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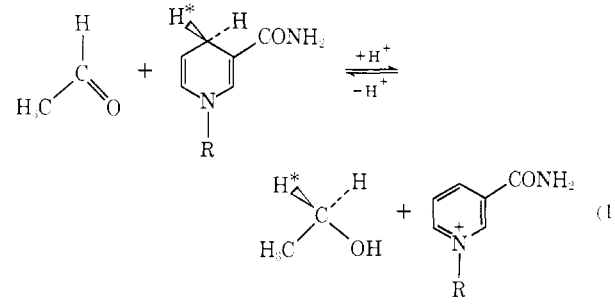
Model Reactions Which Establish a Facile Reduction of Pyridoxal Phosphate and Analogs by 1,4-Dihydropyridines†

Seiji Shinkai‡ and Thomas C. Bruice*

ABSTRACT: A kinetic study of the reaction of pyridine-4-carboxaldehydes with 1,4-dihydropyridines is reported. 3-Hydroxypyridine-4-carboxaldehyde (PyrCHO), but not pyridine-4-carboxaldehyde, is reduced to the corresponding alcohol by N^1-(*n*-propyl)-1,4-dihydronicotinamide (NPrNH) in refluxing methanol. Under the same conditions, PyrCHO and pyridoxal are reduced by 2,6-dimethyl-3,5-dicarboethoxy-1,4-dihydropyridine (Hantzsch ester, HE). Pyridoxal phosphate (pyridoxal-P) and pyridoxal are readily reducible by HE and NPrNH in *ca.* 50% aqueous methanol at 30°. The apparent second-order rate constants for the reaction of HE with pyridoxal are comparable at 30° in aqueous methanol and in boiling neat methanol. Inclusion of 5×10^{-3} M EDTA or 1.2×10^{-3} M hydroquinone in buffered aqueous methanol reaction mixtures of NPrNH and pyridoxal-P did not influence the rate, and nmr product analysis of the reaction of HE and pyridoxal in both refluxing CH_3OD and 48% CH_3OD–D_2O (30°) established solvent deuterons not to be incorporated into product pyridoxine. These results establish the reduction to be a non-free-radical mediated direct hydrogen transfer from dihydropyridine to aldehyde which does not require trace metals as catalysts. In separate experiments, metal ion catalysis of the reduction of pyridoxal-P and PyrCHO by HE was established. The order of metal ion catalysis ($Ni^{2+} > Co^{2+} > Zn^{2+} > Mn^{2+} > Mg^{2+} > 0$) was found to be that previously established for complexation to pyridine and salicylaldehyde (Brewer, D, G., and Wong, P. T. T. (1966), *Can. J. Chem. 44*, 1407; Mellor, D. P. and Maley, L. (1947), *Nature (London) 159*, 370; Martell, A. E., and Calvin, M. (1952), "Chemistry of the Metal Chelate Compounds," New York, N. Y., Prentice-Hall, p 546). From the pH dependence of the apparent second-order rate constants for the reaction of pyridoxal-P with HE and NPrNH, the individual second-order rate constants for reduction of the various ionic species of pyridoxal-P were calculated. The rates of dihydropyridine reduction were found to parallel the rates of imine formation (Auld, D. S., and Bruice, T. C. (1967a), *J. Amer. Chem. Soc. 89*, 2083) and transamination (Auld, D. S., and Bruice, T. C. (1967b), *J. Amer. Chem. Soc. 89*, 2090) for like ionic species (Table VI).

The nicotinamide nucleotides, nicotinamide adenine dinulceotide (NAD^+) and its 2′-phosphoric acid derivative ($NADP^+$) with their reduced forms (NADH, NADPH), are coenzymes in a very large number of enzymatic oxidations and reductions (Bruice and Benkovic, 1966). Alcohol dehydrogenases of both yeast and liver, for example, contain Zn^{II} and NADH at the active sites and catalyze the stereospecific transfer of a hydride ion (or its equivalent) to aldehyde substrates (for a review see Popják, 1970). In order to better understand the mechanisms of catalysis of "hydride" transfer from dihydronicotinamides to aldehydes, searches have been made, to no avail, for aldehydes which are reducible in water at ambient temperatures by dihydronicotinamides (Bruice and Benkovic, 1966). Though enzymatic reduction of pyridoxal-P[1] by pyridine nucleotide has been established (Morino and Sakamoto, 1960; Holzer and Schneider, 1961) the



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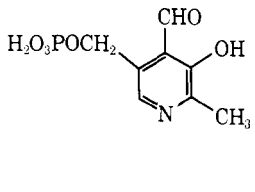
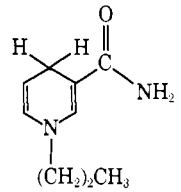
[1] Abbreviations employed are: nicotinamide adenine dinucleotide and its reduced form, NAD^+ and NADH; 2,6-dimethyl-3,5-dicarboethoxy-1,4-dihydropyridines (Hantzsch ester), HE; 1-propyl-3-carbamidopyridinium ion and *N*-propyl-1,4-dihydronicotinamide, $NPrN^+$ and NPrNH; 3-hydroxypyridine-4-carboxaldehyde, PyrCHO; pyridoxal phosphate, pyridoxal-P; 3-hydroxypyridine-4-methanol, $PyrCH_2OH$.

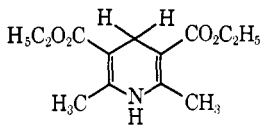
reduction of the 3-hydroxypyridine-4-carboxaldehydes has escaped attention in model studies. We report herein our studies of the facile reduction of pyridoxal-P and analogs by *n*-propylnicotinamide (NPrNH) and 2,6-dimethyl-3,5-dicarboethoxy-1,4-dihydropyridine (HE).



pyridoxal-P



NPrNH



HE

Experimental Section

Materials. 3-Hydroxypyridine-4-carboxaldehyde was from a previous study and was purified as described (Auld and Bruice, 1967a). 3-Hydroxypyridine-4-methanol was prepared by the method of O'Leary and Payne (1971) and the HCl salt was obtained by passing dry HCl through an acetone solution: mp 196–200° (lit. [Heinert and Martell, 1958] mp 198–202°; nuclear magnetic resonance (nmr) δ 4.70 (s, 2 H), 7.97 (d, J = 7 Hz, 1 H, 8.48 (m, 2 H). Pyridoxal hydrochloride was a product of Mann Research Lab and pyridoxal 5′-phosphate was obtained from Sigma Chemical Co. The neutral pyridoxal and pyridoxine were generated from the hydrochlorides according to the method of Metzler and Snell (1955). 1-Propyl-1,4-dihydronicotinamide and 1-propyl-3-carbamidopyridinium iodide were supplied by Dr. P. Y. Bruice (see Paiss and Stein, 1958). 2,6-Dimethyl-,5-dicarboethoxy-1,4-dihydropyridine (HE) and 2,6-dimethyl-3,5-dicarboethoxypyridine were prepared according to the method of Singer and McElvain (1943). Both compounds had melting point and nmr spectra identical with those reported (Singer and McElvain, 1943; Huffman and Bruice, 1967).

Apparatus. Spectrophotometric measurements were made with a Cary 15 spectrophotometer equipped with a thermostated cuvet holder and pH was determined with a Radiometer Model 26 pH meter (30 ± 0.1°) employing a combined-type GK Metrohm electrode.

Kinetics. The kinetic measurements in 52.1 wt % methanolic solution were carried out at 30 ± 0.1° at a calculated μ = 0.01 (with KCl) under anaerobic conditions (N_2 or argon). The 0.02 M buffer solutions of 52.1 wt % methanol were prepared according to the method of Ong *et al.* (1964) and Bates *et al.* (1963) (succinate below pH 7.41, phosphate for pH 7.69–8.34, and borate for pH 9.22). As 1,4-dihydropyridines (especially 1-propyl-1,4-dihydronicotinamide) decompose slowly at low pH initial changes in absorbance were monitored, and after correction for spontaneous disappearance of dihydronicotinamide the zero-order rate constants were computed. The rates of dihydronicotinamide decomposition were negligible when compared to those in the presence of substrate at pH 9.22 for 1-propyl-1,4-dihydronicotinamide and above pH 8.34 for Hantzsch ester allowing second-order computation of the rate constants.

For reactions in refluxing methanol, anaerobic (N_2) dark conditions were employed for which case the 1,4-dihydropyridines were found to be stable. Aliquots (2 ml) of the reaction mixture were withdrawn with time and scanned in the ultraviolet (uv) range at 30° (diluted if necessary). The wavelengths employed for the kinetic analysis were 372 nm for the Hantzsch ester and 354 nm for 1-propyl-1,4-dihydronicotinamide.

The buffered solutions containing 0.01 M EDTA and metal ion were prepared and pH values were determined as before (μ = 0.02 with KCl). To obviate the formation of precipitates the kinetic measurements were conducted in solutions containing 0.015 M metal ion added as the chloride salt. In those instances where conspicuous acceleration of reduction was brought about by metal ion, second-order rather than zero-order computation of rate constants was possible even at pH 7.1.

pK_a' values of pyridoxal-P were determined by potentiometric titration at 30°, μ = 0.01 (with KCl), in 52.1 wt % methanol–H_2O. The apparatus employed was a Radiometer Model 26 pH meter and a Radiometer ABU-1c Auto-burette. From the equivalence of base required, the pyridoxal-P employed in this study was determined to be 96.7% pure. The pK_a' values were calculated by the method of Noyes (1893) and corrected according to Bates *et al.* (1963). Values obtained were <4.05, 4.49, ± 0.02, 7.44 ± 0.03, and 8.41 ± 0.02. Comparison of these pK_a' values to those reported for pyridoxal and 5-deoxypyridoxal (Metzler and Snell, 1955) allows assignment for the pK_a values of N-1–H as 4.49 and 3-OH as 8.41.

Product Analysis. For thin layer chromatographic (tlc) analysis of the reaction poducts silica gel (6060 Silica Gel, Eastman) was employed. Absolute ethanol was found to be suitable for the developing solvent. As absorbants containing fluorescent indicators were used, all reactants and products could be detected through fluorescence. The methanol solution was evaporated *in vacuo*, after the desired period of refluxing, and the residue taken into Me_2SO-d_6 for nmr examination.

Hydrogen Transfer in Deuterated Solvents. Hantzsch ester (691 mg; 2.73 mmol) and neutral pyridoxal (435 mg; 2.60 mmol) were dissolved in 25 ml of refluxing methanol-*d*, and refluxing continued for 72 hr under anaerobic, dark conditions. After cooling, methanol-*d* was evaporated *in vacuo*, and the residue was taken into 40 ml of water, which was extracted with 2 × 30 ml of chloroform. After evaporation of the solvents, 431 mg of an oil was obtained from the water phase, which crystallized slowly in an ice bath, and 694 mg of yellow solid was obtained from the chloroform phase. Nmr examinations established that the former was a mixture of pyridoxal and pyridoxine, and the latter a mixture of Hantzsch ester and 2,6-dimethyl-3,5-dicarboethoxypyridine.

Neutral pyridoxal (130 mg; 0.78 mmol) and well-powdered Hantzsch ester (1.08 g; 4.3 mmol) were dissolved and suspended respectively in 50 ml of a 0.05 M phosphate buffer solution made up in methanol-*d* (48 wt %)–D_2O adjusted to pD 8.25 and stirred under anaerobic (N_2) conditions for 10 days at room temperature. Methanol-*d* was removed at room temperature *in vacuo*, the aqueous residual clarified by filtration, and the filtrate, after being extracted with 2 × 30 ml of chloroform, was taken to dryness *in vacuo* (40°). The solid material obained was extracted with 50 ml of anhydrous methanol, the extract evaporated, and the light yellow solid obtained was dissolved in Me_2SO-d_6 and its nmr spectra recorded.

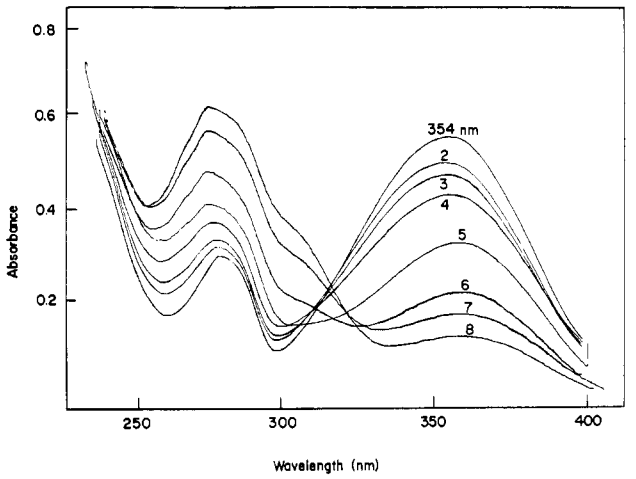


FIGURE 1: Spectral time study for the reaction of 3-hydroxypyridine-4-carboxaldehyde (1.31×10^{-4} M) with 1-propyl-1,4-dihydronicotinamide (7.73×10^{-5} M) in refluxing methanol: 2, 10 min; 3, 30 min; 4, 70 min; 5, 130 min; 6, 220 min; 7, 310 min; 8, 430 min.

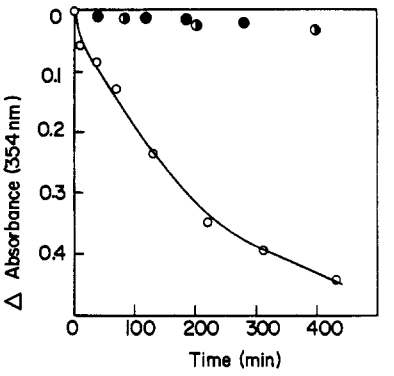


FIGURE 2: Time dependency of absorbance of 1-propyl-1,4-dihydronicotinamide (354 nm) in the presence of pyridinecarboxaldehyde derivatives in refluxing methanol: ○, [PyrCHO] = 1.31×10^{-4} M; [NPrNH] = 7.73×10^{-5} M; ●, [pyridine-4-carboxaldehyde] = 1.25×10^{-4} M; [NPrNH] = 7.73×10^{-5} M; ◑, [NPrNH] = 7.73×10^{-5} M without the presence of aldehyde.

Results

Reactions in Refluxing Methanol. The rate of decrease of absorbance of NPrNH (λ_{max} 354 nm) exhibits a pronounced dependence upon [PyrCHO] as shown in Figure 1. In contrast, no significant change of NPrNH absorbance was observed in the presence of pyridine-4-carboxaldehyde (Figure 2). These results clearly indicate the importance of the 3-hydroxy group to the rate of reduction of the aldehyde group substituted at the 4 position of the pyridine ring of PyrCHO. Disappearance of reactants and appearance of products were monitored *via* tlc studies of the reaction mixture (Table I). Spectral examination of the reaction mixture established the appearance of a product with λ_{max} 270 nm (Figure 1) which may be compared to that of $NPrN^+$ (λ_{max} 264 nm). Since the reaction solutions were not buffered and hydride equivalent reduction of an aldehyde by a 1-substituted dihydronicotinamide produces alkoxide ion, the "pH" of the methanolic solutions would not remain constant. For this reason tight isosobestic points were not obtained for spectral scans taken at periodic times during the reaction (Figure 1). Also, the equilibrium of eq 2 should lead to production of the much less reactive 3-alkoxypyridine-4-carboxaldehyde. Near completion of the reaction, a new shoulder peak appeared at *ca.* 300 nm which is assignable to the absorbance of $PyrCH_2OH^-$, the authentic sample of which showed its λ_{max} at 302 nm. The nmr spectrum of the reaction mixture after 12 hr of refluxing still showed the aldehyde peak of PyrCHO at 10.3 ppm (δ). As shown in Table I, however, the same sample did not give a tlc spot indicative of PyrCHO, establishing that the aldehyde peak observed by nmr must be identified with that of $PyrCHO^-$ which would not develop on the thin layer. At termination of the reaction, solvent was removed *in vacuo* and the residue dissolved in Me_2SO-d_6 and an nmr spectra taken. The nmr spectra showed several new peaks at 7.12 (d, J = 5 Hz) and 8.05 ppm (m), which are explicable as the C_5 proton and C_2,C_6 protons, respectively, of $PyrCH_2OH$ in company with $NPrN^+$ [9.02 (d, J = 8 Hz), 9.25 (d, J = 6 Hz), 9.58 ppm (s), etc]. In combination, the spectral and nmr results clearly support reduction of PyrCHO by NPrNH to provide $PyrCH_2OH^-$ and $NPrN^+$ with establishment of the equilibrium of eq 2.

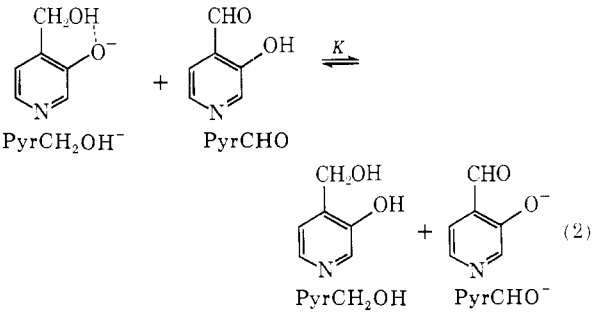


TABLE I: Tlc Study of the Reaction of PyrCHO and NPrNH in Refluxing Methanol.[a]

Sample	R_F		
	Authentic Samples	3-hr Reflux	12-hr Reflux
NPrNH	0.54	0.52	0.55
PyrCHO	0.20	0.18	None
$NPrN^+$	0.0–0.05[b]	0.0–0.02	0.0–0.05
$PyrCH_2OH$	0.26	0.25	0.24

[a] [PyrCHO] = 6.26×10^{-3} M, [NPrNH] = 5.97×10^{-3} M. [b] Iodide salt was used.

For kinetic analysis the material balances shown in eq 3–6 may be employed, where $[PyrCHO]_0$ and $[NPrNH]_0$

$$[PyrCHO]_0 = [PyrCHO] + [PyrCHO^-] + [PyrCH_2OH] + [PyrCH_2OH^-] \quad (3)$$

$$[NPrNH]_0 = [NPrNH] + 2[PyrCH_2OH] + [PyrCH_2OH^-] - [PyrCHO^-] \quad (4)$$

$$[NPrNH]_0 = [NPrNH] + [PyrCH_2OH^-] + [PyrCHO^-] \quad (5)$$

$$K = \frac{[PyrCH_2OH][PyrCHO^-]}{[PyrCH_2OH^-][PyrCHO]} \quad (6)$$

denote the intial concentrations of PyrCHO and NPrNH and K denotes the equilibrium constants of eq 2. From eq 3–6

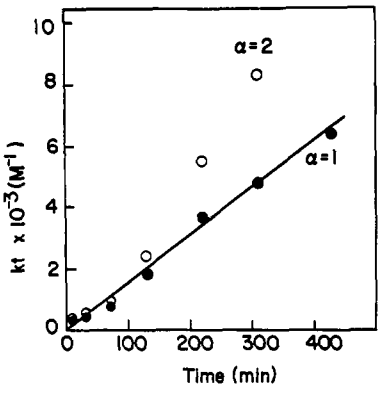


FIGURE 3: Plots of Z *vs.* t for the reaction of NPrNH with PyrCHO: ○, from eq 10; ●, from eq 11; in refluxing methanol.

eq 7 can be deriviated, where $\Delta[\text{PyrCHO}] = [\text{PyrCHO}]_0 - [\text{PyrCHO}]$ and $\Delta[\text{NPrNH}] = [\text{NPrNH}]_0 - [\text{NPrNH}]$. Putting $\alpha = \Delta[\text{PyrCHO}]/\Delta[\text{NPrNH}]$, eq 7 can be rewritten as shown in eq 8. Because in the initial phase of the reaction every molecule of NPrNH (eq 8) consumed requires two molecules

$$K = \frac{(\Delta[\text{PyrCHO}] - \Delta[\text{NPrNH}])^2}{[\text{PyrCHO}](2\Delta[\text{NPrNH}] - \Delta[\text{PyrCHO}])} \quad (7)$$

$$K = \frac{\Delta[\text{NPrNH}](\alpha - 1)^2}{[\text{PyrCHO}](2 - \alpha)} \quad (8)$$

$$1 \leqq \alpha \leqq 2$$

of PyrCHO as reactant and proton source, respectively (eq 2 and 3), $\alpha = 2$. Because the reaction of eq 3 is reversible, α approaches 1.0 as the reaction progresses. The second-order rate equation (9), therefore, was resolved for the two extreme

$$-\frac{d[\text{NPrNH}]}{dt} = k[\text{PyrCHO}][\text{NPrNH}] \quad (9)$$

cases, $\alpha = 2$ and 1. For $\alpha = 2$

$$kt = Z = \frac{1}{2[\text{NPrNH}]_0 - [\text{PyrCHO}]_0} \times \left(\ln \frac{[\text{NPrNH}]_t}{[\text{PyrCHO}]_0/2 - \Delta[\text{NPrNH}]} - \ln \frac{2[\text{NPrNH}]_0}{[\text{PyrCHO}]_0}\right) \quad (10)$$

For $\alpha = 1$

$$kt = Z = \frac{1}{[\text{NPrNH}]_0 - [\text{PyrCHO}]_0} \times \left(\ln \frac{[\text{NPrNH}]}{[\text{PyrCHO}]_0 - \Delta[\text{NPrNH}]} - \ln \frac{[\text{NPrNH}]_0}{[\text{PyrCHO}]_0}\right) \quad (11)$$

The calculation was carried out applying eq 10 and 11 to the experimental results contained in Figure 3. A good linear relationship was observed for the plots obtained from eq 11, as shown in Figure 3, but the plots generated from eq 10 possessed an upward curvature. From the slope of the line of Figure 3, the second-order rate constant for the reaction can be estimated to be 16 M^{-1} mi^{-1} (refluxing methanol).

The reaction of HE with PyrCHO was conducted in the same manner as in the reaction of NPrNH with PyrCHO.

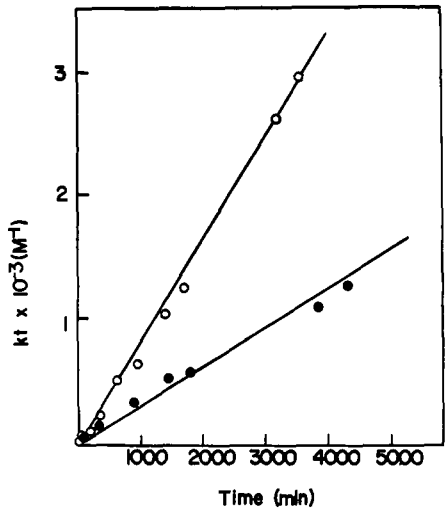


FIGURE 4: Plots of Z *vs.* t for the reaction of Hantzsch ester with pyridinecarboxaldehyde derivatives in the refluxing methanol employing eq 11: ○, 3-hydroxypyridine-4-carboxaldehyde; ●, pyridoxal.

The decrease in absorbance of HE (λ_{max} 372 nm, ϵ 4500) was accompanied by an increase of absorbance at 277 nm. 2,6-Dimethyl-3,5-dicarboethoxypyridine, the oxidized product of HE, has its λ_{max} at 272 nm. In contrast to the reaction with NPrNH, an isosbestic point was observed at 293 nm, providing evidence for a simple A → B process without accumulation of any intermediate or intervention of an additional acid–base equilibria. This is as anticipated from eq 12. Equation 11 can be applied to the rate studies of this reaction and from the linear plot (Figure 4) the second-order rate constant was determined. Reduction of pyridoxal with HE was investigated in the same manner. A summation of the rate data is provided in Table II.

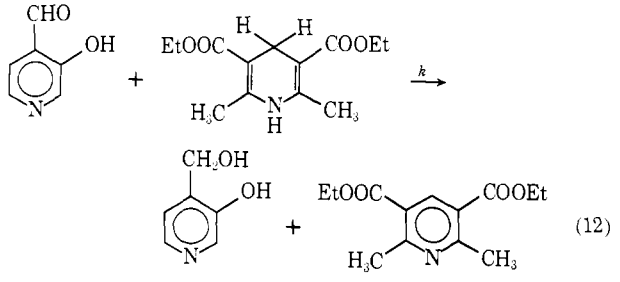


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TABLE II: Second-Order Rate Constants for Reduction of Pyridine-4-carboxaldehydes by Dihydropyridines in Refluxing Methanol.[a]

Aldehyde	Dihydropyridine	k (M^{-1} min^{-1})
PyrCHO	NPrNH	16
PyrCHO	HE	0.74
Pyridoxal	HE	0.28
4-Pyridinecarboxaldehyde	NPrNH	~0

[a] 0.77×10^{-4}–1.48×10^{-4} M NPrNH and HE; 1.25×10^{-4}–1.75×10^{-4} M aldehydes.

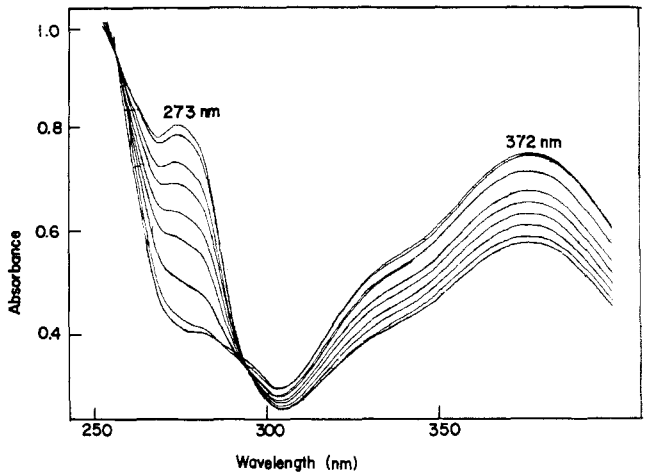


FIGURE 5: Spectral time study for the reaction of Hantzsch ester (8.6×10^{-5} M) with pyridoxal 5′-phosphate (7.60×10^{-5} M) (30°, 0.01 M KCl, pH 8.34, 52.1 wt % methanol).

TABLE III: Apparent Second-Order Rate Constants, k_{app} (M^{-1} min^{-1}), for the Reactions of 1,4-Dihydroxypyridines and Pyridinecarboxaldehyde Analogs.[a]

	HE[d] with			
pH	PyrCHO[e]	Pyridoxal[e]	Pyridoxal-P[e]	NPrNH[d] with Pyridoxal-P[e]
7.15[b]	~0			2.6
7.41			0.76	2.5, 2.6[c]
7.69[b]				2.3
7.90			0.65	2.2
8.34	~0	0.22	0.48	1.7
9.22			0.15	0.5

[a] 30°, 0.01 M KCl, 52.1 wt % methanol. [b] 30°, 0.01 M KCl, 52.1 wt % methanol, [EDTA] = 5×10^{-3} M. [c] 30°, 0.01 M KCl, 52.1 wt % methanol, [hydroquinone] = 1.2×10^{-3} M. [d] 0.86×10^{-4}–1.60×10^{-4} M HE and NPrNH. [e] 0.76×10^{-4}–1.20×10^{-4} M PyrCHO, pyridoxal-P, and pyridoxal-P.

3-Hydroxypyridine-4-carboxaldehyde analogs are known to have two species, neutral and dipolar, even in methanol solution (Matsushima and Martell, 1967). We have not taken these equilibria into account due to a lack of knowledge concerning K_e in refluxing methanol. Therefore, the second-order rate constants of Table II pertain to total aldehyde present. As it is reasonable to consider the neutral species to be more reactive than the dipolar one, the second-order rate for the former should be greater than indicated by the calculated constants of Table II.

The reaction of pyridoxal-P with HE was also examined in refluxing methanol. The absorbance of HE decreased abruptly and a species absorbing at about 290 nm was formed. 1,4-Dihydropyridines are known to be susceptible to acid-catalyzed (H_3O^+, CH_3COOH) methanolysis (Cornforth *et al.*, 1962). The lowest pK_a of neutral pyridoxal-P is reported to be about 3.4 (Anderson and Martell, 1964), so that undissociated pyridoxal-P should be a strong enough acid to catalyze the methanolysis of HE. Moreover, the new λ_{max} at 290 nm cannot be due to formation of 2,6-dimethyl-3,5-carboethoxypyridine but is attributable to the methanolysis product. Due to this complexity, it was not possible to determine the second-order rate constant for reaction of HE with pyridoxal-P in refluxing methanol.

Reactions in Aqueous 52.1 Wt % Methanolic Solution. Reactions in this solvent were carried out at 30° employing buffers (Experimental Section) to maintain constant pH. The peculiar solvent ratio employed was chosen for convenience since standard buffer compositions are available in this solvent (Experimental Section). The ultraviolet absorbance scans for the reaction of HE and pyridoxal-P at pH 8.34 are shown in Figure 5. Examination of Figure 5 reveals the absorbance maximum of HE, 372 nm, decreases slowly, giving rise to a new absorbance maximum at 273 nm. Furthermore, tight isosbestic points are maintained at 256 and 293 nm. This result established that HE reduces pyridoxal-P at 30° in aqueous methanol in an A → B process without the build-up of absorbing intermediates or the intervention of side reactions. The apparent second-order rate constant k_{app} can be defined as shown in eq 13,

$$-\frac{d[HE]}{dt} = k_{app}[\text{pyridoxal-P}_T][HE] \quad (13)$$

where [pyridoxal-P_T] and [HE] denote the total concentrations of pyridoxal-P and Hantzsch ester, respectively. The value of k_{app} was determined by graphical analysis (eq 11) of the time-dependent decrease of optical density at 372 nm. At pH values below 7.90 slow disappearance of HE due to solvolytic reactions comes into play. At pH values below 7.90, the apparent second-order rate constants were, therefore, determined by zero-order kinetics. The reaction of NPrNH, λ_{max} 362 nm, with pyridoxal-P was examined in like fashion. Zero-order kinetics were employed for this case below pH 8.34. A summary of the kinetic results is provided in Table III.

Comparison of the results of Tables II and III establishes that reduction of pyridoxal by HE is facilitated on transfer from neat methanol to *ca.* 50% aqueous methanol (μ = 0.01). Thus, the apparent second-order rate constant for the Hantzsch ester–pyridoxal system at 30° in 52.1 wt % methanolic solution is almost the same as that in refluxing methanol. No reaction of PyrCHO with HE could be detected in aqueous methanol at pH 7.15 and 8.34. The pK_a of the 3-hydroxyl group of PyrCHO is much lower than for the other pyridinecarboxaldehydes (4.05 at 30°) (French *et al.*, 1965), so that at pH 7.1–8.3 it exists predominantly in the unreactive anionic state. Since pyridoxal and pyridoxal-P are known to have pK_a values above 8 (Ahrens *et al.*, 1970), a considerable mole fraction of these compounds should exist as the reactive neutral species.

Alcohol dehydrogenase is known (Drum *et al.*, 1969) to contain zinc at the active sites which plays a decisive role in the mechanism of NADH reduction, presumably (Mildvan, 1970) by complexation to the aldehyde carbonyl group. To assess the possible contribution of heavy metal salt impurities present in the KCl and buffer salts employed, the kinetic studies were also run in the presence of 5×10^{-3} M EDTA. The results (Table III) establish that the k_{app} values were not affected significantly by the addition of EDTA. The absence of unwanted trace metal ion catalysis is also supported by the fact that PyrCHO was inactive toward HE between pH 7.1 and 8.3. In this pH region, the anionic species of PyrCHO is capable of interacting with metal ions. The pH–rate profiles for the reaction of pyridoxal-P with HE and

TABLE IV: Second-Order Rate Constants for the Reaction of Pyridoxal 5′-Phosphate with 1,4-Dihydropyridines.[a]

Second-Order Rate Constants (M^{-1} min^{-1})	1,4-Dihydropyridines	
	NPrNH	HE
k_1	17	6.4
k_2	2.7	0.83
k_3	$\sim 10^{-1}$	$\sim 2 \times 10^{-2}$

[a] 30°, 0.01 M KCl, 52.1 wt % methanol in water.

NPrNH are shown in Figure 6. The decrease of the apparent second-order rate constants in going to high pH is in agreement with the suggestion that ionization of the 3-hydroxyl group greatly reduces susceptibility to reduction by 1,4-dihydropyridines. Since the rate points obtained in the presence of EDTA fall on the same curve as in its absence, there appears to be no effect of EDTA on the apparent second-order rate constants. The rate measurement was also conducted in the presence of 1.2×10^{-3} M hydroquinone, a typical radical inhibitor, in order to ascertain if the reductions were free radical in nature (Dittmer and Fouty, 1964). The rate constant (Table III) being the same, within experimental error, with or without inhibitor allows the conclusion that the reduction of pyridoxal-P does not proceed *via* a free-radical mechanism and a "hydride transfer" mechanism is involved.

The pH dependence of k_{app} may be employed to determine the individual second-order rate constants for reduction of the ionic species of pyridoxal-P providing that the state of ionization of the phosphate ester moiety does not effect the rate of reduction. This assumption allows consideration of but three ionic species (eq 14). Equation 19 follows from

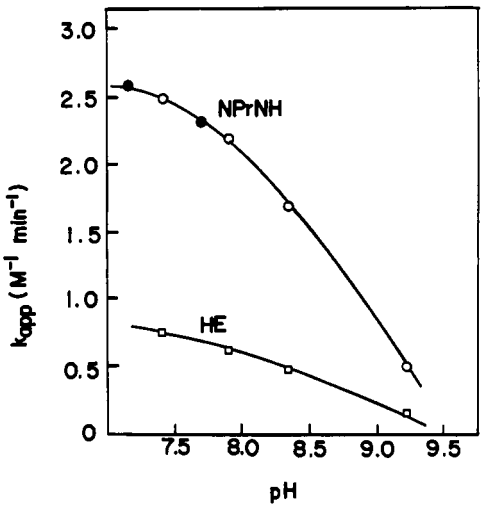
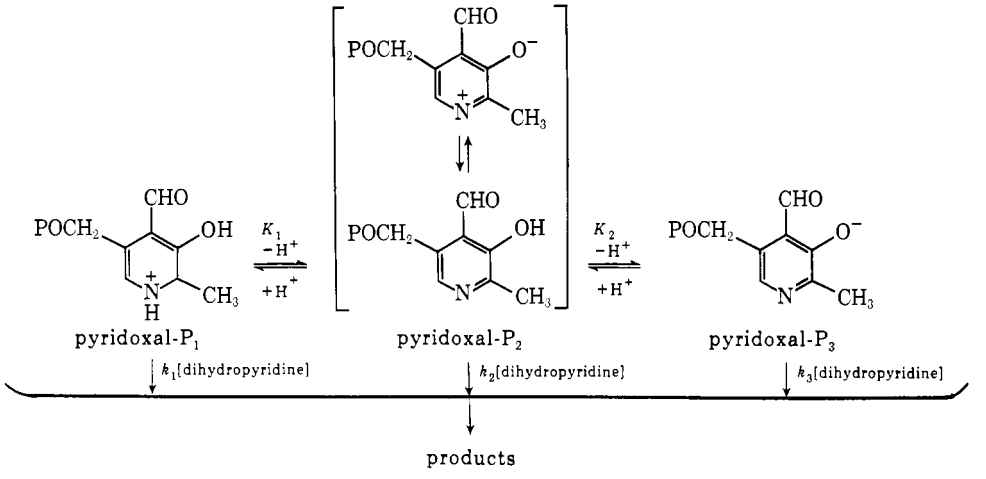


FIGURE 6: pH–rate profiles for the reaction of pyridoxal 5′-phosphate (0.86×10^{-4} –1.20×10^{-4} M) with (30°, 0.01 M KCl, 52.1 wt % methanol) *N*-propyl-1,4-dihydronicotinamide (○) and Hantzsch ester (□) (0.76×10^{-4}–1.6×10^{-4} M). The shaded points pertain to results obtained in the prexence of 5×10^{-3} M EDTA.

$$k_{app}[\text{pyridoxal-P}_T] = k_1[\text{pyridoxal-P}_1] + k_2[\text{pyridoxal-P}_2] + k_3[\text{pyridoxal-P}_3] \quad (18)$$

$$k_{app} = \frac{k_1 a_H^2 + k_2 a_H K_1 + k_3 K_1 K_2}{a_H^2 + a_H K_1 + K_1 K_2} \quad (19)$$

as determined with the glass electrode (Ong *et al.*, 1964; Bates *et al.*, 1963). The ionization constants, K_1 and K_2, were determined in the same solvent at the temperature employed in the kinetic studies (Experimental Section). The



(14)

eq 15–18, where a_H is the corrected hydrogen ion activity

$$[\text{pyridoxal-P}_T] = [\text{pyridoxal-P}_T] + [\text{pyridoxal-P}_2] + [\text{pyridoxal-P}_3] \quad (15)$$

$$K_1 = \frac{[\text{pyridoxal-P}_2]a_H}{[\text{pyridoxal-P}_1]} \quad (16)$$

$$K_2 = \frac{[\text{pyridoxal-P}_3]a_H}{[\text{pyridoxal-P}_2]} \quad (17)$$

k_{app} values obtained at pH 7.41, 7.90, and 8.34 were employed for the calculation of k_1, k_2, and k_3. The curves of Figure 6 were generated from eq 19 employing the constants of Table IV. The rate constants of Table IV establish pyridoxal-P_1 as the most reactive species toward 1,4-dihydropyridines and pyridoxal-P_3 as hardly reactive at all.

Metal Ion Acceleration. From the foregoing it is concluded that dihydropyridine reduction of pyridoxal-P and analogs is most facile at low pH where the mole fraction of internally hydrogen-bonded aldehyde species (by the 3-HO group) is maximal. At these pH values, hydrolysis of dihydropyridine

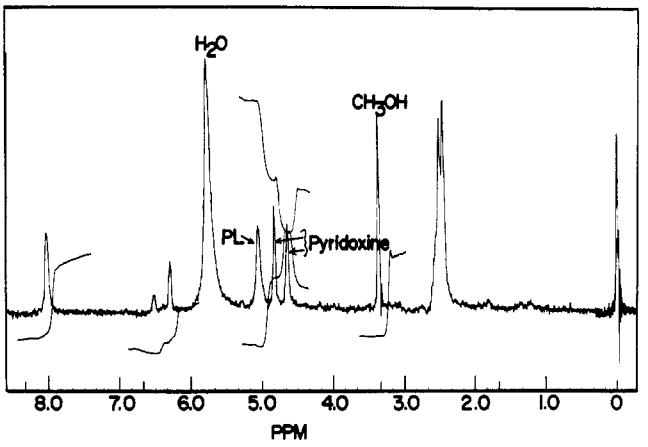


FIGURE 7: Nmr spectra of the reaction product from pyridoxal (0.104 M) and Hantzsch ester (0.109 M) in refluxing methanol-*d* (solvent, Me_2SO-d_6). Methanol is used to transfer the products.

TABLE V: Apparent Second-Order Rate Constants (k_{app}) in the Presence of Metal Ions.[a]

Metal	pH	k_{app} (M^{-1} min^{-1}) HE + Pyridoxal-P	k_{app} (M^{-1} min^{-1}) HE + PyrCHO
None	7.10	0.8	~0
Ni^{2+}	7.04	5.8, 6.2[b]	
Co^{2+}	7.08	2.7	
Zn^{2+}	7.10	2.2	0.4
Mn^{2+}	7.05	1.0	
Mg^{2+}	7.12	0.95	

[a] 30°, μ = 0.02 with KCl, 52.1 wt % methanol in water, $[EDTA_T]$ = 0.010 M, $[M_T]$ = 0.015 M. [b] [Hydroquinone] = 1.2×10^{-3} M.

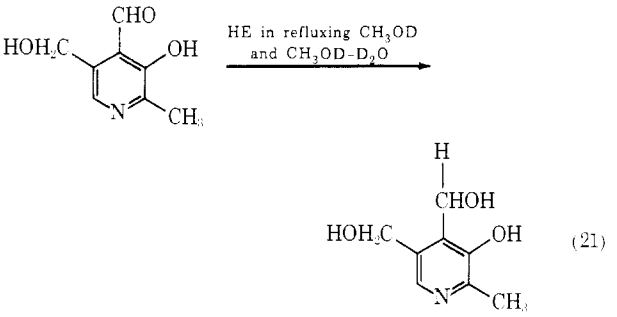
species comes into play. It is inferrable that the 3-HO and 4-CHO groups of pyridoxal-P analogs participate in the formation of six-membered chelate ring structures with metal ions (Longenecker and Snell, 1957). In such complexes, which remain soluble even at pH values above 7 under the coexistence of EDTA, the metal replaces the hydrogen in the internally hydrogen-bonded structure. It was anticipated, therefore, that such complexation would lead to enhanced reducibility. Pyridoxal-P and PyrCHO were stable in the buffer solutions used and Hantzsch ester was found not to be decomposed by the metal ions studied (cupric ion, which is known to be reduced by 1-benzyl-1,4-dihydronicotinamide (Dittmer and Kolyer, 1962) was not employed).

All experiments were carried out in the presence of 0.01 M EDTA which assures the presence of a constant and low concentration of free metal ion as provided by eq 20. Appar-

$$[M] = \frac{[EDTA\text{–}M]}{K_{EDTA}[EDTA]} \quad (20)$$

ent rate constants, determined at a single pH, were calculated from the dependence of the change in absorbance of HE (372 nm) using eq 11 as before. The calculated values of k_{app} are provided in Table V. These results show that metal ions activate 3-hydroxypyridine-4-carboxaldehyde derivatives to reduction by dihydropyridines.

Hydrogen Transfer Reaction in Deuterated Solvents. The product isolation experiments (Experimental Section) provided a mixture of pyridoxal and pyridoxine from the reaction of pyridoxal and HE in methanol-*d*. The nmr spectra of the mixture were analyzed by comparison to spectra taken of authentic samples of pyridoxine and pyridoxal. The nmr spectra of the authentic samples were measured in Me_2SO-d_6, which established the position of the methylene hydrogens of pyridoxine to be at δ 4.62 and 4.83 ppm and those of pyridoxal to be at δ 5.00 ppm. The nmr spectrum of the reaction mixture (Figure 7) from runs in refluxing methanol-*d* possessed singlet peaks at δ 4.63, 4.82, and 5.03 ppm. Therefore, the two singlet peaks, which had almost the same integral intensities (18:19), at the higher magnetic field are assignable to the 4- and 5-methylene groups of pyridoxine. The reaction product isolated from 48 wt % methanol-*d* also indicated the same intensities (16:16). These results reveal that no deuterium was incorporated in the 4-methylene group of pyridoxine (eq 21) and provide clear evidence that reduc-



(21)

tion occurs *via* direct hydrogen atom transfer in both methanol and *ca.* 50 wt % aqueous methanol.

In making the products analysis, it was esential to employ reactants at much higher concentrations than for kinetic studies. The course of the product analysis studies was monitored by withdrawal of aliquots, dilution, and spectrophotometric examination. The apparent second-order rate constants determined at these high concentrations (*ca.* 0.1 M) were found to be smaller than those obtained in the kinetic studies (Table II). For example, the constant for the reaction of HE with pyridoxal in refluxing methanol was estimated to be about 10^{-3} M^{-1} min^{-1}. Rate constants determined at [pyridoxal] = 0.01–0.08 M in refluxing methanol when plotted *vs.* [pyridoxal] provided a Michaelis–Menten-like behavior (k_{cat} = 1.5×10^{-4} min^{-1}, K_m = 2.7 mM). The nonlinear dependence of rate on reactant concentration could be due to many factors such as formation of aggregations of zwitterions (Sensi and Gallo, 1954, 1955), changes in ionic strength, activities, and pH.

Discussion

In contrast to the inability of 1,4-dihydropyridines to reduce all but the most activated carbonyl carbons, liver alcohol dehydrogenase exhibits a rather broad specificity for aldehydes of widely varying structure (Sund and Thorell, 1962). The versatility of the enzyme is most likely due, in great part, to association of the carbonyl oxygen of the substrate, as a ligand, with enzyme-bound Zn^{II} (Drum and Vallee, 1970). Such an association would create a partial

positive charge upon the carbonyl carbon and increase its susceptibility to hydride (or equivalent) attack. Highly electron deficient carbonyl compounds which undergo reduction by dihydropyridines include quinone (Wallenfels and Gellrich, 1959) and hexachloroacetone (Dittmer and Fouty, 1964). Thioketones, due to their polar nature, are also reducible by 1,4-dihydropyridines (Abeles *et al.*, 1957). To the present, there had been no report of reduction of an aldehyde by a 1,4-dihydropyridine in aqueous solution at ambient temperature.

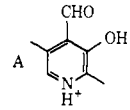
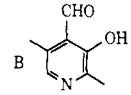
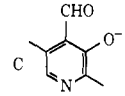
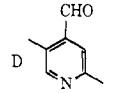
One would anticipate that an aldehyde, in which the carbonyl oxygen was a part of a metal chelate or hydrogen-bonded bridge and whose carbonyl carbon was electron deficient, would be readily reducible by NADH and its analogs. That internal hydrogen bonding can facilitate reduction by 1,4-dihydropyridines was shown by Abeles *et al.* (1957). Thus, *o*-hydroxythiobenzophenone is about twice as susceptible to reduction as benzophenone and 28 times as susceptible as *p*-hydroxythiobenzophenone. For carbonyl compounds the influence of a neighboring phenolic hydroxyl group is more pronounced than with thioketones. In fact, in refluxing dioxane benzaldehyde and *m*-nitrobenzaldehyde are inert toward HE, but reduction of salicylaldehyde is detectable (Pandit and Mas Cabré, 1971). In the salicylaldehyde system, the proton donation to the carbonyl oxygen by the ortho hydroxyl group appears to be of greater importance than electron withdrawal from the carbonyl carbon by the meta nitro group. An example of promotion of susceptibility to reduction by metal complexation at the carbonyl oxygen has been offered by Creighton and Sigman (1971) who reported the reduction of 1,10-phenanthroline-2-carboxaldehyde by NPrNH in acetonitrile to take place only in the presence of Zn^{II}.

Pyridoxal phosphate, pyridoxal, and 3-hydroxypyridine-4-carboxaldehyde possess electron-deficient carbonyl groups which are hydrogen bonded to a neighboring phenolic hydroxyl group. The facility of reduction of pyridoxal-P and analogs by 1,4-dihydropyridines would be expected to far exceed the reducibility of substituted salicylaldehydes. This conclusion follows from early studies of the transamination reaction. Thus, the ability of 3-hydroxypyridine-4-carboxaldehydes to enter into non-metal-mediated transamination reactions with α-amino acid is dependent upon electron withdrawal from the aldehyde moiety by the pyridine ring and internal hydrogen bonding by the 3-hydroxyl group (Bruice and Benkovic, 1966). Salicylaldehydes are apparently incapable of entering into a transamination with amino acids (Ikawa and Snell, 1954). The electron withdrawing ability of a pyridine nitrogen is often considered akin to that of a nitro group. However, 4-nitrosalicylaldehyde reacts with glutamic acid *via* an oxidative deamination reaction in which the nitro group acts as an oxidizing agent. Other salicylaldehydes exhibit no reactivity with glutamic acid even in the presence of Al^{III} (Ikawa and Snell, 1954). In addition, the well-known ability of metal ions to act as catalysts in the transamination reaction (Metzler and Snell, 1952) through formation of a chelate structure would suggest that metal ions should also catalyze the reduction of 3-hydroxypyridinecarboxaldehydes.

The enumerated expectations have been borne out in the present study, which establishes that pyridoxal and 3-hydroxypyridine-4-carboxaldehyde, but not pyridine-4-carboxaldehyde, are reduced by NPrNH and HE in boiling methanol. The resemblance to the requirements of the transamination reaction is marked. Thus, pyridoxal (Bruice and Topping, 1963) and 3-hydroxypyridine-4-carboxaldehyde (Thanassi *et al.*, 1965; Auld and Bruice, 1967a,b) but not pyridine-4-carboxaldehyde undergo non-metal-mediated transamination with amino acids (ambient temperature in H_2O). At 30° in 52.1 wt % methanol–water, pyridoxal and pyridoxal phosphate are readily reduced by HE and *N*-propylnicotinamide. Addition of EDTA has no effect on the rate of reduction, ruling out essential contribution of trace metal ion. The reaction is not inhibited or altered by addition of excess hydroquinone and solvent protons are not incorporated into the pyridoxine product in either boiling methanol or aqueous methanol. Thus, the reduction is not free radical in nature (Dittmer and Fouty, 1964) but involves the direct transfer of hydrogen from dihydropyridine to the pyridinecarboxaldehyde group. Reduction of pyridoxal by 1,4-dihydropyridines is facilitated on transfer from neat methanol to *ca.* 50% aqueous methanol (μ = 0.01). This is to be anticipated for a reaction whose transition state is more polar than initial state. Thus, Abeles *et al.* (1957) found that reduction of neutral thioketones is accelerated by transfer to polar solvents while Huffman and Bruice (1967) report that rates of reduction of cationic phenyl-α-methylindolidenemethanes by HE are accelerated 600-fold on transfer from ethanol to acetonitrile. In the latter investigation, it was noted that the absorbance of the indolidenium salt underwent a 100-fold change in extinction accompanying solvent transfer. The parallel changes in rates and extinction were interpreted as due to greater solvation of the initial state in the more polar solvent.

The data of Table VI provide a comparison of relative rate constants for imine formation, water general base catalyzed transamination, and NPrNH (52.1 wt % MeOH–H_2O) reduc-

TABLE VI: Comparison of the Relative Rates (30°) for NPrNH Reduction, Imine Formation with Alanine, and General Base (by H_2O) Catalyzed Prototropy of Alaninimine.

	Imine Formatn	Transamination with H_2O Gen Base Catal	NPrNH Reductn
A 	1.64×10^4 [a]	1.55×10^5 [b]	1.7×10^2 [c]
B 	8.6×10^1 [a]	8.8 [b]	2.7×10^1 [c]
C 	1 [a]	1 [b]	~1 [c]
D 	≪1 [d]	No reaction [d]	No reaction [c]

[a] Auld and Bruice, 1967a. Employing alanine and 3-hydroxypyridine-4-carboxaldehyde. [b] Auld and Bruice, 1967b. Employing alanine and 3-hydroxypyridine-4-carboxaldehyde. [c] This study employing pyridoxal-P and NPrNH. [d] French and Bruice, 1964.

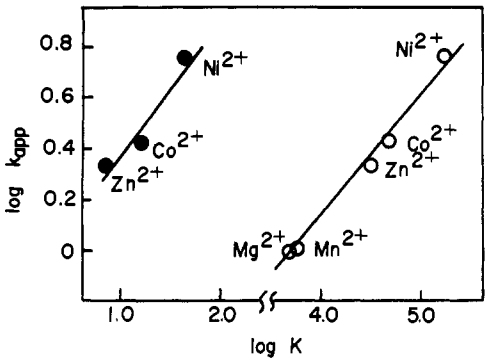
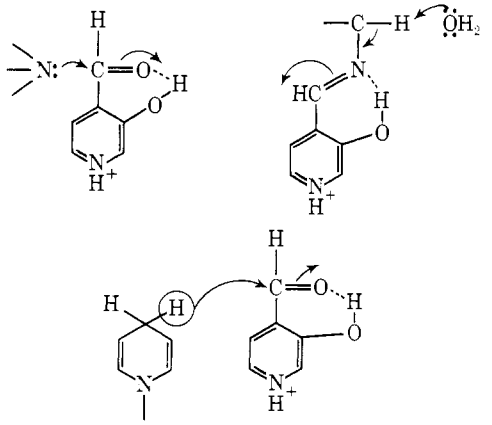


FIGURE 8: Plot of the log of the apparent second-order rate constants (log k_{app}) calculated on the basis of the total concentration of HE and pyridoxal-P (pH ~7.15) *vs.* the log of the stability constants (log K) for metal ions with salicylaldehyde (○) and pyridine (●). (Reactions were carried out in 52.1 wt % methanol–H_2O with [EDTA] = 0.01 M and [metal ion] = 0.015 M, 30°, μ = 0.02 with KCl).

tion of pyridine-4-carboxaldehyde species (all at 30°). For each of these reactions, nucleophilic attack is completely or partially rate determining. The parallel nature of the effect

SCHEME I



of structure modification on the relative rate constants for the three reactions is most marked and therefore finds explanation through common phenomena. (For a summary of deductions concerning structural effects on imine formation and transamination, see Auld and Bruice (1967c).) Unexplained is the greater reactivity of species C compared to D. Thus, in both C and D internal hydrogen bonding by the 3-OH group is absent as is the positive charge on the pyridine nitrogen. The 3-O^- group of C would be anticipated to decrease susceptibility of the 4-CHO group to nucleophilic attack compared to the 4-CHO group of D. Therefore, the reason for the greater reactivity of C over D is not at all apparent. It is possible that the degree of hydration of the aldehyde function of D is less than that of C so that a greater mole per cent of D is present in a reactive form.

The reduction of pyridoxal-P and PyrCHO by HE (52.1 wt % methanol–H_2O) is accelerated by metal ions. In the experiments described in this study, metal ions were employed at 0.015 M and the solution buffered by EDTA at 0.01 M. From a knowledge of the stability constants of EDTA–metal complex (Schwarzenbach and Ackermann, 1947; Schwarzenbach *et al.*, 1954), it follows that a metal ion concentration equivalent to EDTA employed is sequestered and not available as a catalyst. Thus, the rate enhancements brought about (Table V) by the presence of 0.015 M metal ion are due to the 0.005 M unsequestered ions. In Figure 8 there is plotted the log of the apparent second-order rate constant (Table V) for metal ion catalysis *vs.* the log of the stability constants for complexing of the metal ions by pyridine and salicylaldehyde (Brewer and Wong, 1966; Mellor and Maley, 1947; Martell and Calvin, 1952). The linearity of the plots of Figure 8 establishes that the same factors which determine the stability constants for metal ion complexation in H_2O determine the efficiency of metal ion catalysis. The various modes of metal ion complexation with 3-hydroxypyridine-4-carboxaldehyde species are provided in Scheme I.

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Mechanism of Warfarin Resistance. Warfarin and the Metabolism of Vitamin K_1†

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ABSTRACT: If warfarin exerts its anticoagulant effect by causing an accumulation of phylloquinone oxide, an inhibitor of vitamin K, resistance to warfarin could be due to a mutation which renders the conversion of the oxide to vitamin K less sensitive to warfarin. Metabolic studies with ^{3}H-labeled vitamin K_1 and [^{3}H]phylloquinone oxide have provided evidence that resistance is due to the inability of the anticoagulant to inhibit the oxide–K_1 conversion. Homozygous resistant rats had elevated vitamin K requirements and were resistant to warfarin at 0.3 mg/100 g body weight but prothrombin synthesis was blocked by 5 mg/100 g body weight. Phylloquinone oxide stimulated prothrombin synthesis in resistant rats given warfarin but was ineffective in Sprague-Dawley rats treated with anticoagulant. In Sprague-Dawley rats warfarin (0.1 mg/100 g body weight) increases the oxide:K_1 ratio and decreases the concentration of vitamin K_1 in the liver but in resistant animals given ^{3}H-labeled vitamin K_1 the same dose of warfarin did not increase the oxide:K_1 ratio and had little effect on the amount of ^{3}H-labeled vitamin K_1 in the liver. In Sprague-Dawley rats given [^{3}H]phylloquinone oxide, warfarin almost completely blocks the conversion of [^{3}H]-phylloquinone oxide to ^{3}H-labeled vitamin K_1 and increases the oxide:K_1 ratio and the amount of [^{3}H]phylloquinone oxide in the liver. However, in resistant rats injected with [^{3}H]phylloquinone oxide, warfarin had little effect on the oxide:K_1 ratio or on the amount of unmetabolized [^{3}H]-phylloquinone oxide in the liver. If a dose of warfarin (5 mg/100 g body weight) sufficient to block prothrombin synthesis was administered to resistant rats, the conversion of [^{3}H]-phylloquinone oxide to tritiated vitamin K_1 was clearly inhibited and the amount of unmetabolized [^{3}H]phylloquinone oxide in the liver increased. The results are consistent with the idea that resistance is due to a mutation which alters the enzyme system which converts oxide to K_1 so that it is no longer inhibited by warfarin. The metabolic studies also suggest that the altered enzyme was less effective in catalyzing the oxide–K_1 conversion which could account for the high vitamin K requirements. However, the oxide was as effective as K_1 in stimulating prothrombin synthesis in hypoprothrombinemic resistant rats.

The mechanism of warfarin resistance is of great interest because of the spread of warfarin-resistant rats in Europe and the United States (Greaves, 1970; Jackson and Kaukeinen, 1972) and because of the insight it may give into the mechanism of action of the anticoagulant. Coumarin anticoagulants are not only important rodenticides but are widely used in the treatment of thromboembolic disease. Vitamin K_1, whose stimulus of clotting protein synthesis is blocked by warfarin, shuttles between the active vitamin and an inactive metabolite, phylloquinone oxide, in the liver (Bell and Matschiner, 1970, 1972; Matschiner *et al.*, 1970; Bell *et al.*, 1972). Warfarin traps the vitamin as the inactive oxide by inhibiting its conversion back to vitamin K_1. We have proposed that warfarin exerts its effect by increasing the concentration of oxide which acts as an inhibitor of the vitamin (Bell and Matschiner, 1972) and predicted that resistance to warfarin could be due to a mutation which renders the conversion of the oxide to vitamin K_1 insensitive to warfarin. Zimmerman and Matschiner (1972) have reported that the conversion of phylloquinone oxide to vitamin K_1 by liver preparations is in-

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