Semicarbazone Formation from Pyridoxal, Pyridoxal Phosphate, and Their Schiff Bases

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Pyridoxal and pyridoxal phosphate semicarbazone formation is subject to nucleophilic catalysis by primary and secondary amines. The reaction of pyridoxal phosphate with semicarbazide, over the *p*H range 7.3 to 14, proceeds through acid-catalyzed, base-catalyzed, and uncatalyzed reactions. In contrast, the reaction of the Schiff base formed from pyridoxal phosphate and methylamine, which serves as a model for "trans-Schiffization" reactions involved in pyridoxal phosphate–dependent enzymatic reactions, is nearly independent of *p*H over this range. The Schiff base is inherently thirtyfold more reactive than pyridoxal phosphate toward semicarbazide, but is much less reactive than protonated Schiff bases, as judged from studies on morpholine-catalyzed pyridoxal phosphate semicarbazone formation. The latter reaction proceeds via a cationic imine intermediate, $\rangle\!=\!\overset{+}{\mathrm{N}}\!\langle$, a model for protonated Schiff bases. Since the Schiff bases are more reactive than the free aldehyde, the existence of the coenzyme as the Schiff base is advantageous to pyridoxal phosphate–dependent enzymes. The uncatalyzed reaction of aspartate with pyridoxal phosphate at *p*H 8.5 is several orders of magnitude too slow to account for the over-all rate of substrate utilization by glutamic-aspartate transaminase under these conditions. Since the reactivities of pyridoxal phosphate and its Schiff bases toward semicarbazide are not greatly dissimilar, the "trans-Schiffization" between aspartate and enzyme-bound pyridoxal phosphate must be catalyzed by this enzyme.

Pyridoxal–dependent reactions, which mimic pyridoxal phosphate–dependent enzymatic reactions, proceed via a reaction path involving a Schiff base between substrate and pyridoxal (Metzler *et al.*, 1954; Metzler, 1957; Braunstein and Shemyakin, 1952, 1953). Pyridoxal phosphate is bound to the protein moiety of several enzymes in the form of a Schiff base, involving the ϵ-amino group of a lysine residue (Fischer and Krebs, 1959; Fischer *et al.*, 1958; Turano *et al.*, 1961). This and other evidence suggests that a "trans-Schiffization" is involved in the catalytic process of pyridoxal phosphate–dependent enzymes (Snell and Jenkins, 1959).

It has recently been shown that aniline-catalyzed benzaldehyde semicarbazone formation proceeds via the reaction path shown in equations (1) and (2) (Cordes and Jencks, 1962a). The second step, a "trans-Schiffization," is nearly

$$\mathrm{Ph{-}CHO} + \mathrm{Ph{-}NH_2} \underset{}{\overset{\mathrm{H^+}}{\rightleftharpoons}} \mathrm{Ph{-}\underset{\mathrm{H}}{\overset{|}{C}}{=}N{-}Ph} + \mathrm{H_2O} \quad \text{slow} \quad (1)$$

$$\mathrm{Ph{-}\underset{\mathrm{H}}{\overset{|}{C}}{=}N{-}Ph} + \mathrm{NH_2NHCONH_2} \longrightarrow \mathrm{Ph{-}\underset{\mathrm{H}}{\overset{|}{C}}{=}NNHCONH_2} + \mathrm{NH_2{-}Ph} \quad \text{fast} \quad (2)$$

instantaneous in dilute, slightly acidic aqueous solution. Consequently, semicarbazone formation via "trans-Schiffization" occurs several orders of magnitude faster than from the free aldehyde under these conditions. This finding raises the possibility that the existence of enzyme-bound pyridoxal phosphate as the Schiff base may contribute to the over-all efficiency of the enzymatic process.

The present communication is concerned with the reactions of semicarbazide with pyridoxal, pyridoxal phosphate, and their Schiff bases. The reaction of semicarbazide with pyridoxal phosphate Schiff bases serves as a model for enzymatic "trans-Schiffization." Semicarbazide was chosen for this study because the absorption maximum of pyridoxal and pyridoxal phosphate semicarbazones (370 mμ) is different from that of the pyridoxal Schiff bases (near 415 mμ) and pyridoxal itself (320, 390 mμ), so that the reactions may readily be followed spectrophotometrically.

EXPERIMENTAL

Materials.—Pyridoxal and pyridoxal phosphate were obtained from commercial sources and were used without further purification. All other reagents, except reagent grade inorganic salts, were recrystallized or redistilled before use.

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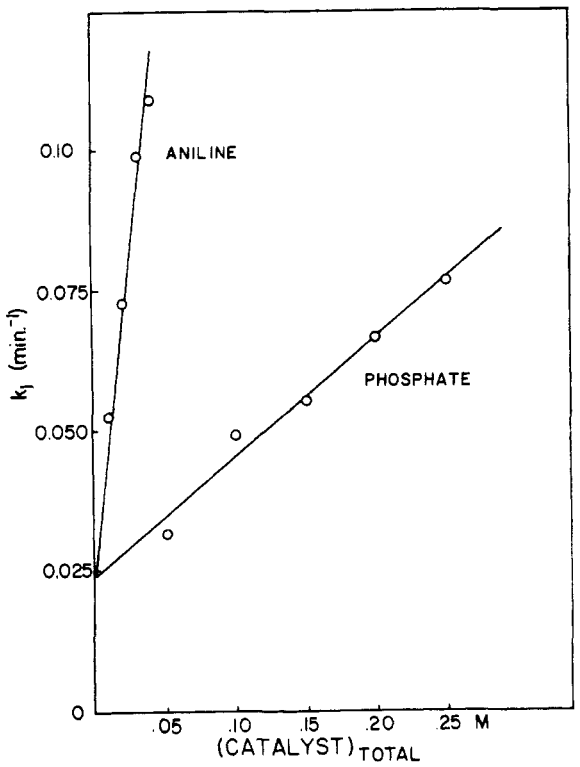


FIG. 1.—First-order rate constants for pyridoxal semicarbazone formation as a function of the total concentration of aniline and phosphate at 25° and *p*H 6.8 in the presence of 0.05 M semicarbazide; ionic strength 0.50; 370 mμ.

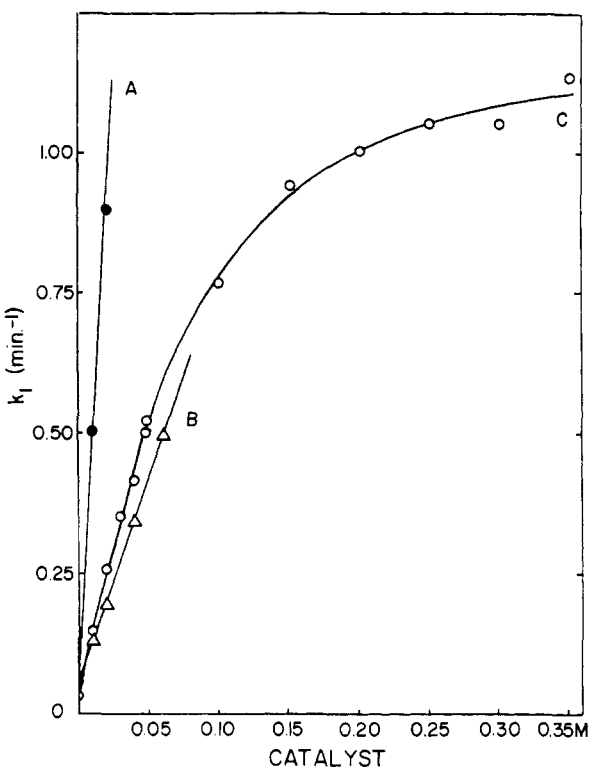


FIG. 2.—First-order rate constants for the formation of pyridoxal phosphate semicarbazone as a function of the concentration of morpholine (curve A) and proline (curve B) and for the formation of pyridoxal semicarbazone as a function of the concentration of morpholine (curve C) at 25° and *p*H 8.6. Semicarbazide concentration 0.005 M in experiments involving pyridoxal phosphate and 0.05 M in experiments involving pyridoxal; ionic strength 0.50; 370 mμ.

Pyridoxylideneethylamine (m.p. 112–114°) was prepared by a known method (Heyl *et al.*, 1952). Other Schiff bases were prepared by mixing the appropriate reagents in aqueous solution and were not isolated. Aqueous solutions of pyridoxal and pyridoxal phosphate were stored at 2° and were stable for a few days at this temperature. Solutions of nitrogen bases were prepared just prior to use. Glass-distilled water was used throughout.

Kinetic measurements were carried out spectrophotometrically at 25° as previously described (Jencks, 1959). The ionic strength of all reaction mixtures was maintained at 0.50 by the addition of KCl. The *p*H values were measured with a glass electrode and the Radiometer PHM-4b *p*H meter.

RESULTS

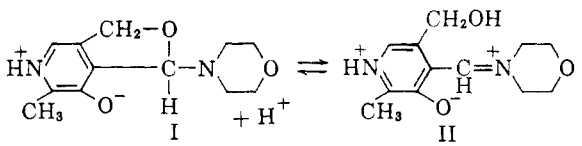
In Figure 1, the first-order rate constants for pyridoxal semicarbazone formation are shown as a function of phosphate and aniline concentration at *p*H 6.84 and 25° in the presence of 0.05 M semicarbazide. The first-order rate constants increase linearly with the concentration of both catalysts. On the basis of the total concentration of all ionic species present in solution, aniline is approximately tenfold more effective than phosphate, which presumably functions as a general acid catalyst for pyridoxal semicarbazone formation (Conant and Bartlett, 1932; Cordes and Jencks, in preparation). On the basis of the concentration of acidic species present in solution, aniline is several orders of magnitude more effective than phosphate as a catalyst, suggesting that aniline functions as a nucleophilic, rather than as a general acid, catalyst (equations 1 and 2) in pyridoxal semicarbazone formation.

In Figure 2, the first-order rate constants for pyridoxal semicarbazone formation at *p*H 8.6 are shown as a function of the concentration of the secondary amine, morpholine. At low morpholine concentrations, the first-order rate constants increase linearly with catalyst concentration, then level off, and eventually become almost independent of catalyst concentration. This result suggests that, at high morpholine concentrations nearly all of the pyridoxal is rapidly converted to a morpholine addition product (I). The rate of morpholine-catalyzed pyridoxal semicarbazone formation was found to be accurately first-order in respect to semicarbazide concentration over the concentration range 0.025 to 0.10 M, indicating

TABLE I
PSEUDO-FIRST-ORDER RATE CONSTANTS FOR SEMICARBAZONE FORMATION FROM PYRIDOXAL, PYRIDOXAL PHOSPHATE, AND THEIR IMINES IN WATER AT 25°[a]

Substrate X	Substrate Y	Amine (M)	pH	Semicarbazide (M)	k_{obs} (min.$^{-1}$)
H	O		6.84	0.070	0.073[b]
H	Valine	0.51	6.84	0.067	3.70[b]
H	Ethylamine		6.86	0.070	2.87[b]
Phosphate	O		7.34	0.033	1.66[b]
Phosphate	Lysine	0.20	7.34	0.033	2.10[b]
Phosphate	O		8.61	0.005	0.053[c]
Phosphate	Glycine	0.05	8.70	0.005	0.415[c]
Phosphate	Lysine	0.04	8.70	0.005	0.166[c]
Phosphate	Ornithine	0.06	8.72	0.005	0.244[c]
Phosphate	Methylamine	0.05	8.71	0.005	0.231[c]
Phosphate	O		10.05	0.0067	0.020[d]
Phosphate	O		10.75	0.0067	0.014[e]
Phosphate	Methylamine	0.20	10.75	0.0067	0.432[e]

[a] Measured spectrophotometrically at 370 mμ. Ionic strength maintained at 0.50 with KCl. [b] In 0.10 M phosphate buffer. [c] In 0.05 M triethylenediamine (DABCO) buffer. [d] In 0.05 M carbonate buffer. [e] In 0.10 M carbonate buffer.



I II

that the rate-determining step in morpholine-catalyzed pyridoxal semicarbazone formation occurs after the formation of the pyridoxal-morpholine addition compound. A reciprocal plot of k_1 against morpholine concentration gives an estimated equilibrium constant for addition compound formation at pH 8.6, K_{eq} = [addition compound]/[pyridoxal] [morpholine], of 24 M^{-1}. This value is in fair agreement with a value of K_{eq} of 30 M^{-1} determined spectrophotometrically at 390 mμ and pH 8.6. The addition of increasing concentrations of morpholine to pyridoxal at pH 8.6 results in a progressive decrease in absorption at 390 mμ and a progressive increase in absorption at 310 mμ. The absorption at 390 mμ is due to the free aldehyde form of pyridoxal, whereas that at 310 mμ is similar to that of the internal hemiacetal form of pyridoxal (Metzler and Snell, 1955; Nakamoto and Martell, 1959). The addition of 0.1 M morpholine, pH 8.5, which causes a 75% decrease in the absorption of pyridoxal at 390 mμ, causes less than a 2% change in the absorption of pyridoxal phosphate at 390 mμ.

First-order rate constants for pyridoxal phosphate semicarbazone formation at pH 8.6 are shown in Figure 2 as a function of the concentration of morpholine and proline. In both cases, in contrast to the results obtained with pyridoxal, the rate constants increase linearly with catalyst concentration. Morpholine is a considerably more effective catalyst for the reaction with pyridoxal phosphate than for that with pyridoxal. On the basis of the total concentration of all species, morpholine is approximately fivefold more effective than proline as a catalyst for pyridoxal phosphate semicarbazone formation.

Pyridoxal phosphate semicarbazone formation is also subject to nucleophilic catalysis by the primary amines lysine, ornithine, glycine, and methylamine under slightly alkaline conditions. In contrast to the catalysis of pyridoxal semicarbazone formation by primary and secondary amines and to the catalysis of pyridoxal phosphate semicarbazone formation by secondary amines, the first-order rate constants for pyridoxal phosphate semicarbazone formation are essentially independent of catalyst concentration above 0.02 M. Therefore, under these conditions, pyridoxal phosphate is converted nearly quantitatively to the Schiff base in a rapid pre-equilibrium step. In a second, slow step, semicarbazide reacts with the Schiff base to yield the semicarbazone. The first-order rate constants for semicarbazone formation from pyridoxal, pyridoxal phosphate, and several of their Schiff bases were measured at amine concentrations sufficient to convert at least 90% of the aldehydes to the Schiff bases (Table I). At pH 6.84, pyridoxal semicarbazone formation proceeds forty to fifty times more rapidly from pyridoxal Schiff bases than from pyridoxal itself. On the other hand, pyridoxal phosphate semicarbazone formation proceeds only one to eight times faster from the Schiff bases than from the free aldehyde at pH 7.34 and 8.7. At pH 10.75, the pyridoxal phosphate–methylamine Schiff base is 30 times more reactive toward

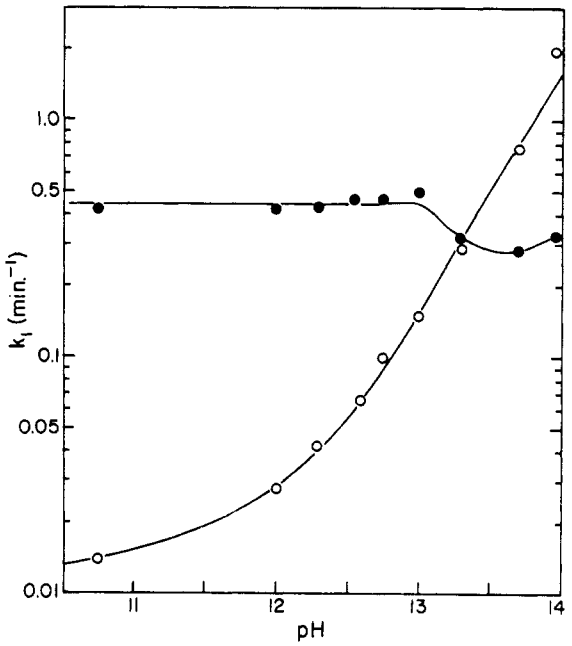


FIG. 3.—First-order rate constants for pyridoxal phosphate semicarbazone formation from pyridoxal phosphate, o, and the Schiff base of pyridoxal phosphate with methylamine, ●, as a function of *p*H at 25°. Semicarbazide concentration 0.0067 M; ionic strength 0.50; 370 mμ.

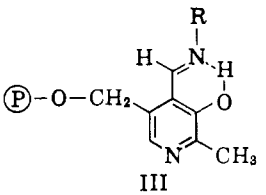
semicarbazide than pyridoxal phosphate.

The first-order rate constants for semicarbazone formation from pyridoxal phosphate and its methylamine Schiff base are plotted against *p*H in the range *p*H 10.7 to 14 in Figure 3. The rate of semicarbazone formation from the Schiff base is almost independent of *p*H from 8.7 to 14 (Table I, Fig. 3). The pyridoxal phosphate–methylamine Schiff base undergoes a change in ionic species, from a yellow to a colorless form, above *p*H 13. On the other hand, the rate of the reaction of pyridoxal phosphate with semicarbazide increases linearly with hydroxide ion concentration above *p*H 12.5. This reaction also proceeds with acid catalysis, as indicated by the increased rates at *p*H 8.7 and 7.3, and through an uncatalyzed reaction, since the rate constants at intermediate *p*H values are greater than those calculated from the rates of the acid and base catalyzed reactions.

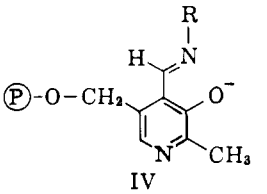
A pseudo first-order rate constant for Schiff base formation from pyridoxal phosphate and excess aspartate (0.05 M) in 0.05 M triethylenediamine buffer, *p*H 8.50, of 1.50 min.$^{-1}$ was observed, from which a second-order rate constant, based on the total concentration of aspartate, of 30 M^{-1} min.$^{-1}$ was calculated.[1]

Discussion

The conversion of pyridoxal phosphate Schiff bases from the hydrogen-bonded (III) to the anionic species (IV) is accompanied by a shift in



III



IV

absorption maximum from approximately 415 mμ to near 330 mμ (Metzler, 1957; Christensen, 1958). Similar behavior of the absorption spectrum has been observed for several pyridoxal phosphate–containing enzymes, including glutamic-aspartic transaminase (Jenkins and Sizer, 1957, 1959; Lis *et al.*, 1960; Jenkins *et al.*, 1959), glutamic acid decarboxylase (Shukuya and Schwert, 1960), cystathionase (Matsuo and Greenberg, 1958), and phosphorylase b (Kent *et al.*, 1958), suggesting that enzyme-bound pyridoxal phosphate is present in the form of a Schiff base.[2] Sodium borohydride reduction of phosphorylase b, glutamic-aspartic transaminase, and cystathionase (Fischer and Krebs, 1959; Turano *et al.*, 1961; Fischer *et al.*, 1958), followed by chemical degradation, has resulted in the isolation of pyridoxal phosphate bound in stable covalent linkage to the ε-amino group of a lysine residue. Pyridoxal phosphate–dependent reactions involve formation of a Schiff base between substrate and pyridoxal phosphate (Metzler *et al.*, 1954; Metzler, 1957). Hence, the first covalent bond-forming reaction with pyridoxal phosphate–dependent enzymes may be visualized as a "trans-Schiffization." The relative reactivity of pyridoxal phosphate and its Schiff bases toward nucleophilic reagents is, therefore, of interest from the standpoint of the enzymatic reactions.

Pyridoxal phosphate derivatives may be divided into four groups, based on reactivity toward nucleophilic reagents: (1) the free aldehyde; (2) the free Schiff base; (3) the Schiff base with a proton hydrogen bonded to the imino nitrogen atom; (4) the protonated Schiff base, $\rangle C{=}\overset{+}{N}\langle$.

Spectral evidence indicates that, under neutral and mildly alkaline conditions, pyridoxal phos-

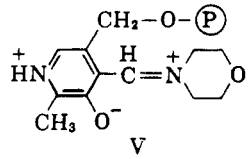
[1] In the presence of 0.10 M borate buffer, the second-order rate constant for the reaction of excess aspartate with pyridoxal phosphate at *p*H 8.5 is approximately 5 M^{-1} min.$^{-1}$. This low value is a consequence of the partial conversion of pyridoxal phosphate to a borate complex. The addition of borate to pyridoxal phosphate at *p*H 8.5 causes a decrease in absorption at 390 mμ and an increase in absorption at 292 mμ. The equilibrium constant for formation of the pyridoxal phosphate–borate addition compound at *p*H 8.5, based on the total concentration of all ionic species present in solution, was measured spectrophotometrically at 390 mμ in 0.0045 to 0.027 M borate and a value of 126 M^{-1} was obtained.

[2] Illingworth *et al.* (1958) have suggested, on the basis of the failure of isonicotinic acid hydrazide to inhibit phosphorylase b, that the Schiff base between pyridoxal phosphate and this enzyme exists only as a transient intermediate.

phate Schiff bases possess a strong hydrogen bond between the phenolic hydroxyl group and the imino nitrogen atom (III) (Metzler, 1957). Under sufficiently alkaline conditions, at *p*H 10–13, the phenolic proton is lost and the pyridoxal phosphate Schiff bases are converted from the hydrogen bonded to the free form (IV). Although little direct information pertinent to the relative reactivities of the hydrogen bonded and free Schiff bases toward nucleophilic reagents is available, it is probable, by analogy with other reactions of imines, that the hydrogen bonded form, with a partially protonated imino nitrogen atom, is considerably more reactive than the free Schiff base.

As indicated by the data in Table I and Figure 3, the reaction of pyridoxal phosphate with semicarbazide exhibits acid, base, and uncatalyzed reactions typical of systems of this type (Anderson and Jencks, 1960; Cordes and Jencks, 1962b). The reaction of pyridoxal phosphate with isonicotinic acid hydrazide also exhibits acid-catalyzed and uncatalyzed reactions (Bonavita and Scardi, 1959). On the other hand, the reaction of the pyridoxal phosphate–methylamine Schiff base with semicarbazide is nearly *p*H independent from *p*H 8.7 to 14, and, therefore, proceeds only through an uncatalyzed reaction. The reactions of these two substrates with semicarbazide differ then in two important respects. First, the inherent over-all reactivity of the hydrogen bonded Schiff base toward semicarbazide is about thirty-fold greater than that of the free aldehyde, as judged by the relative magnitude of the uncatalyzed reactions. Second, the free aldehyde has available to it both acid and base catalyzed reaction pathways not available to the Schiff base. Thus, while "internal general acid catalysis," due to partial protonation of the imino nitrogen atom in the hydrogen bonded Schiff base, increases substrate reactivity, it prevents formation of a still more reactive species, the protonated Schiff base.

Morpholine-catalyzed pyridoxal phosphate semicarbazone formation must proceed via condensation of morpholine with the aldehyde to give the cationic amine, V, a model for protonated Schiff bases, followed by attack of semicarbazide on V to give the semicarbazone. Since only trace amounts of V are presumably present at equilibrium, semicarbazone formation from the cationic imine must be extremely rapid, several orders of magnitude faster than from the free aldehyde or the free or hydrogen bonded Schiff bases.



V

In summary, the finding that the reaction of pyridoxal phosphate with semicarbazide is subject to nucleophilic catalysis by primary amines indicates that, even though the catalysis is only moderate, it is an advantage to pyridoxal phosphate–dependent enzymes to have the coenzyme present as the Schiff base. If the enzyme can protonate the Schiff base to give a compound with the $\rangle C{=}\overset{+}{N}\langle$ structure, similar to that of the intermediate in the morpholine-catalyzed reaction, a considerably greater rate enhancement will result.

For glutamic-aspartic transaminase, a minimum second-order rate constant for the reaction of aspartate with enzyme-bound pyridoxal phosphate may be calculated. From the maximum velocity (380 μmoles of substrate/min./mg of enzyme, *p*H 8.5) (Jenkins and Sizer, 1957), the Michaelis constant for aspartate (0.0039 M) (Jenkins *et al.*, 1959), and the molecular weight of the enzyme (110,000) (Jenkins *et al.*, 1959), an estimated minimum second-order rate constant at *p*H 8.5, based on the total concentration of aspartate of 7.7×10^6 M^{-1} $min.^{-1}$ is obtained. This is several orders of magnitude greater than the maximum second-order rate constant, also based on the total concentration of aspartate at *p*H 8.5, of approximately 30 M^{-1} $min.^{-1}$ for the reaction of aspartate with pyridoxal phosphate. Only a slightly higher value would be expected for the reaction of aspartate with a pyridoxal phosphate Schiff base under these conditions. Therefore, the reaction of free aspartate and pyridoxal phosphate Schiff bases is too slow to account for the over-all rate of the enzymatic reaction, and, consequently, the "trans-Schiffization" between aspartate and enzyme-bound pyridoxal phosphate must be catalyzed by glutamic-aspartic transaminase, possibly by conversion of the pyridoxal phosphate–enzyme Schiff base to the much more reactive, protonated species. However, glutamic-aspartic transaminase reacts with alanine (0.1 M) at *p*H 8.0 with a first-order rate constant of 0.20 $min.^{-1}$ (Jenkins, 1961), a value similar to that for the non-enzymatic reaction of pyridoxal phosphate with aspartate.

Although these calculations suggest that glutamic-aspartic transaminase must catalyze the "trans-Schiffization" step as well as the tautomerization step, it is likely that the tautomerization step, which involves the removal of a carbon-bound proton, is the rate-determining step of the enzymatic reaction. Banks *et al.* (1961) have recently shown that pyridoxal-dependent nonenzymatic transamination involves rapid preequilibrium Schiff base formation, followed by a rate-determining tautomeric rearrangement.

Enzymatic reactions require pyridoxal phosphate, rather than pyridoxal, as coenzyme. The requirement for a phosphate group has generally been ascribed to a binding phenomenon. However, the greater reactivity of pyridoxal phosphate than of pyridoxal toward nucleophilic reagents, because of the existence of the latter compound largely in the form of the unreactive internal hemiacetal, provides a further chemical advan-

tage for pyridoxal phosphate as a coenzyme. Equilibrium constants for Schiff base formation are one to two orders of magnitude greater with pyridoxal phosphate than with pyridoxal (Metzler, 1957; Christensen, 1958; Matsuo, 1957) and pyridoxal phosphate is one to two orders of magnitude more reactive than pyridoxal toward semicarbazide (Table I) and toward a variety of carbohydrazides (Wiegand, 1956). In addition, catalysis of pyridoxal reactions by morpholine, which must involve the intermediate $\rangle C{=}\overset{+}{N}\langle$, is highly effective in the case of pyridoxal phosphate but is less effective and levels off in concentrated morpholine solutions in the case of pyridoxal. This levelling off, and the spectral changes observed upon the addition of morpholine to pyridoxal, suggest that an unreactive cyclic aminoacetal (I) is formed from pyridoxal and morpholine. A similar structure has been suggested for the addition product of pyridoxal with sarcosine (Metzler, 1957).[3]

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[3] Christensen (1958) has suggested that the pyridoxal-sarcosine addition product formed at *p*H 11.3 is a non-hydrogen-bonded Schiff base, rather than the internal hemiacetal, since the addition compound is not formed in detectable amounts at *p*H 7.5. This objection does not apply to the pyridoxal-morpholine addition compound since pyridoxal can be converted almost completely to the addition compound at *p*H 8.6 within a few seconds. Formation of the compounds to which Christensen assigned non-hydrogen-bonded structures requires several hours.