×10^{-3} M NADH and the corresponding salt (1.5 M with the exception of sulfite, which was 0.2 M). The temperature was kept at 34.4°. Readings were made in uncovered 1-mm light path cuvets against appropriate controls (NADH omitted) in a Zeiss PMQ II spectrophotometer. [b] As HSO_3^-, $H_2AsO_4^-$, $H_2PO_3^-$, $H_2PO_4^-$. Estimated from the pH and the corresponding pK values. Sulfates and chlorides were considered as fully ionized. [c] These pK' values were determined titrimetrically at 26° in a recording titrator (Radiometer, Copenhagen). A 0.1 N solution of arsenic acid was titrated with 0.3 N NaOH ($pK_2' \simeq 6.72$) and a 0.1 N Na_2SO_3 solution was titrated with 0.1 N HCl ($pK_2' \simeq 6.83$). [d] A pK' value of 6.50 (Robertson and Boyer, 1956) was used. [e] An Na_2HPO_2 solution was adjusted to the desired pH value with HCl. [f] A KH_2PO_4 solution was adjusted to the desired pH value with 3 M imidazole solution.

low $H_2PO_4^-$ concentrations, at pH 6.62. The reaction with 1,4-dihydronicotinamide ribonucleotide (Table IV, No. 1) proceeded twice as fast as with NADH. In comparison to NADH, the reaction rate with NADPH was somewhat faster, but there was no difference of the rates between NADH and dihydronicotinamide–hypoxanthine dinucleotide (Table IV). These results indicate dependence of the reaction rate on the accessibility of the reaction site to the proton donor. They will be discussed in this context together with interpretations of nuclear magnetic resonance spectra in a following paper (see footnote 2).

Results obtained with NADH analogs differently substituted at position 3 of the dihydropyridine moiety are also shown in Table IV (No. 4, 6, 7, and 8). It is apparent from these results that maximal decreases in absorbance occur at a wavelength which coincides with that of the peak corresponding to the dihydropyridine moiety of the analog. In contrast, the wavelength(s) of maximal increase in absorbance varies among the differently substituted dihydropyridine derivatives. The direction of these variations (bathochromic in the series $-CONH_2$, $-COH$, $-COCH_3$) is in agreement with similar variations observed by others (Stock *et al.*, 1961) in the 1-methyldihydropyridine series with the above substituents in the 3 position.

An alleged participation of the phosphates in the structure of *B* (Stock *et al.*, 1961; Burton and Kaplan, 1963; Alivisatos *et al.*, 1964b) was retested by attempts to demonstrate the presence of the anion in the isolated *B*. Thus, in ionophoretic studies at pH 8.0 (Tris–HCl, Alivisatos *et al.*, 1960), ultraviolet-quenching material was detected on paper, in the pathway of application of *B*, moving toward the positive electrode in a manner identical with that of NADH. It should be noted that, at this pH, NADH bears two negative charges. The hypothetical adduct with phosphate should bear a minimum of 3.5 negative charges at pH 8.0. When the spontaneous interaction was carried out in solutions containing ^{32}P-labeled orthophosphate, there was no indication of radioactivity associated with the ultraviolet-quenching spot in either ionopherograms (see above) or descending paper chromatograms (Rathlev and Rosenberg, 1956). Attempts were also made to adsorb material corresponding to *B* on Norit A directly from the reaction mixture or after 10-fold dilution with deionized water (at pH 8.0 with Tris). In each case, the adsorbed material was eluted with a mixture of 50%

TABLE IV: Wavelengths of Maximal Changes and k_1 Values of the Time-Dependent Decrease of Absorbancy at the Spectral Peaks Corresponding to the Dihydropyridine Moiety of Various NADH Analogs in 1.5 M Orthophosphate, pH 6.62, at 34.4°.[a]

No.	Component	Structure of Side Chain	Maximal Changes of Absorbance[b] Decrease at λ	Maximal Changes of Absorbance[b] Increase[c] at λ	$k_1 \times 10^5$ (M^{-1} min^{-1})
1	NMNH[d]	$-CONH_2$	(338)	269	4088
2	NADPH[f]	$-CONH_2$	340	277	2177
3	NHDH[e]	$-CONH_2$	340	277	1821
4	NADH[f]	$-CONH_2$	340	277	1726
5	NADH[g]	$-CONH_2$	340	280	1719
6	Dihydrothionicotinamide-AD[f]	$-CSNH_2$	398	239, *273*, 330	303
7	Dihydro-3-acetylpyridine-AD[f]	$-COCH_3$	363	297	99
8	Dihydropyridinealdehyde-AD[f]	$-CHO$	(355) (340–357)	290 (285–292)	<6
9	Adenosine diphosphate ribose	—	—	—	0

[a] Experimental conditions for determination of k_1 as in Figure 4. [b] Differential spectroscopy as in Figure 2. [c] Numbers in parentheses define broad maxima of the difference spectra; italic numbers indicate the largest differential peak. [d] Reduced by hydrosulfite (see Methods). [e] Dihydronicotinamide–hypoxanthine dinucleotide. [f] Enzymatically reduced (see Methods). [g] Reduced by borohydride (see Methods).

alcohol and 5% ammonia. It was invariably found free of radioactivity. Such studies suggested that association with the polybasic anion (if any) should be extremely short-lived.

In other experiments, we determined the energies of activation in a number of different systems in which either the polybasic anion (*i.e.*, the acid) or the substituted 1,4-dihydropyridine were varied. Results are gathered in Table V together with the frequency factors of the Arrhenius equation.

TABLE V: Energies of Activation and Frequency Factors for the Interaction of NADH with Orthophosphate and Other Anions [Formation of *B*].[a]

No.	Anion	Activation Energy (cal/mole)	Frequency Factor $\times 10^{-7}$ (M^{-1} sec^{-1})
1	$H_2PO_4^-$	16,976	4.36
2	$H_2AsO_4^-$	15,335	2.57
3	$H_2PO_3^-$	15,139	0.68

[a] Experimental conditions as described in the text and in Figures 8 and 9.

Enzymatic Studies

Many of the properties of the so-called "DPNH-X" (Chaykin *et al.*, 1956) were reminiscent of the presently studied intermediate, *B*, occurring during the spontaneous NADH–orthophosphate interactions. Previous investigations (Stock *et al.*, 1961) also pointed toward this identity. In our studies, aliquots from an NADH–orthophosphate mixture were reincubated with an enzyme preparation first described by Meinhart *et al.* (1956) in the presence of ATP and magnesium ions. Sampling of aliquots was scheduled on the basis of the expected composition of the mixture, as predicted from the known course of the reaction (see kinetic analysis). It was shown that only the intermediate *B* may be reverted to NADH, as indicated by the increase in absorbance at 340 mμ. Thus, at the early stages of the NADH–orthophosphate reaction (*i.e.*, 0–70 min at 34.4°), the rate of the enzymatically catalyzed increase in absorbance at 340 mμ was proportional to the concentration of *B* (see Figure 10). Later, however, the enzymatic reaction slowed down, despite larger accumulation of *B* (compare slopes of curves from aliquots obtained after preincubation of the NADH–orthophosphate mixture for 60, 90, or 152 min in Figure 5). This was attributed to the conversion of "DPNH-X" to the primary acid modification product of NADH (Rafter *et al.*, 1954) (see Discussion). In our experiments the identity of the material showing increased absorbance at 340 mμ with NADH was demonstrated by its reoxidation with acetaldehyde in the

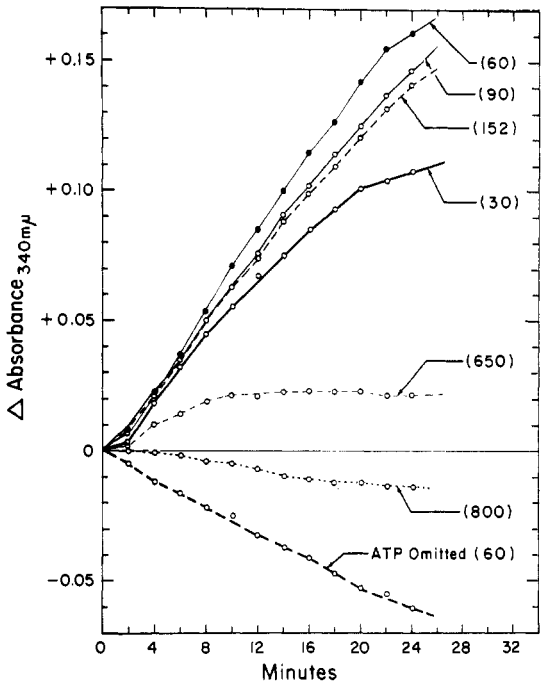


FIGURE 10: Rates of the enzymatic conversion of the intermediate *B* to NADH. NADH (1 mM) and 1.5 M orthophosphate, pH 6.62, were incubated at 34.4°. Aliquots (0.1 ml) of this mixture were withdrawn at times indicated in the figure and reincubated with enzyme (0.262 mg of protein) (Gornall *et al.*, 1949), ATP (3 mM), and $MgCl_2$ (1 mM) in a volume of 1 ml. The increase in absorbance at 340 mμ was followed in a DK-1 Beckman recording spectrophotometer in 10-mm light path cuvets. The reference cell was identical with the experimental with NADH omitted. The initial absorbances differed in each case, according to the concentration of *A* in the sample.

presence of alcohol dehydrogenase. The decrease in absorbance observed in experiments from which ATP was omitted (Figure 10) was attributed to continuation of the spontaneous NADH–orthophosphate interactions, even though the reactants in the enzymatic test were diluted 10-fold. It is probable that in such instances proteins present in the yeast preparation (Meinhart *et al.*, 1956) act catalytically, promoting the spontaneous reaction, as is the case with glyceraldehyde 3-phosphate dehydrogenase (Rafter *et al.*, 1954). However, it is difficult to account for the comparatively small drop in absorbance observed in enzymatic assays of aliquots obtained after the spontaneous reaction progressed considerably (*e.g.*, 800-min sample in Figure 10). In accordance with findings of Meinhart *et al.* (1956), it was not possible to reverse the reaction catalyzed by the yeast enzyme preparation, *i.e.*, to achieve a conversion of ADP to ATP at the expense of NADH in the presence of magnesium ions and orthophosphates.

It was of interest to examine whether *B* could serve, in an appropriate system, as a generator of ATP from ADP. Certain similarities of the spontaneous reactions studied in our laboratory and those described by Griffiths (1964) suggested the use of antimycin A-treated mitochondrial preparations in the presence of a glucose–hexokinase trap in our assays. As mentioned in methods, the mitochondria were preexposed to hypotonic solutions in order to permit permeability of NADH or its products from the milieu (Tapley and Cooper, 1956). Such experiments performed with aliquots from reaction mixtures obtained as early as 60 min after the onset of the spontaneous reaction were unsuccessful (however, see Discussion).

Discussion

Studies reported in this communication stem from our previous findings on NAD interactions with orthophosphate and other anions (Ungar *et al.*, 1961). However, in contrast to our previous studies, present observations fall into the general category of the instability of NADH in acid environment (Warburg *et al.*, 1935; Euler *et al.*, 1936; Karrer and Warburg, 1936). Changes of the ultraviolet absorption spectrum of NADH in the presence of acid were first described by Haas (1936). The mechanism of this acid degradation was studied in detail with model dihydropyridine derivatives (Wallenfels *et al.*, 1959; Anderson and Berkelhammer, 1958; Stock *et al.*, 1961), and it was discussed by Kosower (1962, p. 166).

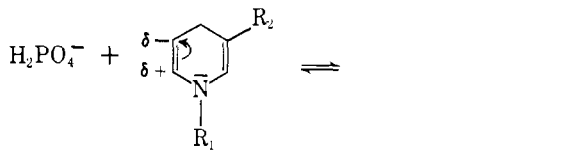
Acid degradation of dihydropyridines proceeds relatively fast. In contrast to this, direct donation of protons from the orthophosphates to the dihydropyridine proceeds at relatively slow rates. Under these conditions, the process is slower when NADH is used in the reaction, as contrasted to dihydronicotinamides with simpler substituents in the N^1 position (see below). Rates achieved in this manner permitted a detailed kinetic approach of the over-all process.

The kinetics of two consecutive second-order reactions have been the object of numerous investigations (Frost and Pearson, 1961, p. 178.) Such studies were often hampered by the difficulty of devising analytical expressions for the concentrations of the consecutive components (Chien, 1948; French, 1950). In our studies, graphical evaluation of the spectrophotometric data suggested that under our conditions the NADH–orthophosphate system probably involved three consecutive second-order reactions. Analysis of such a problem would probably be prohibitive under other circumstances. In attempting its solution, advantage was taken of the pseudo-first-order character of the reactions (Abel, 1906). However, a most important factor was that each successive rate constant in this sequence proved much smaller than the previous. Thus, each step was essentially completed before the subsequent step started (Knolbauch, 1898).

The graphical procedure reported here was found suitable in rendering an accurate picture of the composition of the mixture at any given time in the course of this reaction. Kinetic analysis was instrumental in

elucidating the role of "polybasic anions" (*e.g.*, orthophosphates) as that of proton donor in the reaction (see below). Any further participation of the anions, as such, in these reactions would be incidental. In the following we will discuss our findings within the framework of previous work on the subject.

In accordance with the enamine (R—CH=CH—N<) structure of the dihydropyridine, its acid degradation most probably involves an initial β-protonation (Anderson and Berkelhammer, 1958).



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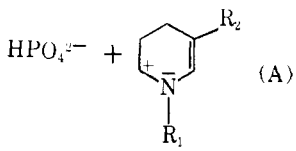


12 (A)

In acidic watery milieu, this initial protonation occurs very rapidly (Anderson and Berkelhammer, 1958; Stock *et al.*, 1961; Burton and Kaplan, 1963). However, when the hydrogen ion concentration is very low (approximately pH 7.0), the over-all reaction rate from *A* (corresponding to NADH) to *B* (partly corresponding to the "primary acid product" of NADH) (see below) is too slow to be measured with accuracy (see Results, and Stock *et al.*, 1961). It is reasonable to assume that under such circumstances β-protonation of the dihydropyridine is the rate-limiting step in the formation of the so-called "primary acid product" of NADH. This view is further supported by the observation that, at neutrality, the presence of fully dissociated anions, like Cl^- or SO_4^{2-}, would hardly influence the exceedingly slow rate of degradation of NADH. Hydroxide ions are equally ineffective, as judged from the relative stability of NADH in alkaline environment.

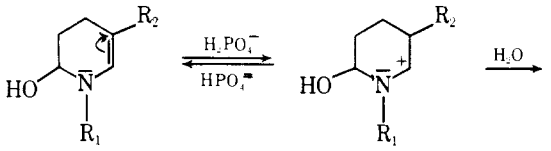
In contrast to the above, conversion of *A* to *B* in the vicinity of neutrality proceeds at appreciable rates only in the presence of a number of acid anions, *i.e.*, proton donors, provided that the numerical values of the p*K* values of their dissociation are not far from the pH values in the reaction mixture. Such acid anions include, among others, $H_2PO_4^-$, $H_2AsO_4^-$, $H_2PO_3^-$ (Table I), $H_2P_2O_4^{2-}$, and citrate (Stock *et al.*, 1961), all effective in the vicinity of neutrality, and others, like thioglycol (Stock *et al.*, 1961), effective at higher pH values. In all such instances, correlation of the reaction rate to the hydrogen ion concentration is very poor (see also Stock *et al.*, 1961, p. 204). However, this rate always depends on the concentration of the acid, in a manner suggesting a mole/mole interaction of NADH with the acid (Table I).

In previous studies (Stock *et al.*, 1961), the promoting effect of the acids on the reaction rate was attributed to their (incidental) anionic nature and their concomitant ability to act as nucleophiles. Thus, it was implicitly assumed that the rate-limiting step in the conversion of *A* to *B* is the one following protonation (presumably at position 5 of the dihydropyridine). This step would involve the reaction of an intermediate carbonium ion [*i.e.*, compound 12] with a nucleophilic species. The above-mentioned nucleophilic substitution could perhaps be rate limiting in the presence of high hydrogen ion concentrations[6] (Anderson and Berkelhammer, 1958), but, at pH 6.0 or above, the arguments mentioned above are against such a hypothesis.

In a preliminary communication (Alivisatos *et al.*, 1964b) we recognized that the primary function of the acid in this reaction is that of a proton donor. In that report (Alivisatos *et al.*, 1964b), we also suggested the possibility of a concerted action of the acid, supplying concomitantly the proton and the anion at positions 5 and 6 of the dihydropyridine. Such concerted action would probably be operative in certain reactions of model dihydropyridine derivatives with acids in organic solvents (Wallenfels *et al.*, 1959), provided that spatial conditions would be favorable. However, our recent studies on the structure of *B* failed again to demonstrate phosphate or other anions (with the exception of sulfite, see footnote 5) as a part of this structure, and this is in agreement with previous investigations (Chaykin *et al.*, 1956; Stock *et al.*, 1961). Working at considerably higher hydrogen ion concentrations, Anderson and Berkelhammer isolated from 1-benzyl-3-acetyl-1,4-dihydropyridine a product with a hydroxyl at the 6 position (21), and they interpreted their results as due to solvent attack on the intermediate carbonium ion (12) (Anderson and Berkelhammer, 1958). There is no reason to postulate a different (from the above) course in spontaneous reactions of 1,3-substituted 1,4-dihydropyridines with orthophosphates, etc., at low H^+ concentrations. Apparently, water molecules in the immediate surrounding of the α carbon have a greater chance to participate in this reaction.

Transformation of *B* at neutrality also depends on the concentration of the acid anion (see Results). This is again interpreted as due to a direct transfer of a proton from the acid to the ring (22). Degradation of *B* is characterized by a loss of absorbance in the 260–290 mμ region. As in the "secondary acid modification product" (Chaykin *et al.*, 1956), the residual absorption of the mixture at 280 mμ could be accounted for by the absorbtion of the adenine moiety of NADH. Most probably these transformations could be formulated as follows.

[6] Anderson and Berkelhammer (1958) assumed that β-protonation of 1,3-substituted 1,4-dihydropyridines in acid media proceeds so rapidly that it reaches thermodynamic equilibrium with the nonprotonated form before the rate-limiting second step occurs. They further assumed that changes of the substituent at the 3 position would affect the equilibrium constant, hence influence indirectly the over-all rate of the reaction. Under our proposal, at low hydrogen ion concentrations β-protonation is the rate-limiting step and changes of the substituent at the 3 position would affect the rate directly. However, in both instances, the net effect would be a slowdown of the reaction.



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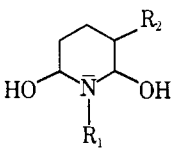
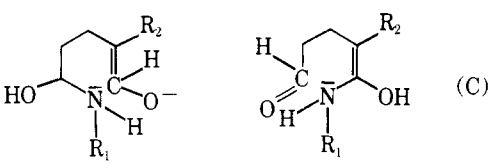


23 (B)

According to the above, *C* has a structure (23) proposed by the group of Pfleiderer (Stock *et al.*, 1961).

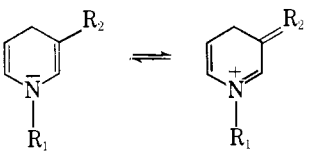
Subsequent transformation of *C* to *D* is accompanied by increased absorption at 280 mμ. Actually, *D* has the same molar absorbtivity as *B*, indicating the reappearance of a double bond, probably at the 2,3 position. This compound is probably similar to or identical with a product previously obtained in Pfleiderer's laboratory (Stock *et al.*, 1961) by acidification (pH 3.4) of the "secondary acid modification product" [*i.e.*, most probably (23)] and subsequent mild alkalinization (pH 7.7). It was suggested (Stock *et al.*, 1961) that protonation of the nitrogen at position 1 of compound 23 leads to ring opening in the 1,2 position, with concomitant formation of an aldehydic group at position 2. According to this view, the chromophore absorbing at 280 mμ is an enolate ion (31) formed in slightly alkaline solution. This is at variance with the observation (Burton and Kaplan, 1961) that, while



31 32 (C)

2,6-diketo-3,5-dicarbethoxy-*n*-heptane does not show significant absorbance$_{290}$ in alkaline solution, structure 32 is highly absorbing in the vicinity of 280 mμ. However, in our studies of nuclear magnetic resonance spectra of NADH–$D_2PO_4^-$ mixtures in D_2O (see footnote 2) we failed to detect any aldehydic group in such mixtures. It follows that *D* is probably similar to but not identical with compound 32. More experimentation is required in order to clarify this matter.

Kosower (1962, p. 166) pointed out that β-protonation of 1,4-dihydropyridines may be regarded as an instance of enamine nucleophilicity. With this in mind, our results may be interpreted as due to a direct attack of the nucleophilic carbon at position 5 of the 1,4-dihydropyridine upon the acid (*e.g.*, the $H_2PO_4^-$). Direct transfer of the proton from the (undissociated) acid anion to the β-carbon would be favored in the vicinity of the p*K'* of the acid. On the other hand, the rate of this transfer would be retarded in the presence of additional proton acceptors, like ammonia or imidazole. Both expectations were fulfilled, as shown in the Results. The transfer would also depend on the degree of nucleophilicity of the β-carbon. Factors influencing the latter would be expected to affect the velocity of the $A \rightarrow B$ reaction (see footnote 6). This is reflected in changes of the velocity constant, k_1, observed in the series: 3-aldehydedihydropyridine-AD, 3-acetyldihydropyridine-AD, thiodihydronicotinamide-AD, and NADH (Table IV). In these experiments, k_1 increased gradually, in the above order, to over 300-fold. Thus, the rate of the reaction is accelerated as the effectiveness of the substituent (R_2) at position 3 of the dihydropyridine to act as an electron sink decreases and structures similar to 42 prevail.



41 42 (D)

It follows that, when other conditions (*i.e.*, pH, nature, and concentration of the acid anion and temperature) are kept constant, k_1 may serve as a measure of the nucleophilicity of the enamine carbon in 1,3-substituted 1,4-dihydropyridines which participates in the first of the consecutive reactions. Further, the ratio of the two second-order velocity constants, k_1/k_2, remained roughly constant in NADH, despite wide variations of the experimental conditions. This ratio is an expression of the relative nucleophilicity of the two carbon atoms in the ring which are involved in the two consecutive reactions (presumably, the carbon atoms at positions 5 and 3 of the dihydropyridine, in that order). It is expected from the above that under the same standard conditions the k_1/k_2 ratio of the two second-order velocity constants could serve as an indicator of the influence of substituents on the carbon participating in the second reaction.

Differences observed among the energies of activation of the reactions of NADH with orthophosphates or other proton donors (Table V) reflect very roughly the differences among the rate constants observed with the various acids (Table VI). Undoubtedly, more experimental material is required for a sound interpretation of such results.

In confirmation of previous observations (Alivisatos *et al.*, 1964a,b; Stock *et al.*, 1961), 1-methyl-3-carboxamido-1,4-dihydronicotinamide and NMNH reacted much faster than NADH with acids in neutral watery milieu. This was related to the folded state of NADH in solution (Alivisatos *et al.*, 1964) and will be discussed in a following publication (see footnote 2). The reaction rate of orthophosphates with a mixture of all three isomer forms of reduced NAD [*i.e.*, the 1,2-NADH, 1,4-NADH, and 1,6-NADH, all in nearly isomolar quantities (Chaykin and Meissner, 1964)], was the same as that of 1,4-NADH. These results are

TABLE VI: k_1/k_2 Ratios under Different Conditions of pH and Temperature in the Presence of Various Acid Anions.[a]

No.	Anion	Concentration (M)		pH	Temperature (°C.)	k_1/k_2
		Total	Acid Anion			
1	Phosphate	1.50	1.05	6.33	34.4	29
2	Phosphate	1.50	1.05	6.33	43.9	33
3	Phosphate	1.50	0.82	6.62	34.4	30
4	Phosphate	1.50	0.82	6.62	43.9	36
5	Phosphate	1.50	0.52	6.98	34.4	27
6	Phosphate	1.25	0.68	6.62	43.9	29
7	Phosphate	1.25	0.68	6.62	34.4	29
8	Phosphate	1.00	0.55	6.62	34.4	30
9	Phosphate	1.00	0.55	6.62	43.9	27
10	Phosphate	0.75	0.41	6.62	43.9	31
11	Phosphate	0.25	0.14	6.62	43.9	27
12	Arsenate	1.50	0.82	6.62	43.9	28
13	Arsenate	1.50	0.82	6.62	34.4	31
14	Phosphite	1.50	0.65	6.62	34.4	28

[a] Conditions as in Figure 8. The acid-anion concentrations were calculated with the aid of the Hasselbach equation from the pH and the total salt concentration (no corrections for temperature or ionic strength).

difficult to interpret at present. The possibility of acid-catalyzed rearrangements (Kosower, 1962, p. 211). proceeding in the neutral milieu by mechanisms similar to those discussed above cannot be excluded. However, a sound interpretation should rest upon experiments performed with the isolated isomers.

The spontaneously produced intermediate, *B*, is very similar to the DPNH-X (Chaykin *et al.*, 1956). Thus, fresh *B*, like DPNH-X, may be reverted to NADH by a yeast enzyme preparation (Meinhart *et al.*, 1956) (see Results). Similar results were reported by others (Stock *et al.*, 1961; Hilvers, 1964). A difference in the reported wavelengths of the differential (relative to NADH) maxima of "DPNH-X" [in the vicinity of 290 mμ (Chaykin *et al.*, 1956)] and of *B* (in the vicinity of 280 mμ, Figure 2) may probably be attributed to impurities of the enzymatically prepared sample[7] and to the use, in our studies, of an optically compensated system (Herskovits and Laskowski, 1961).

The so-called "primary acid modification product" of NADH (Rafter *et al.*, 1954) cannot be reverted to NADH by the yeast enzyme and must be different from both "DPNH-X" and *B*. This product is obtained by short exposure of "DPNH-X" or of *B* to pH 4.0. This pH value is close to the pK' of protonation of the amino group in the 6 position of the adenine moiety of NADH. Considering the folded state of NADH in solution (Weber, 1957, 1958) and a bathochromic shifting of the maximum from reduced NMN (332 mμ) to NADH (339 mμ), it was suggested (Stock *et al.*, 1961) that hydrogen-bond formation occurs between the NH_2 group of adenine and the oxygen of the carboxamido residue in dihydronicotinamide.[8] According to this view (Stock *et al.*, 1961), when NADH is exposed to acids, the amino group shifts from its previous attachment with the carboxamide and interacts intramolecularly with the carbonium ion formed at position 6 of the ring. This corresponds to "DPNH-X." Formation of the "primary acid product" entails breakdown of this association in the vicinity of the pK' of protonation of the amino group, resulting in the irreversible formation of the hydroxylated adduct. This hypothesis (Stock *et al.*, 1961) is favored by our observations that *B*, obtained at later stages of the reaction, is only partly reverted to NADH in the presence of ATP and the enzyme preparation of Meinhart *et al.* (1956) (see Results). It is also supported by our observation (footnote 4) that the apparent $\epsilon_{B(280)}$ increases at the initial stages of the reaction until a constant (limiting) value is obtained. Thus, in our studies *B* may be regarded as the sum total of both forms. As time progresses, "DPNH-X" changes to the "primary acid modification product," which is unreactive in the enzymatic assay. To the same cause may be attributed present failures in efforts to achieve utilization of the products contained

[7] S. Chaykin, personal communication.

[8] Deformations of the NAD and NADH molecules in the presence of high concentrations of orthophosphates (Ungar and Alivisatos, 1961; Cilento and Schreier, 1964) may further influence the interactions between different parts in these molecules. Thus, in the adenine moiety of NAD, the two nuclear magnetic resonance signals arising from the protons in the 2 and 8 positions change upon addition of 1.5 M orthophosphate from δ = 8.45 and 8.60, respectively, to δ = 8.02 and 8.36 respectively. This increased separation of signals corresponds approximately to the signal separation found in NADH (see footnote 2).

in aliquots of NADH–orthophosphate mixtures as precursors in the enzymatic formation of ATP from ADP and inorganic phosphate.

If the proposed mechanism of spontaneous transfer of protons from the acid to the dihydropyridine moiety of NADH is correct, then the role of glyceraldehyde 3-phosphate dehydrogenase as a catalyst in the formation of "DPNH-X" would be an acceleration of this transfer of protons. This view is supported by the results of Meinhart and Heines (1957). In these studies it was demonstrated that one deuterium atom from D_2O in the milieu is entering the "DPNH-X." This can be interpreted as a result of a spontaneous exchange of protons and deuterons between the acid and the D_2O. It would be of interest to investigate the possibility of a similar acceleration of the protonation of the "primary acid modification product" by the same enzyme.

A practical corollary of our studies is that in *in vitro* studies involving dehydrogenases, etc., or in nonenzymic systems containing reduced coenzymes buffers involving acidic anions should be avoided or used with prudence.

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