Relation of Structure to Activity of Pyridoxal Analogs as Substrates for Pyridoxamine Pyruvate Transaminase*

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ABSTRACT: To define the role of individual functional groups of pyridoxal in enzymatic transamination, the substrate specificity of pyridoxamine pyruvate transaminase, which catalyzes reaction 1, has been examined by kinetic methods. Neither the 2-methyl group nor the 5-hydroxymethyl group of pyridoxal plays a role in the transamination reaction *per se*, since 2-norpyridoxal, ω-methylpyridoxal, 5-deoxypyridoxal, and 3-hydroxypyridine-4-aldehyde replace pyridoxal in reaction 1b and undergo transamination at a similar rate. The corresponding amines show similar activity in reaction 1a. The substituent at position 2 of the analogs strongly affects binding to the enzyme. The dissociation constants (and K_m values) for norpyridoxal, ω-methylpyridoxal, and 3-hydroxypyridine-4-aldehyde are substantially higher than those for pyridoxal or 5-deoxypyridoxal. Removal of the 5 substituent affects binding to a lesser extent. The affinity of norpyridoxal and 3-hydroxypyridine-4-aldehyde for the transaminase are of similar magnitude. Phosphorylation at the 5′ position to yield pyridoxal 5′-phosphate or pyridoxamine 5′-phosphate greatly decreases the affinity for the enzyme. Within the limits of variation studied here, alterations in the 2 substituent of the pyridine substrate did not greatly affect binding of the second substrate, alanine or pyruvate; such binding was greatly reduced by phosphorylation or removal of the 5-hydroxymethyl group. but not by its conversion into a methyl group. *O*-Methylpyridoxal, 3-deoxypyridoxal, and pyridine-4-aldehyde were neither substrates nor competitive inhibitors of pyridoxamine pyruvate transaminase. A phenolic group in the 3 position is thus required for efficient binding. The Schiff's base formed nonenzymatically between alanine and pyridine-4-aldehyde inhibits reaction 1 but does not undergo transamination, indicating that a 3-phenolic group also is essential for the enzymatic transamination reaction. *N*-Methylpyridoxal and 4-nitrosalicylaldehyde, which simulate the reactions of pyridoxal in nonenzymatic systems, are neither substrates nor competitive inhibitors of pyridoxamine pyruvate transaminase. The minimum structural requirements for enzymatic transamination in this system are met by 3-hydroxypyridine-4-aldehyde and are exactly the same as those required for nonenzymatic transamination in aqueous solutions. These findings indicate that the contributory role of individual functional groups of pyridoxal to transamination is similar in both enzymatic and nonenzymatic reactions. Observed differences in compounds that undergo transamination in the nonenzymatic and enzymatic systems appear to result only from structural requirements imposed by the enzyme for formation of the enzyme–substrate complex. Comparative values for the dissociation constant and the rate of formation of the ω-methylpyridoxal–enzyme complex obtained from kinetic data or by direct spectrophotometric measurement show that the species which absorbs at 430 mμ (presumably a hydrogen-bonded Schiff's base of the pyridoxal analog with enzyme) is not an intermediate in enzymatic transamination. By extension, it is assumed that the corresponding complexes formed by interaction of pyridoxal and other analog substrates with enzyme (λ_{max} 415–430 mμ) are not true intermediates but are in rapid equilibrium with the true intermediates.

pyridoxamine + pyruvate $\underset{b}{\overset{a}{\rightleftharpoons}}$ pyridoxal + L-alanine (1)

Reaction 1, catalyzed by pyridoxamine pyruvate transaminase, also proceeds nonenzymatically at a very much slower rate (Snell, 1945) which can be accelerated

pyridoxamine + pyruvate $\underset{b}{\overset{a}{\rightleftharpoons}}$ pyridoxal + alanine (1)

severalfold by addition of appropriate metal ions. Investigations of the structural specificity of aldehydes other than pyridoxal for participation in similar ion-catalyzed, nonenzymatic reactions showed that the structural features of pyridoxal essential for the reaction are a formyl group adjacent to a phenolic group and conjugated to the electron-withdrawing heterocyclic nitrogen of the pyridine ring (Metzler *et al.* 1954). Based on these model studies, a mechanism for transamination and other pyridoxal-catalyzed reactions that

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assigned no catalytic function to substituents at the 2, 5, or 6 position of pyridoxal was suggested (reviews, Snell, 1958; Braunstein, 1960; Guirard and Snell, 1964).

Now that an enzyme is available that catalyzes reaction 1, the structural features of pyridoxal required for enzymatic transamination can be determined and compared directly to those for nonenzymatic transamination. Kinetic studies reported herein show that the structural requirements for enzymatic transamination are the same as those for the nonenzymatic reaction; substituents at the 2 and 5 positions of the pyridine ring affect the binding of substrates but not the catalytic act *per se*. These studies also permit exclusion of certain spectrophotometrically observable enzyme–substrate complexes as intermediates in the reaction. Such results should also apply to transaminases that require pyridoxal phosphate as coenzyme since the mechanism of reaction 1 resembles closely that of a half-reaction catalyzed by such enzymes (Ayling and Snell, 1968).

Experimental Procedures

Substrate Analogs. The synthesis of 2-norpyridoxal and ω-methylpyridoxal (Mühlradt *et al.*, 1967), ω-methylpyridoxamine (Ikawa and Snell, 1954a), and 5-deoxypyridoxal (Mühlradt and Snell, 1967) has been described elsewhere. 2-Norpyridoxamine and also a separate sample of 2-norpyridoxal were gifts from Dr. M. Karpeisky (Florentiev *et al.*, 1967). 5-Deoxypyridoxamine (Heyl *et al.*, 1953), 3-hydroxypyridine-4-aldehyde (Heinert and Martell, 1959), and *p*-nitrosalicylaldehyde (Bavin *et al.*, 1950) were gifts from Drs. K. Folkers, A. Martell, and M. Ikawa, respectively. 3-*O*-Methylpyridoxal and *N*-methylpyridoxal were gifts from Drs. A. Pocker and E. H. Fischer. 3-Hydroxy-4-aminomethylpyridine and 3-deoxypyridoxal have not been described previously and were synthesized as described below. Other substrate analogs tested were from commercial sources. Many commercial samples of pyridoxamine phosphate and pyridoxal phosphate contain small amounts of free pyridoxamine or pyridoxal and give misleading results in kinetic studies if used directly. Chromatographically purified samples (Peterson *et al.*, 1953) were free from such impurities. A sample of pyridine-4-aldehyde (K & K Laboratories) contained small amounts of an unidentified impurity which underwent transamination; pyridine-4-aldehyde hydrate from the same source was free of this substance.

3-Hydroxy-4-aminomethylpyridine. This compound was prepared by catalytic reduction of the oxime of 3-hydroxypyridine-4-aldehyde (*cf.* Ikawa and Snell, 1954a), and also by enzymatic transamination of 3-hydroxypyridine-4-aldehyde with alanine. In the latter preparation, 1 mmole of the aldehyde, 10 mmoles of L-alanine, and 1 mg of crystalline pyridoxamine pyruvate transaminase in a volume of 10 ml were incubated with shaking at pH 8.5 and 25°. After 2 hr the reaction had reached equilibrium and about 90% of the 3-hydroxypyridine-4-aldehyde was converted into 3-hydroxy-4-aminomethylpyridine. The product of both synthetic procedures was separated from the reaction mixture by passing over a column of Dowex 50-X8 (200–400 mesh) in the H^+ form. Undesired products and excess reactants were eluted with 1 N HCl. 3-Hydroxy-4-aminomethylpyridine, detected by its spectrum (λ_{max} 287 mμ) and reaction with ninhydrin, was eluted with 3 N HCl, lyophilized, dissolved in a minimum of hot 95% ethanol, and precipitated by addition of ether. The products obtained by the two procedures gave identical kinetic constants when used in place of pyridoxamine as substrates for pyridoxamine pyruvate transaminase in reaction 1a, and had identical melting points (179°) and other physical properties. *Anal.* Calcd for $C_6H_8N_2O \cdot 2HCl$: C, 36.6; H, 5.1; N, 14.2. Found: C, 36.9; H, 5.3; N, 14.1. Other results were λ_{max} in 1 N HCl: 287 mμ (ε 6750) and 223 mμ (shoulder, ε 3700); λ_{max} in 1 N NaOH: 299 mμ (ε 5600) and 240 mμ (ε 9450). The spectrum of 3-hydroxy-4-aminomethylpyridine is compared with that of 2-norpyridoxamine and pyridoxamine in Figure 1.

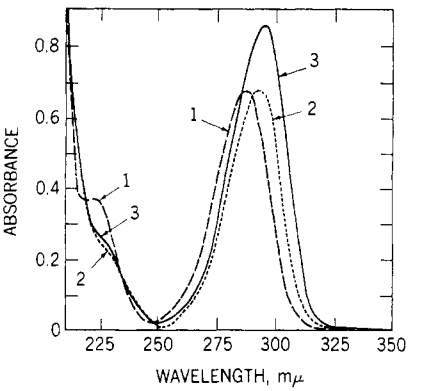


FIGURE 1: Absorption spectrum of 3-hydroxy-4-aminomethylpyridine (curve 1), 2-norpyridoxamine (curve 2), and pyridoxamine (curve 3), each at a concentration of 0.1 mM in 1 N HCl.

3-Deoxypyridoxal and 3-Deoxyisopyridoxal. These analogs were synthesized by partial oxidation of 2-methyl-4,5-bis(hydroxymethyl)pyridine, a gift from Dr. R. G. Jones (Kornfeld and Jones, 1951). The starting material (2 mmoles) in 10 ml of water was stirred at room temperature with 2 mmoles of MnO_2, prepared by heating $MnCO_3$ at 300° for 12 hr. Sulfuric acid (2 mmoles) was added over a period of 1 hr, maintaining the pH at 4.0. After stirring an additional 2 hr at room temperature, about 30% oxidation to aldehyde had occurred, and the mixture was filtered. The reaction was stopped before completion to prevent further oxidation of the desired monoaldehydes. Aldehydes were monitored by thin-layer chromatography with ethyl acetate as solvent and 2,4-dinitrophenylhydrazine as the developing spray. Two major products, designated A (R_F 0.12) and B (R_F 0.34), were separated from the main reaction mixture by countercurrent distribution between chloroform and water. Product A remained in the water phase, but moved more slowly than the starting material, which moved with the water front; product B was very quickly extracted by chloroform, and remained in the first few tubes. The ratio of the two products under the conditions of synthesis is A:B 6:1. On paper electrophoresis at pH 5.3 (pyridine–acetic acid–acetone–

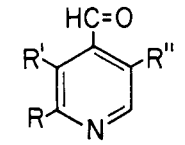
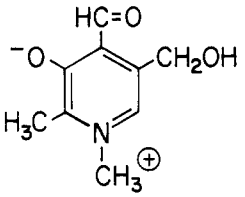
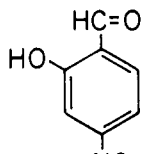
Structure	Name	Substituents R	R'	R''	Active as Substrate
 I – IX	I. Pyridoxal	CH_3	OH	CH_2OH	Yes
	II. ω-Methylpyridoxal	CH_3CH_2	OH	CH_2OH	Yes
	III. Norpyridoxal	H	OH	CH_2OH	Yes
	IV. 5-Deoxypyridoxal	CH_3	OH	CH_3	Yes
 X	V. 3-Hydroxy-4-pyridine aldehyde	H	OH	H	Yes
	VI. Pyridoxal phosphate	CH_3	OH	$CH_2OPO_3^{=}$	(Yes)
	VII. 3-Deoxypyridoxal	CH_3	H	CH_2OH	No
	VIII. Pyridine-4-aldehyde	H	H	H	No
 XI	IX. O-Methylpyridoxal	CH_3	OCH_3	CH_2OH	No
	X. N-Methylpyridoxal	(see column I)			No
	XI. 4-Nitrosalicylaldehyde	(see column I)			No

FIGURE 2: Structure of pyridoxal analogs and their activities as substrates of pyridoxamine pyruvate transaminase. Compounds I–V are excellent, VI is a very poor substrate, and VII–XI are inactive as substrates.

H_2O, 4:2:15:80, 25 V/cm and 50 mA), A migrates 4 cm/hr and B 5 cm/hr, both toward the negative pole, indicating that neither compound contains a carboxyl function. In the phenylhydrazine assay for pyridoxal (Wada and Snell, 1961), the reaction of phenylhydrazine with B is complete within 1 hr at room temperature, whereas that with A is only about 15% complete in this time. The color yields eventually reached equivalent values, however, indicating that the products are the two monoaldehydes corresponding to the starting material. From the relative electrophoretic mobilities of the two isomers A is assumed to be 3-deoxyisopyridoxal (2-methyl-4-hydroxymethyl-5-formylpyridine) and B to be 3-deoxypyridoxal. This assignment is derived from a comparison with pyridine-3-aldehyde (pK = 3.8) and pyridine-4-aldehyde (pK = 4.77; Nakamoto and Martell, 1959), where the latter compound would carry the greater charge at pH 5.3 and migrate at the faster rate. The assignment of structure is tentative only, but is not crucial to this investigation since neither A nor B is a substrate or inhibitor of pyridoxamine pyruvate transaminase. The absorption spectrum of A in 1 N HCl (λ_{max} 268 mμ (ϵ 6300)) was similar to that of the starting material; that of B in 1 N HCl showed λ_{max} 272 mμ (ϵ 6250) with a shoulder at 295 mμ (ϵ 2800).

Kinetic Measurements. Initial rates of reaction 2, where A represents pyridoxamine or its analogs and C represents pyridoxal or its analogs, were made in both the forward (f) and reverse (r) reactions. Procedures were identical with those described for reaction 1 (Ayling and Snell, 1968), except that since all pyridoxal analogs differed from pyridoxal in having either higher extinction coefficients, higher Michaelis constants, or both, initial velocity measurements could be made in cuvets of 1-cm light path. The rate of formation or utilization of pyridoxal analog was determined from the absorbance change at 390 mμ, except for 3-hydroxypyridine-4-aldehyde. In reaction 2f the rate of formation of the latter compound was measured at 385 mμ, the absorbance maximum, but in the reverse reaction, 2r, measurements were made at 440 mμ, so that concentrations greater than K_m could be used. The rate of nonenzymatic transamination with the highest concentrations of any unphosphorylated substrate was less than 1% of that observed in the enzymatic reaction. The amount of enzyme used to catalyze the reactions of analog substrates varied, depending on the extinction coefficient of the analog and its affinity for the enzyme, from 0.5 μg for 5-deoxypyridoxal to 10 μg for ω-methylpyridoxal. The specific activity of the enzyme under standard assay conditions (Ayling and Snell, 1968) was 25 μmoles of pyridoxal formed/mg of protein per min. Measurements were made in a Gilford multichannel recording spectropho-

$$\text{A} + \text{pyruvate} \underset{\text{r}}{\overset{\text{f}}{\rightleftharpoons}} \text{C} + \text{L-alanine} \qquad (2)$$

tometer with the cuvet chamber thermostated at 25°. All reactions were at pH 8.85 in 0.05 M sodium pyrophosphate buffer.

Results

Substrate Specificity of Pyridoxamine Pyruvate Transaminase. The structures and activities as substrates of the various pyridoxal analogs tested are summarized in Figure 2. Compounds I–VI were active as substrates in reaction 2r, as were the corresponding amines in reaction 2f; compounds VII–XI were inactive both as substrates and as inhibitors under the conditions summarized in Table I. Compounds I–VI, active as substrates, and also compound VIII were studied further to assess in greater detail the effect of structure on the capacity to undergo transamination.

Kinetic Parameters for Substrates of Pyridoxamine Pyruvate Transaminase. From initial velocity measurements of reaction 2, double-reciprocal plots similar to those shown in Figure 3A,B for 5-deoxypyridoxal were obtained for compounds I–V, Figure 2, and also for the corresponding amines. In each of these plots, all of the lines intersected at a single point in the second quadrant (*cf.* Ayling and Snell, 1968). In a plot of $1/v$ *vs.* 1/[C] (*e.g.*, Figure 3A,B) this point projected to the abscissa is numerically equal to the constant $-K_{Ala}/K_{Ala,C}$ or, in a plot of $1/v$ *vs.* 1/[A], to $-K_{pyr}/K_{pyr,A}$. The ordinate is intersected at $1/V'$, where V' is the apparent maximal velocity of the reaction. Secondary reciprocal plots of V' against concentration of the second substrate (*e.g.*, Figure 3C,D) showed a linear relationship in each case, from which values for the true maximum velocity, V, and the K_M value for each substrate were obtained.[1] These results are similar in all respects to those previously found for pyridoxal and pyridoxamine (Ayling and Snell, 1968), and have been analyzed in the same way. The kinetic parameters obtained with each analog active as substrate are summarized in Table II.

Velocity Constants. When pyridoxamine and pyridoxal are substrates, reactions 2f and 2r follow the mechanism shown in (3), where EXY is a rate-determining ternary complex (Ayling and Snell, 1968). Assuming that this mechanism also holds for the reactions with

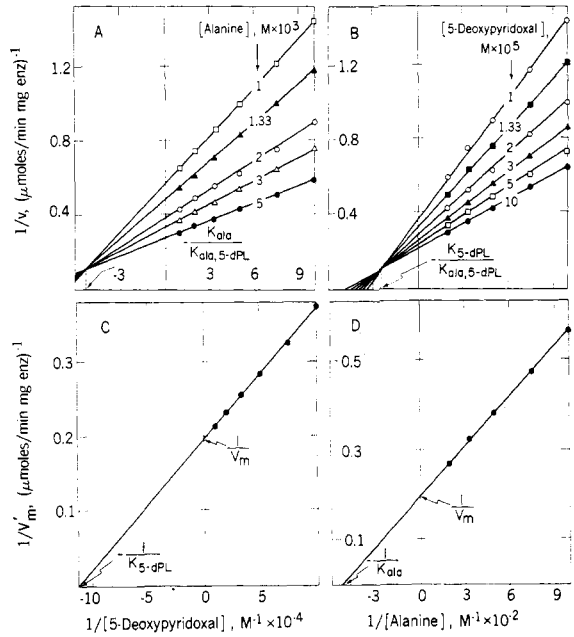


FIGURE 3: Initial velocities of reaction of 5-deoxypyridoxal with L-alanine in reaction 2r catalyzed by pyridoxamine pyruvate transminase. (A,B) Double-reciprocal plots at various levels of substrates. Reaction mixtures contained substrate as indicated in 0.05 M sodium pyrophosphate buffer (pH 8.85) and 0.5 μg of enzyme/ml. The reaction was followed spectrophotometrically at 25° and 390 mμ, an absorption maximum of 5-deoxypyridoxal. (C,D) Secondary plots of apparent maximal velocities (from A,B) as a function of substrate concentration.

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA \underset{k_4(-\text{pyr})}{\overset{k_3(+\text{pyr})}{\rightleftharpoons}} EXY \underset{k_6(+\text{Ala})}{\overset{k_5(-\text{Ala})}{\rightleftharpoons}} EC \underset{k_8}{\overset{k_7}{\rightleftharpoons}} E + C \quad (3)$$

analogs of pyridoxal and pyridoxamine, each of the velocity constants in (3) can be calculated from the data of Table II; their values are summarized in Table III.

Equilibrium Constants of Reaction 2. Equilibrium constants for reaction 2, calculated from kinetic data of Table II by use of the appropriate Haldane equation for the mechanism shown in (3), are compared with directly measured values for each of the substrate pairs, A and C, in Table IV. Directly measured values are the average of four measurements in 0.05 M sodium pyrophosphate buffer (pH 8.85) at 25°, using varying initial concentrations of substrates. The final concentrations of reactants at equilibrium were determined from the change in absorbance at 390 mμ resulting from change in concentration of the aldehyde, as described earlier for the reaction with pyridoxal (Ayling and Snell, 1968). A comparison of the values determined in this way with the corresponding values calculated from steady-state kinetic data is an indication of the accuracy of the kinetic data.

Dissociation Constants of Enzyme–Substrate Complexes. K_{diss} for the complexes formed between enzyme and the analog substrates, A and C of reaction 2, have been calculated from kinetic data and are listed in the last column of Table II. Except for 5-deoxypyridoxal and 5-deoxypyridoxamine, these values are much higher

[1] Values of K_{Ala} (Table II) are such that less than 10 mM L-alanine was required for their determination with all pyridoxal analogs except 3-hydroxypyridine-4-aldehyde. Under these conditions the double-reciprocal plots were linear in all cases. Higher concentrations of alanine caused substrate inhibition of reaction 2r at low pyridoxal concentrations and substrate activation with 2-norpyridoxal as substrate. Indirect evidence indicates that these effects result from differential binding of the free substrates as opposed to the Schiff's bases between aldehyde and alanine formed nonenzymatically at high alanine concentrations. Estimates of the dissociation constants of these Schiff's bases (*cf.* Metzler, 1957) showed that their concentration was unimportant under conditions used for determining values of K_{Ala} for pyridoxal and norpyridoxal (Table II) but becomes significant with 3-hydroxypyridine-4-aldehyde. No deviations from linearity of reciprocal plots occurred with the latter substrate even at high alanine concentrations, indicating that the binding properties of this substrate were unchanged by Schiff's base formation.

TABLE I: Conditions of Testing Inactive Analogs as Substrates or Inhibitors of Reaction 2r.[a]

Compound C	Initial Reactant Concn during Test as Substrate in Reaction 2r: C[b] (mM)	Ala (M)	E[b] (μg/ml)	Inhibitor of Reaction 1b[c]: C (mM)
O-Methylpyridoxal	<2.5	0.01–0.1	20	2.5
3-Deoxypyridoxal	<5.0	0.01	30	5.0
3-Deoxyisopyridoxal	<5.0	0.01	30	5.0
Pyridine-4-aldehyde	<30	0.10	25	10
N-Methylpyridoxal	<10	0.01–0.5	20	10
4-Nitrosalicylaldehyde	<5.0	0.01–0.5	1000	1

[a] All measurements were made in 0.05 M sodium pyrophosphate buffer at pH 8.85 and 25°. The extent of reaction was determined spectrophotometrically (see text) or, for substrate activity of the first four analogs, by assay for pyruvate formed with an excess of lactate dehydrogenase and DPNH. The activity of lactate dehydrogenase was not affected by these compounds. [b] E represents pyridoxamine pyruvate transaminase. In column 2, < signifies that concentrations up to, but not higher than, that indicated were tested. [c] These tests were run in the presence of 0.02–0.2 mM pyridoxal, 2–10 mM L-alanine, and 1.0 μg/ml of pyridoxamine pyruvate transaminase.

TABLE II: Kinetic Parameters and Dissociation Constants for Substrates A and C in Reaction 2 Catalyzed by Pyridoxamine Pyruvate Transaminase.[a]

Substrate A	Reaction 2f: A + pyr → C + L-Ala K_A (mM)	K_{pyr} (mM)	$K_{A,pyr}$ (μM²)	V_f (sec⁻¹)	K_{diss} (mM)
Pyridoxamine	0.013	0.35	0.024	12.6	0.07
ω-Methylpyridoxamine	0.48	0.40	8.5	18.3	21
5-Deoxypyridoxamine	0.014	0.42	0.082	4.9	0.20
2-Norpyridoxamine	0.16	0.52	5.9	19.7	12
3-Hydroxy-4-aminomethylpyridine	0.35	7.4	45	2.6	6.0

Substrate C	Reaction 2r: C + L-Ala → A + pyr K_C (mM)	K_{Ala} (mM)	$K_{C,Ala}$ (μM²)	V_r (sec⁻¹)	K_{diss} (mM)
Pyridoxal	0.012	1.6	0.032	10.5	0.02
ω-Methylpyridoxal	1.3	0.58	4.3	5.95	7.5
5-Deoxypyridoxal	0.009	1.9	0.036	7.95	0.019
2-Norpyridoxal	0.59	2.7	5.9	10.0	2.2
3-Hydroxypyridine-4-aldehyde	0.89	64.5	394	24.0	6.1

[a] All kinetic constants were evaluated by the graphical procedures shown in Figure 3 for 5-deoxypyridoxal. K_A, K_C, K_{pyr}, and K_{Ala} are the K_m values for these specific substrates in reaction 2; $K_{A,pyr}$ and $K_{C,Ala}$ the corresponding combined constant, and V_f and V_r the maximum velocity of reactions 2f and 2r, respectively. K_{diss} of amines was calculated from the relationship $K_{diss} = K_{A,pyr}/K_{pyr}$, and of aldehydes from the relationship $K_{diss} = K_{C,Ala}/K_{Ala}$. Experimental procedures are described in the text.

than those observed for pyridoxal and pyridoxamine. As a consequence, relatively high concentrations of substrate are required for formation of measurable concentrations of the enzyme–substrate complexes, EA and EC, and since the spectral shifts arising from binding are relatively small (<40 mμ), the high background absorbance of the unbound substrate interferes with accurate measurement of the enzyme–substrate complexes. For this reason, direct measurements of dissociation constants were attempted only with 5-deoxypyridoxal and ω-methylpyridoxal.

5-DEOXYPYRIDOXAL. Concentrations of 5-deoxypyridoxal between 0.002 and 0.1 mM were incubated with 1.4 mg of pyridoxamine pyruvate transaminase in 1 ml of 0.05 M sodium pyrophosphate buffer at pH 8.85 and 25°. The increase in absorbance at 430 mμ resulting from the E–C complex was measured against a blank containing equivalent concentrations of 5-deoxypyri-

TABLE III: Comparative Velocity Constants for Reaction 2 Catalyzed by Pyridoxamine Pyruvate Transaminase with Analogs of Pyridoxamine and Pyridoxal.[a]

Velocity Constant	Substrate A, C				
	PM, PL	ω-Methyl-PM, ω-Methyl-PL	5-Deoxy-PM, 5-Deoxy-PL	Nor-PM, Nor-PL	HAMP, HPA
k_1 (M^{-1} sec^{-1})	9.5×10^5	3.8×10^4	3.4×10^5	1.2×10^5	7.5×10^3
k_2 (sec^{-1})	66.0	8×10^2	68.0	1.4×10^3	45.0
k_3 (M^{-1} sec^{-1})	4.7×10^4	5.3×10^4	2.7×10^4	4.7×10^4	7.1×10^3
k_4 (sec^{-1})	12.5	6.0	9.0	10.0	51.0
k_5 (sec^{-1})	44.0	40.0	6.9	42.0	2.66
k_6 (M^{-1} sec^{-1})	3×10^4	7.9×10^4	7.3×10^3	1.9×10^4	3.9×10^2
k_7 (sec^{-1})	18.0	34.0	17.0	37.0	165.0
k_8 (M^{-1} sec^{-1})	9×10^5	4.5×10^3	8.9×10^5	1.7×10^4	2.7×10^4

[a] PM = pyridoxamine, PL = pyridoxal, HAMP = 3-hydroxy-4-aminomethylpyridine, HPA = 3-hydroxypyridine-4-aldehyde. The constants were calculated as described by Ayling and Snell (1968) for pyridoxamine and pyridoxal, which are included for comparison.

doxal. The method of calculation has been described previously (Ayling and Snell, 1968). The dissociation constant determined by this method was 9 μM; that from steady-state kinetics was 19 μM (Table V).

The rate of formation (k_8) of this complex under these same conditions was measured in a Gibson-Durrum stopped-flow apparatus by recording the rate of change of absorbance at 430 mμ as the enzyme–5-deoxypyridoxal complex was formed. Concentrations of enzyme and substrate near 20 μM were used. The data, which fit a second-order rate equation for a reversible reaction, were analyzed in the same way described previously for pyridoxal (Ayling and Snell, 1968). From the value for k_8 thus obtained and the directly determined value of K_{diss} ($=k_7/k_8$), the value for k_7 was calculated. These values (Table V) are within experimental error of those calculated from kinetic measurements, and provide additional evidence that reaction 2 proceeds by the mechanism shown in 3.

ω-METHYLPYRIDOXAL. Complex formation between this analog and enzyme was studied because of the observation (Dempsey and Snell, 1963) that its transamination, unlike that of pyridoxal, proceeded without formation of a yellow enzyme–aldehyde adduct. Further investigation showed that such an adduct (λ_{max} 430 mμ) was formed, but only very slowly. In the stopped-flow apparatus, with concentrations of 1.3 mM ω-methylpyridoxal and 0.0173 mM enzyme, no reaction could be detected using total sweep times of from 20 msec to 10 sec. However, with a setting of 50 sec/sweep, the reaction recorded in Figure 4A, which covers a total time of 7.5 min, was observed. The dissociation constant of the complex was determined using conditions of titration similar to those described for 5-deoxypyridoxal except that higher concentrations of enzyme (7.5 mg/ml) and of ω-methylpyridoxal (0.1–2.0 mM) were employed. After each addition of ω-methylpyridoxal the reaction mixture was incubated for 20 min to ensure that equilibrium was reached before measuring the absorbance change at 430 mμ. The dissociation constant determined in this way is 1.3 mM. Using this value for K_{diss} the data of Figure 4A, plotted according to a pseudo-first-order rate equation for a reversible reaction, give a rate of binding, k_8, of 1.53 M^{-1} sec^{-1}, and a value of k_7 ($=k_8K_{diss}$) of 0.002 sec^{-1}. These values, which were quite reproducible, are not in agreement with the corresponding kinetic values (Table V), and the rate of formation of this complex is less than 0.001 that necessary to account for the rate of the over-all transamination reaction. We conclude that the 430-mμ complex formed with ω-methyl-

TABLE IV: Comparison of Equilibrium Constants of Reaction 2 Calculated from Steady-State Kinetics with Directly Measured Values.[a]

Substrates C and A, Reaction 2[b]	Equilibrium Constant	
	Kinetic	Directly Measured
PL–PM[c]	1.58[c]	1.21 ± 0.05
ω-Methyl-PL–ω-methyl-PM	1.53	1.26 ± 0.05
5-Deoxy-PL–5-deoxy-PM	0.27	0.17 ± 0.02
Nor-PL–Nor-PM	1.96	1.96 ± 0.05
HPA–HAMP	0.96	0.70 ± 0.1

[a] The kinetic equilibrium constants were calculated from steady-state kinetic data using the Haldane equation

$$K_{eq} = \frac{V_f \cdot K_{C,Ala}}{V_r \cdot K_{A,pyr}} = \frac{k_1k_3k_5k_7}{k_2k_4k_6k_8} = \frac{[C][Ala]}{[A][pyr]}$$

The procedure for direct measurement of K_{eq} is described in the text. [b] See Table II for abbreviations. [c] Data for pyridoxal and pyridoxamine are taken from Ayling and Snell (1968).

TABLE V: Comparison of Dissociation and Rate Constants Calculated from Steady-State Kinetics with Those Measured Directly.

Substrate	Constant	From Kinetic Measurements[a]	Measured Directly[b]	λ^c (mμ)
Pyridoxal	k_7/k_8	2×10^{-5} M	1×10^{-5} M	415
	k_8	0.9×10^6 M^{-1} sec^{-1}	1.2×10^6 M^{-1} sec^{-1}	415
	k_7	18 sec^{-1}	12 sec^{-1}	415
5-Deoxypyridoxal	k_7/k_8	1.9×10^{-5} M	0.9×10^{-5} M	430
	k_8	9×10^5 M^{-1} sec^{-1}	7.2×10^5 M^{-1} sec^{-1}	430
	k_7	17 sec^{-1}	6.5 sec^{-1}	430
ω-Methylpyridoxal	k_7/k_8	7.5×10^{-3} M	1.3×10^{-3} M	430
			6.7×10^{-3} M	325
	k_8	4.5×10^3 M^{-1} sec^{-1}	1.53 M^{-1} sec^{-1}	430
			7.5×10^3 M^{-1} sec^{-1}	325
	k_7	34 sec^{-1}	0.002 sec^{-1}	430
			50 sec^{-1}	325

[a] Data are from Tables II and III. [b] See text for description of procedures. [c] The wavelength designated is that at which the direct measurements of the indicated constant was made (see text).

pyridoxal is an abortive complex not involved in the enzymatic reaction.

In addition to the peak at 430 mμ, the difference spectrum of enzyme plus ω-methylpyridoxal *vs.* ω-methylpyridoxal shows absorption at 325 mμ (Figure 5). The dissociation constant of this complex, determined as described earlier for the 430-mμ complex, was 6.7 mM, in agreement with the value calculated from steady-state kinetics (Table V). The rate of formation of the 325-mμ complex is shown in Figure 4B. To decrease the background absorbance, the enzyme concentration was higher and substrate concentration was lower than that in the reaction measured at 430 mμ. The rate of binding (k_8) calculated from this trace is 7500 M^{-1} sec^{-1} in reasonable agreement with the value of 4500 M^{-1} sec^{-1} calculated from steady-state kinetics.

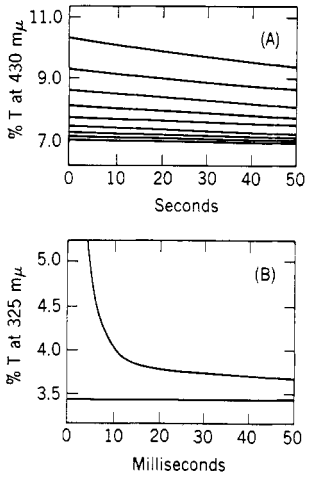


FIGURE 4: Rate of complex formation between ω-methylpyridoxal and pyridoxamine pyruvate transaminase as measured at (A) 430 mμ and (B) 325 mμ in 0.05 M sodium pyrophosphate buffer (pH 8.85) at 25°. (A) Initial concentrations of reactants were 1.3 mM ω-methylpyridoxal and 0.017 mM enzyme. The reaction was recorded by continually retracing across the oscilloscope screen, the uppermost curve representing the first sweep. (B) Initial concentrations were 0.2 mM ω-methylpyridoxal and 0.1 mM enzyme. The lower line is the per cent transmission at the end of reaction.

Pyridoxal Phosphate and Pyridoxamine Phosphate. Titration of enzyme with pyridoxal phosphate results in very slow formation of a complex, presumably the hydrogen-bonded Schiff's base, absorbing maximally at 430 mμ. K_{diss} for this complex is 0.7 mM and its rate of formation (k_8) is about 1 M^{-1} sec^{-1}. At normal concentrations of enzyme (up to 60 μg) and L-alanine (0.01 M), no substrate activity of pyridoxal phosphate was observed in reaction 2r at concentrations up to 0.5

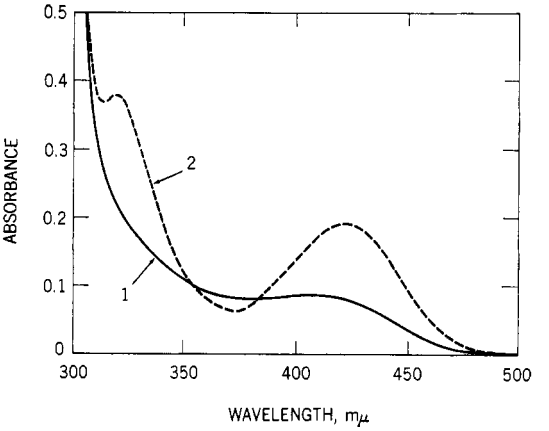


FIGURE 5: Absorption spectrum of pyridoxamine pyruvate transaminase in absence (curve 1) and presence (curve 2) of ω-methylpyridoxal. Curve 1: enzyme (7.5 mg) per milliliter of 0.05 M sodium pyrophosphate (pH 8.85). Curve 2: same as curve 1 plus 0.3 mM ω-methylpyridoxal read against a buffer blank containing 0.3 mM ω-methylpyridoxal. The spectrum was taken 15 min after the addition of ω-methylpyridoxal.

mM. However, when 1 mg of enzyme was used, *i.e.*, a concentration over 1000 times that used to demonstrate the reaction with pyridoxal, a reaction occurred at very high (1 M) alanine concentrations. The rate of the enzymatic reaction under these conditions was only about 25 times that of the corresponding nonenzymatic reaction and was of the same order of magnitude as the rate of complex formation between enzyme and pyridoxal phosphate.

In previous studies (Dempsey and Snell, 1963), commercially available pyridoxamine phosphate appeared to be a reasonably good substrate for pyridoxamine pyruvate transaminase in reaction 2f. This activity apparently resulted chiefly from contamination with free pyridoxamine. Chromatographically purified pyridoxamine phosphate shows activity only under extreme conditions similar to those used for pyridoxal phosphate, *i.e.*, with enzyme at about 1 mg/ml and pyruvate above 0.1 M. Because of the large excess of pyruvate or alanine used, it was possible to run both the transamination of pyridoxamine phosphate (by reaction 2f) and of pyridoxal phosphate (by reaction 2r) to completion, thus demonstrating that the very low activities of these compounds as substrates did not result from trace impurities.

Inhibition of Reaction 1b by Pyridine-4-aldehyde. Crystalline pyridine-4-aldehyde hydrate is neither a substrate nor an inhibitor of pyridoxamine pyruvate transaminase under the conditions of Table I. However, at similar concentrations of alanine and pyridoxal, an anomalous type of inhibition of reaction 1b which depended on both the alanine and pyridoxal concentrations occurred when the pyridine-4-aldehyde concentration was increased to 0.1 M. At alanine concentrations below 2 mM, the inhibition is linear, slight, and noncompetitive with respect to pyridoxal (Figure 6A); the K_I value is 0.04 M. At higher alanine concentrations (>10 mM) inhibition by high concentrations of pyridine-4-aldehyde is progressively greater than that predicted from the effects of lower concentrations of the inhibitor (Figure 6B). Under these conditions the concentrations of alanine and pyridine-4-aldehyde are such that nonenzymatic Schiff's base formation between them becomes significant. If this base has a higher affinity for the enzyme than free pyridine-4-aldehyde, as might be expected from its similarity to the potent inhibitor, pyridoxylalanine (Dempsey and Snell, 1963), the observed inhibition pattern would be produced. Similar observations could not be made with other inactive pyridoxal analogs because of limited availability, limited solubility, or the presence of high absorbance at 390 mμ (absent in pyridine-4-aldehyde) which precluded accurate spectrophotometric determinations of the disappearance of pyridoxal.

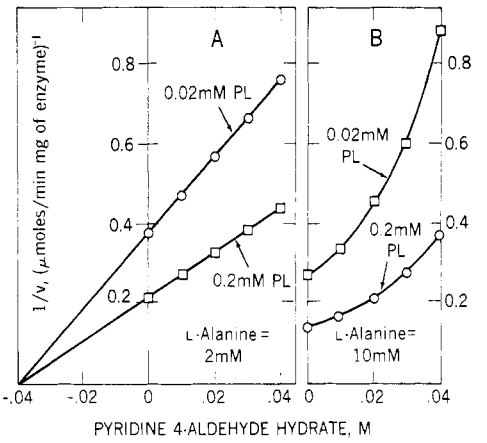


FIGURE 6: Inhibition of reaction 1b by high concentrations of pyridine-4-aldehyde at different concentrations of pyridoxal and alanine. Reaction mixtures contained 1.3 μg of pyridoxamine pyruvate transaminase per ml of 0.05 M sodium pyrophosphate buffer (pH 8.85) together with pyridoxal (PL), L-alanine, and inhibitor at the indicated concentrations.

Discussion

Effect of Structure on Rate of Catalysis. Not only are ω-methylpyridoxal, norpyridoxal, 5-deoxypyridoxal, 3-hydroxypyridine-4-aldehyde, and the corresponding amines all active as substrates for pyridoxamine pyruvate transaminase, but the maximum velocity of reaction with each of these substrates is of the same order of magnitude (Table II). 3-Hydroxypyridine-4-aldehyde, which lacks substituents at both the 2 and 5 positions, undergoes transamination even faster than does pyridoxal. It is therefore apparent that the 2 and 5 substituents of the pyridine ring are neither essential for nor contribute in any major way to the transitions that occur during the transamination reaction. Since 5-deoxypyridoxal and 3-hydroxypyridine-4-aldehyde are active substrates for pyridoxamine pyruvate transaminase, it is clear that the ability to form a hemiacetal does not contribute to the reactivity of pyridoxal in reaction 1. Such a contribution would not be expected if, as concluded elsewhere (Ayling and Snell, 1968), the reaction of pyridoxal as substrate is comparable to the action of pyridoxal phosphate as coenzyme in other transaminases, since in the latter case hemiacetal formation is not possible. Lack of a hydroxymethyl group at position 5 slows the forward reaction (amine → aldehyde) somewhat, but not the reverse reaction, thus shifting the equilibrium position toward the left (Table II and IV). The slightly higher maximum velocities observed for reaction 2f when ω-methylpyridoxamine or norpyridoxamine replaces pyridoxamine are not a result of a greater rate of catalysis but result from an increase in k_7, the rate of dissociation of the corresponding aldehyde from the enzyme (Table III).

Effect of Structure on Binding of the Pyridine Substrates A and C. The affinity of pyridoxal or pyridoxamine analogs for pyridoxamine pyruvate transaminase is greatly reduced by alterations at position 2 of the pyridine ring. The lack of a methyl group at this position (*e.g.*, in 2-norpyridoxamine, 3-hydroxy-4-aminomethylpyridine, and the corresponding aldehydes) or its change to an ethyl group (in ω-methylpyridoxamine and ω-methylpyridoxal) markedly raises the Michaelis and dissociation constants of these substrates (Table II). As seen from the velocity constants (Table III), the change in K_m results from a greatly reduced rate of binding to the enzyme, since the rate constants, k_1 and k_8, are very small for these substrates. This reduction in rate of bind-

ing, together with an increase in k_2 and k_7, the rate of dissociation of the enzyme–substrate complex, results in very high dissociation constants for these analogs. The reduced affinity of these substrates for enzyme is most probably due to steric rather than to charge effects since, although the pK_2 values of 2-norpyridoxal and 3-hydroxypyridine-4-aldehyde (which reflect chiefly the ionization of the pyridinium nitrogen; Metzler and Snell, 1955) are lower than that of pyridoxal, that of ω-methylpyridoxal is not (Table VI). Since either increasing or decreasing the size of the 2 substituent decreases binding of the substrate, the binding site of the enzyme appears to provide a rather specific receptor for a methyl group at position 2 of the pyridine ring.

TABLE VI: pK Values of Pyridoxal and Its Analogs.

Compound	pK_1	pK_2
Pyridoxal	4.15,[a] 4.2,[b] 4.23[d]	8.8,[a] 8.66,[b] 8.7[d]
Pyridoxamine	3.31,[b] 3.54[d]	7.9,[b] 8.21[d]
ω-Methylpyridoxal[a]	4.15	8.85
2-Norpyridoxal[a]	3.9	7.85
5-Deoxypyridoxal[b]	4.17	8.14
3-Hydroxypyridine-4-aldehyde[c]	4.05	6.77
Pyridoxal 5′-phosphate[d]	4.14	8.69
O-Methylpyridoxal[b]	4.75	
Pyridine-4-aldehyde[c]	4.77	

[a] Determined in this laboratory as part of a senior thesis by Miss Mary Yue by the spectrophotometric procedure of Metzler and Snell (1955). [b] Metzler and Snell (1955). [c] Nakamoto and Martell (1959). [d] Williams and Neilands (1954).

The substituent at position 5 of the pyridine ring does not appear to be involved in the binding of pyridoxal or pyridoxamine to enzyme. The Michaelis and dissociation constants of 5-deoxypyridoxal are very similar to those of pyridoxal; the constants for the corresponding amines are also alike. Similarly, the binding constants for 2-norpyridoxal and 2-norpyridoxamine are quite close in value to those of 3-hydroxypyridine-4-aldehyde and 3-hydroxy-4-aminomethylpyridine, respectively, which lack a substituent at position 5. In this respect, the binding of pyridoxal analogs to pyridoxamine pyruvate transaminase differs greatly from the binding of pyridoxal phosphate by apoenzymes dependent on this coenzyme. For example, the affinity of glutamate–oxaloacetate apotransaminase for pyridoxal phosphate is over 1000 times that for pyridoxal, although addition of either substance at appropriate concentrations permits catalysis of the over-all transamination reaction (Wada and Snell, 1962).

The very slow rate of transamination of pyridoxamine phosphate as compared to pyridoxamine by pyridoxamine pyruvate transaminase is the converse of the situation which exists in a clostridial transaminase, for which pyridoxamine phosphate is the preferred substrate and pyridoxamine is transaminated very slowly (Ogata *et al.*, 1968). This enzyme presumably will closely resemble pyridoxamine pyruvate transaminase in its mechanism of action.

Effect of Structure of Pyridoxal Analogs on Binding of Cosubstrates of Pyridoxamine Pyruvate Transaminase. It is clear from eq 3 that the cosubstrates of reaction 2, pyruvate or alanine, bind not to the unmodified enzyme, but rather to a site in close juxtaposition to bound A or C. Any alteration in the binding site caused by combination with a pyridoxal analog should therefore be reflected in a changed affinity for the cosubstrate. The affinity for pyruvate in reaction 2f or for alanine in reaction 2r is changed only moderately (less than threefold) by variations in the 2 substituent of the pyridine substrate, A or C, whether this affinity is measured by the K_m values (Table II) or by the dissociation constants (k_4/k_3 for pyruvate; k_5/k_6 for alanine; Table III) for these cosubstrates. Changing the 5 substituent of the pyridine substrate from CH_2OH to CH_3 also changes these values relatively little; however, replacement of CH_2OH by H very greatly decreases the affinity for the cosubstrate. Whether this decrease results from steric factors alone or is connected in part with the decreased basic strength of the heterocyclic nitrogen (*cf.* the pK_2 values for pyridoxal, 2-norpyridoxal, and 3-hydroxypyridine-4-aldehyde, Table VI) is not certain. However, the K_M value of the cosubstrate with 2-norpyridoxal is not affected in spite of the low pK_2 value. This suggests that steric factors play a more important role especially since the K_m values for pyruvate or alanine are too large to measure when A or C is pyridoxamine phosphate or pyridoxal phosphate, respectively.

Pyridoxal Analogs without Substrate Activity. 4-Nitrosalicylaldehyde (Ikawa and Snell, 1954b) and *N*-methylpyridoxal (Johnston *et al.*, 1963) simulate the reactions of pyridoxal in nonenzymatic reactions but are inactive as substrates of pyridoxamine pyruvate transaminase. Since they are also inactive as inhibitors, we assume that their ineffectiveness as substrates results from their inability to bind at the active site of the enzyme and does not indicate that differences exist in the nature of the participation of substituent groups in the enzymatic as opposed to the nonenzymatic reaction.

Since *O*-methylpyridoxal and 3-deoxypyridoxal serve neither as substrates nor as inhibitors of pyridoxamine pyruvate transaminase, the phenolic group at the 3 position is essential for binding, either directly or because of its very large effect on basicity of the pyridine nitrogen (*cf.* pK_2 for pyridoxal and 3-hydroxypyridine-4-aldehyde *vs.* pK_1 for *O*-methylpyridoxal and pyridine-4-aldehyde, Table VI). Whether it is also necessary for enzymatic transamination *per se*, as it is for the nonenzymatic reaction (Metzler *et al.*, 1954; Thanassi *et al.*, 1965), is not determined by these data. However, the inhibitory effects of pyridine-4-aldehyde on reaction 1 at high alanine concentrations (Figure 6) indicate that a ternary complex must be formed between enzyme, pyridinealdehyde, and alanine, and yet no reaction occurs.

It is likely, therefore, that the phenolic group is mandatory for the enzymatic reaction as well as for binding. Results of French *et al.* (1965) in nonenzymatic systems implicate the 3-hydroxyl group as a catalyst for dehydration of the carbinolamine intermediate formed initially on addition of amino acid to pyridoxal. In the enzymatic reaction, a similar tetrahedral intermediate must precede formation of both ii and EXY in Figure 7, and the 3-hydroxyl group of the pyridine substrate may participate in its conversion into these latter intermediates.

Rates of Association and Dissociation of Enzyme–Analog Substrate Complexes. Although values obtained by direct measurements at 415 or 430 mμ of the dissociation constant (k_7/k_8) and of the rate constant, k_8, for reaction of enzyme with pyridoxal and 5-deoxypyridoxal agree rather well with the corresponding kinetic values (Table V), those of ω-methylpyridoxal do not. Furthermore, the complex absorbing at 430 mμ, which is considered to be the hydrogen-bonded Schiff's base of ω-methylpyridoxal with enzyme, was formed too slowly to be a reaction intermediate. Redetermination of these constants at 325 mμ gave values in agreement with the kinetic values (Table V). These results indicate that the 430-mμ species (iii, Figure 7) cannot be the intermediate which reacts with alanine but is instead the product of a reversible side reaction which is not significant in the over-all reaction when alanine is present.[2] By extension, the similar complexes formed with pyridoxal (λ_{max} 415 mμ) and 5-deoxypyridoxal (λ_{max} 430 mμ) and possibly also with pyridoxal phosphate (λ_{max} 430 mμ) are most likely not those involved in the transamination reaction even though the directly measured values for k_7/k_8 are in reasonable agreement with the corresponding constants calculated from kinetic data (Table V). Since the rate of formation of iii does give an accurate value of k_8 for pyridoxal and 5-deoxypyridoxal, attainment of equilibrium between ii and iii (Figure 7) must be rapid in these cases compared with the rate of formation of ii from i, so that the rate of formation of iii is governed by that of ii. This is in contrast to the situation with ω-methylpyridoxal, where the transition from ii to iii is much slower than the over-all reaction.

When ω-methylpyridoxal is substrate, the value of K_{diss} for complex ii determined from steady-state kinetics ($=k_7/k_8$) is 7.5 mM. According to the model of Figure 7, the corresponding value measured directly at 430 mμ, 1.3 mM, would be $(k_y/k_x)(k_7/k_8)$, and hence k_x/k_y = 5.7. At equilibrium in the absence of alanine, therefore, approximately 85% of the product formed on addition of ω-methylpyridoxal to enzyme is the slowly formed iii, and about 15% would be ii. Similar calculations may be applied to pyridoxal and 5-deoxypyridoxal, where the directly measured value of k_8 agrees reasonably well with the kinetic value, but the directly measured values of K_{diss} are about half the kinetic values (Table V). In these cases such calculations indicate that about one-third of the complex with enzyme (in the absence of alanine) is the active species, ii, and about two-thirds is iii.

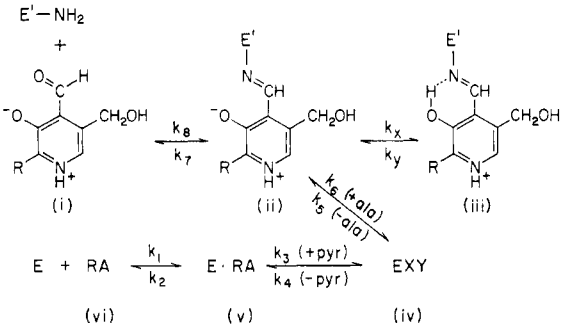


FIGURE 7: Postulated structures of complexes formed by pyridoxamine pyruvate transaminase (E'–NH_2 or E) with pyridoxal and its analogs and their relationship to the transamination reaction, 2r. Structure i is pyridoxal or ω-methylpyridoxal when R = CH_3 or CH_2CH_3, respectively; in 5-deoxypyridoxal, R = CH_3 and CH_2OH has been replaced by CH_3. RA represents the corresponding amines. Compound iii represents the complex absorbing at 415–430 mμ.

The demonstration that the species absorbing at 415–430 mμ is not an intermediate in the over-all transamination reaction is in agreement with the conclusion (Jenkins and Sizer, 1959) that the form of glutamate–oxaloacetate transaminase absorbing maximally at 430 mμ does not participate in the transamination reaction. In tryptophanase-catalyzed reactions, similarly, the form of the enzyme absorbing maximally at 420 mμ is not that which participates in the tryptophanase reaction (Morino and Snell, 1967a). Since at the pH optimum of these reactions the amino acid substrate is predominantly positively charged, it seems logical that a species corresponding to ii (Figure 7), which carries a negative charge on the phenolic group, should be involved in interaction with the amino acid rather than the hydrogen-bonded species iii. It is not clear, however, that this mode of interaction is followed generally in pyridoxal-dependent reactions, since maximum activity of glutamate decarboxylase (Shukuya and Schwert, 1960), arginine decarboxylase (Blethen *et al.*, 1968), and D-serine dehydrase (Dupourque *et al.*, 1966) is observed at pH values where absorbance in the 415-mμ region also is near maximal. From the results with ω-methylpyridoxal discussed earlier, however, it is obvious that the nature of the reactive species of these enzymes cannot be inferred from spectral data alone.

We have emphasized that reactions catalyzed at the substrate level by pyridoxamine–pyruvate transaminase resemble those undergone at the coenzyme level in pyridoxal phosphate dependent transaminases (Ayling and Snell, 1968). The demonstration that the 2 and 5 substituents of pyridoxal are nonessential for the former enzyme thus correlates well with earlier observations that the 2-methyl group of pyridoxal phosphate is not required for its coenzymatic action (Morino and Snell, 1967b), and that free pyridoxal substitutes inefficiently

[2] We picture the reactive intermediate as ii, Figure 7, although such structures usually have absorption maxima near 360 mμ (Johnston *et al.*, 1963; Jenkins, 1961). The position of λ_{max} (325 mμ, Figure 5) indicates that it may instead be a tetrahedral intermediate in which an unidentified adduct, XH, saturates the azomethine bond of ii.

for pyridoxal phosphate in the action of glutamate–oxaloacetate transaminase (Wada and Snell, 1962).

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