The Effects of Various Vitamin B_6 5′-Phosphate Derivatives on the Structure and Activities of L-Aspartate β-Decarboxylase*

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ABSTRACT: Pyridoxal 5′-phosphate and other vitamin B_6 5′-phosphate derivatives (2-norpyridoxal, 2-nor-2-ethylpyridoxal, 2-nor-2-*n*-butylpyridoxal, 3-*O*-methylpyridoxal, *N*-methylpyridoxal, pyridoxine, pyridoxamine, and 4′-deoxypyridoxine) induce association of the low molecular weight apoenzyme ($s_{20,w}$ = 6 S) of L-aspartate β-decarboxylase to a form of the enzyme of much higher molecular weight ($s_{20,w}$ = 19 S). Close to 1 mole of vitamin B_6 derivative is bound per minimal catalytic unit, in general with high affinity. Association does not require Schiff base formation between vitamin B_6 derivative and enzyme. The *N*-methylpyridoxal 5′-phosphate–enzyme, shown here not to have such a Schiff-base linkage, catalyzes at low but significant rates the transamination and the β-decarboxylation of D-aspartate.

The product of D-aspartate decarboxylation is L-alanine. These findings suggest that absence of a Schiff-base linkage between coenzyme and enzyme leads to a less restricted substrate binding site, and are in accord with intermediate formation of a ketimine lacking an asymmetric α-carbon atom. With other vitamin B_6 derivatives, the enzyme exhibits strict L specificity. Replacement of the 2-methyl of pyridoxal by a hydrogen atom, ethyl group, or *n*-butyl group results in greatly decreased decarboxylase and desulfinase activities. The K^m value for cysteinesulfinate is not affected by replacement of the C_2-methyl by a hydrogen atom or ethyl group. The decarboxylase and desulfinase activities of the 3-*O*-methylpyridoxal and *N*-methylpyridoxal forms of the enzyme are also relatively low. In contrast, the L-aspartate–pyruvate transaminase activities observed with the 2-nor, 2-nor-2-ethyl, 2-nor-2-butyl, 3-*O*-methyl, and *N*-methyl derivatives are much higher than or 65–76% as high as that of the pyridoxal 5′-phosphate–enzyme. The substantial transaminase activity catalyzed by the *N*-methylpyridoxal 5′-phosphate–enzyme suggests that little catalytic advantage can be ascribed to a trans-Schiffization mechanism.

In the course of our earlier studies on L-aspartate β-decarboxylase (Tate and Meister, 1968), we found that resolution of the holoenzyme ($s_{20,w}$ = 19 S) at pH 8.0 led to formation of an apoenzyme of much lower molecular weight ($s_{20,w}$ = 6 S); when the 6S apoenzyme was incubated with pyridoxal 5′-phosphate, prompt association occurred with formation of the active 19S holoenzyme.[1] In the present work, we have explored the effects of eight additional vitamin B_6 5′-phosphate derivatives, all of which induce association of the 6S apoenzyme to a 19S form of the enzyme. These vitamin B_6 5′-phosphate derivatives, which bind with high affinity to the enzyme, are bound to about the same extent as pyridoxal 5′-phosphate. The present studies indicate that Schiff base formation between cofactor and the enzyme is not necessary for the association phenomenon or for the transaminase activity exhibited by the enzyme. The various vitamin B_6 5′-phosphate derivatives have different effects on the decarboxylase and transaminase activities of the enzyme, and with one derivative (*N*-methylpyridoxal 5′-phosphate) the enzyme exhibits a significant decrease in optical specificity.

Experimental Section

Materials

L-Aspartate β-decarboxylase was isolated from *Alcaligenes faecalis* (strain N) as described previously (Tate and Meister, 1968).

Pyridoxal 5′-phosphate, pyridoxamine 5′-phosphate, 4′-deoxypyridoxine 5′-phosphate, pyridoxamine dihydrochloride, and pyridoxal were purchased from California Corp. for Biochemical Research. 2-Norpyridoxal 5′-phosphate, 2-nor-2-ethylpyridoxal 5′-phosphate, and 2-nor-2-butylpyridoxal 5′-phosphate (synthesized by Dr. M. Y. Karpeiski) were gifts from Dr. Esmond E. Snell, and 3-*O*-methylpyridoxal 5′-phosphate and *N*-methylpyridoxal 5′-phosphate were gifts from Dr. Anna Pocker, University of Washington, Seattle. Pyridoxine 5′-phosphate was synthesized as described (Peterson and Sober, 1954). [^{32}P]Pyridoxamine 5′-phosphate was prepared by phosphorylation of pyridoxamine dihydrochloride with a mixture of [^{32}P]H_3PO_4 and P_2O_5 according to the method of Peterson *et al.* (1953). We are grateful to Dr. K. Makino for a generous sample of pyridoxal 5′-sulfate.

The structures of the various vitamin B_6–phosphate

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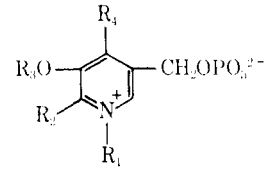
[1] Preliminary studies by Bowers *et al.* (1968) indicate that the molecular weights of the 6S and 19S forms are, respectively, about 110,000 and 720,000.

TABLE I: Vitamin B_6 5′-Phosphate Derivatives.



Vitamin B_6 5′-Phosphate Derivative	R_1	R_2	R_3	R_4	λ_{max} (mμ) Free	λ_{max} (mμ) +Enzyme
Pyridoxal	H	CH_3	H	CHO	330,388	358
2-Norpyridoxal	H	H	H	CHO	330,388	358
2-Nor-2-ethylpyridoxal	H	C_2H_5	H	CHO	330,390	358
2-Nor-2-*n*-butylpyridoxal	H	$n-C_4H_9$	H	CHO	330,393	370
3-*O*-Methylpyridoxal	H	CH_3	CH_3	CHO	279,313	299
N-Methylpyridoxal	CH_3	CH_3	H	CHO	330,398	330,398
Pyridoxamine	H	CH_3	H	CH_2NH_2	327	322
Pyridoxine	H	CH_3	H	CH_2OH	293,324	
4′-Deoxypyridoxine	H	CH_3	H	CH_3	284,314	314

derivatives used in this study are summarized in Table I.

DL-Aspartate-1-^{14}C, L-aspartate-4-^{14}C, sodium pyruvate-1-^{14}C, and [^{32}P]H_3PO_4 were obtained from the New England Nuclear Corp. D-Aspartate-1-^{14}C was obtained by prolonged incubation of DL-aspartate-1-^{14}C with the decarboxylase; the residual D-aspartate-1-^{14}C was isolated by chromatography on Dowex 1-acetate. Crystalline hog kidney D-amino acid oxidase was kindly provided by Dr. Daniel Wellner.

Methods

Enzyme Activities. L-Cysteinesulfinate desulfinase activity and L-aspartate–pyruvate transaminase activity were determined as described previously (Tate and Meister, 1968). The rate of decarboxylation of L-aspartate was measured at 37° in 0.5 ml of assay solution containing 0.1 M sodium acetate buffer (pH 5.5), 1 mM sodium α-ketoglutarate, and 5 mM L-aspartate-4-^{14}C. The reaction was initiated by the addition of enzyme; aliquots (0.1 ml) of the reaction mixture were pipetted, at various time intervals, into liquid scintillation bottles which contained 0.1 ml of 0.5 N HCl. This procedure immediately stops the enzymatic reaction and liberates dissolved $^{14}CO_2$. After 10 min, 10 ml of liquid scintillation medium (Bray, 1960) was added and the radioactivity of the remaining L-aspartate-4-^{14}C was determined. One unit of enzyme is defined as that amount which liberates 1 μmole of CO_2/min under these conditions.

Protein concentration was estimated by the method of Lowry *et al.* (1951). The value taken for the molecular weight of the minimal catalytic unit of the enzyme is 50,000 (Wilson and Meister, 1966; Tate and Meister, 1968).

Isolation of the Apoenzyme. The holoenzyme is readily resolved in the presence of high substrate and salt concentrations (Wilson and Meister, 1966). The following procedure was used to resolve the enzyme; under these conditions the apoenzyme is obtained completely free of the holoenzyme. A solution (2 ml) of the holoenzyme (5 mg/ml) in 1 M sodium acetate buffer containing 0.1 M L-glutamate (pH 5.5) was dialyzed at 25° against 250 volumes of the same buffer for 24 hr. The enzyme solution, which contained the apoenzyme as well as a small amount of the holoenzyme, was then applied to the top of a Sephadex G-200 column (2 × 65 cm) prepared in 0.05 M Tris-acetate buffer (pH 8) containing 2 mM Na_2EDTA; elution was carried out at 25° with this buffer. Under these conditions, the apoenzyme exists in a form having a much lower molecular weight ($s_{20,w}$ = 6 S) than the holoenzyme ($s_{20,w}$ = 19 S) (Tate and Meister, 1968); the latter therefore emerges ahead of the 6S apoenzyme. The fractions containing the apoenzyme (total, about 18 ml) were combined and concentrated to about 6 ml by ultrafiltration through dialysis tubing *in vacuo*.

Polyacrylamide Gel Electrophoresis. Electrophoresis was performed using 4% gels at 26°, essentially as previously described (Tate and Meister, 1968) using 0.05 M Tris-acetate buffer (pH 8). The 6S and 19S forms of the enzyme exhibit characteristic electrophoretic mobilities, as reported earlier. Gel electrophoresis is therefore a convenient method for examining the ability of a coenzyme analog to induce association of the 6S apoenzyme to the 19S form. The validity of this approach has been proven by analytical ultracentrifugation studies. In the gel electrophoresis studies, the apoenzyme (0.1 mg in 0.1 ml of 0.05 M sodium acetate buffer (pH 6)) was mixed with 0.01 ml containing 50 mμmoles of the vitamin B_6 analog. After incubation at 25° for 30 min, 20 μl of this solution was layered on the gel and electrophoresis was begun.

Spectra. The enzyme derivatives were prepared by incubating the apoenzyme (1 mg/0.5 ml of 0.05 M sodium acetate buffer, pH 6) with 0.05 ml of a solution containing 250 mμmoles of the coenzyme analog. The

$$\text{pyridoxamine 5'-phosphate}_F = \frac{(\text{pyridoxamine 5'-phosphate}_I)(C_F)}{C_p + C_F} \quad (1)$$

$$\text{pyridoxamine 5'-phosphate}_B = \frac{(\text{pyridoxamine 5'-phosphate}_I)(C_p - C_F)}{C_p + C_F} \quad (2)$$

unbound coenzyme was removed by gel filtration through a Sephadex G-25 column (coarse grade; 1 × 25 cm) prepared in 0.05 M sodium acetate buffer (pH 6). The absorption spectrum of the enzyme derivative was recorded at 25° with a Cary Model 15 recording spectrophotometer. The fluorescence measurements were made at 23–27° with an Aminco-Bowman spectrofluorometer.

Measurement of Pyridoxamine 5'-Phosphate Binding. The binding of pyridoxamine-^{32}P 5'-phosphate to the apoenzyme was measured by equilibrium dialysis, performed essentially as described by Myer and Schellman (1962), using Lucite cells (The Chemical Rubber Co., Cleveland, Ohio) having a total capacity of 2 ml. The cell was divided into two equal compartments by a semipermeable membrane of 3-cm diameter (cut from cellulose dialysis tubing). Prior to use, the membranes were boiled in 0.01 M EDTA for 1 hr and then washed thoroughly with deionized water; this process was repeated three times, and the membranes were stored in 0.1 M sodium acetate buffer (pH 6). In a typical experiment, 0.8 ml of 0.1 M sodium acetate buffer (pH 6) containing 1.4 mg of the apoenzyme was placed in one compartment of the dialysis cell and 0.8 ml of pyridoxamine-^{32}P 5'-phosphate solution dissolved in the same buffer was placed in the other compartment. The cell was sealed and placed on a reciprocating shaker; dialysis was carried out for 20 hr at 25°. Under these conditions, equilibrium was achieved within 12–15 hr; 0.05-ml aliquots were removed from each compartment and mixed with 10 ml of liquid scintillation medium. The samples were counted with a Nuclear-Chicago liquid scintillation counter. The concentrations of free pyridoxamine 5'-phosphate (pyridoxamine 5'-phosphate$_F$) and of bound pyridoxamine 5'-phosphate (pyridoxamine 5'-phosphate$_B$) were determined from the relationships given by eq 1 and 2 where pyridoxamine 5'-phosphate$_I$ is the initial molar concentration of pyridoximine-^{32}P 5'-phosphate in one compartment of the cell and C_p and C_F are the determined radioactivities (counts per minute) of the aliquots containing and lacking protein, respectively. In all experiments the pyridoxamine-^{32}P 5'-phosphate introduced initially was accounted for within 2% at the end of the experiment.

Results

Conversion of the 6S Apoenzyme into the 19S Form. As indicated in Table II all of the vitamin B_6 5'-phosphate derivatives used in this study (for structures, see Table I) induced association of the 6S apoenzyme to yield a 19S form of the enzyme. Free pyridoxal was not effective, nor was pyridoxal 5'-sulfate. In general, the affinity of the enzyme for the vitamin B_6 5'-phosphate derivatives is high and in all cases close to 1 mole of derivative is bound per minimal catalytic unit of enzyme.

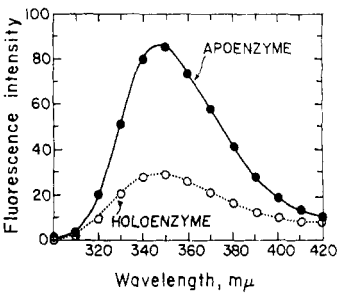


FIGURE 1: Fluorescence emission spectra of the apoenzyme and the holoaspartate β-decarboxylase. Both forms of the enzyme (0.1 mg/ml) were dissolved in 0.075 M sodium acetate buffer (pH 6); the excitation wavelength was 280 mμ.

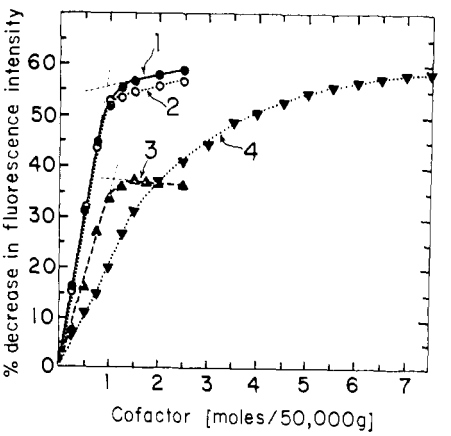


FIGURE 2: Fluorimetric titration of the apoenzyme (0.2 mg/ml in 0.075 M sodium acetate buffer (pH 6)) with pyridoxal 5'-phosphate (curve 1), 2-nor-2-butylpyridoxal 5'-phosphate (curve 2), 3-*O*-methylpyridoxal 5'-phosphate (curve 3), and *N*-methylpyridoxal 5'-phosphate (curve 4). Aliquots of solutions containing the vitamin B_6 5'-phosphate derivatives were added and the fluorescence intensity at 350 mμ was measured after 10 min. The excitation wavelength was 280 mμ.

Binding of Vitamin B_6 5'-Phosphate Derivatives to the Enzyme. FLUORIMETRIC STUDIES. Information concerning the binding of vitamin B_6 5'-phosphate derivatives to the enzyme was obtained from fluorescence measurements. Bertland and Kaplan (1968) have reported that the binding of pyridoxal 5'-phosphate and of pyridoxamine 5'-phosphate to chicken heart soluble glutamate–aspartate transaminase decreases the fluorescence of the tryptophan residues of the enzyme. We have observed similar effects with aspartate β-decarboxylase and the various vitamin B_6 5'-phosphate derivatives. Thus, as shown in Figure 1, both apoenzyme and holo-

TABLE II: Role of Cofactor and Its Analogs on the Structure and Function of L-Aspartate β-Decarboxylase.

Cofactor or Analog	Ability to Catalyze the 6S–19S Reaction	Amt[a] Bound (moles/50,000 g of enyzme)	Activities							
			Decarboxylase		Desulfinase			Transaminase		
			V_{max} (units/mg)	Rel Value	V_{max} (units/mg)	Rel Value	K_m (cysteine-sulfinate)	Sp Act.[g] (units/mg)	Rel Value	K_m (cofactor) M[d]
Pyridoxal 5′-phosphate	+	0.93	122	1	99	1	4.8×10^{-3}	9.2	1	→0[e]
2-Norpyridoxal 5′-phosphate	+	0.94	71.0	0.58	56.5	0.57	4.2×10^{-3}	22.2	2.4	→0
2-Nor-2-ethylpyridoxal 5′-phosphate	+	0.94	24.5	0.2	10.7	0.11	5.2×10^{-3}	13.5	1.5	→0
2-Nor-2-butylpyridoxal 5′-phosphate	+	0.91	5.8	0.05	2.0	0.02		7.0	0.76	→0
3-*O*-Methylpyridoxal 5′-phosphate	+	1.1	6.0	0.05	2.9	0.03		6.0	0.65	→0
N-Methylpyridoxal 5′-phosphate	+	*b*	7.9	0.06	2.9	0.03		6.4	0.7	3.3×10^{-6}
Pyridoxamine 5′-phosphate	+	(0.92),[c] 1.0[f]	0	0	0	0				2.8×10^{-6}
Pyridoxine 5′-phosphate	+	*b*	0	0	0	0		0	0	7×10^{-7}
4′-Deoxypyridoxine 5′-phosphate	+	0.93	0	0	0	0		0	0	→0
Pyridoxal	−	0	0	0	0	0		0	0	

[a] Binding was measured fluorometrically. [b] Presumably 1 mole/50,000 g of enzyme. The analog is weakly bound, thus requiring more than 1 mole/50,000 g of enzyme for saturation of the cofactor sites. [c] Measured in the presence of pyruvate. [d] K_m (cofactor) is arbitrarily defined here as the concentration of the cofactor analog at which 50% of the maximum fluorescence quenching occurs, minus half of the total concentration of enzyme active sites. [e] K_m (cofactor) approaches zero; the vitamin B_6 5′-phosphate derivative is bound by covalent linkage to the apoenzyme. [f] This value was obtained in studies with pyridoxamine-^{32}P 5′-phosphate (see the text). [g] A unit is defined as the amount of enzyme that catalyzes the formation of 1 mμmole of alanine under the conditions of assay.

enzyme exhibit emission maxima at 345 mμ, but the tryptophan fluorescence of the holoenzyme is much lower than that of the apoenzyme. Figure 2 gives fluorimetric titrations of the apoenzyme with pyridoxal 5′-phosphate (curve 1), 2-nor-2-butylpyridoxal 5′-phosphate (curve 2), 3-*O*-methylpyridoxal 5′-phosphate (curve 3), and *N*-methylpyridoxal 5′-phosphate (curve 4). Curves virtually identical with curve 1 (for pyridoxal 5′-phosphate) were obtained in titrations with 2-norpyridoxal 5′-phosphate and 2-nor-2-ethylpyridoxal 5′-phosphate. Sharp breaks were observed in the titration curves with pyridoxal 5′-phosphate, 2-norpyridoxal 5′-phosphate, 2-nor-2-ethylpyridoxal 5′-phosphate, 2-nor-2-butylpyridoxal 5′-phosphate, and 3-*O*-methylpyridoxal 5′-phosphate at a value close to 1 mole of vitamin B_6 5′-phosphate derivative/50,000 g of apoenzyme. The results with pyridoxal 5′-phosphate are in close accord with previous spectrophotometric and optical rotatory dispersion titration data (Wilson and Meister, 1966). These findings indicate that the vitamin B_6 5′-phosphate derivatives bind to the enzyme at the pyridoxal 5′-phosphate binding sites. All of these vitamin B_6 5′-phosphate derivatives except *N*-methylpyridoxal 5′-phosphate (see below) bind to the enzyme with high affinity; the K_m (cofactor) values are extremely low (Table II). It is of interest that all of these vitamin B_6 5′-phosphate derivatives except 3-*O*-methylpyridoxal 5′-phosphate exhibit similar fluorescence intensity at 350 mμ relative to that of the apoenzyme. Although titration with 3-*O*-methylpyridoxal 5′-phosphate also shows a sharp point of inflection, the maximum fluorescence quenching is less than that observed with the other vitamin B_6 5′-phosphate derivatives; this finding seems to be related to the fact that the peak of absorbance of the enzyme-bound 3-*O*-methylpyridoxal 5′-phosphate derivative is shifted to 299 mμ (Table I), which is close to that of tryptophan.

Figure 3 gives fluorimetric titrations of the apoenzyme with pyridoxamine 5′-phosphate (curves 1 and 2), pyridoxine 5′-phosphate (curve 3), and 4′-deoxypyridoxine 5′-phosphate (curve 4). The maximum fluorescence intensities are in all cases not far from those found in the titrations shown in Figure 2. It is evident that the affinity of the enzyme for pyridoxamine 5′-phosphate and pyridoxine 5′-phosphate is lower than for pyridoxal 5′-phosphate. The data obtained with pyridoxamine 5′-phosphate in the prescence of pyruvate (curve 2) were closely equivalent to those found for titration with pyridoxal 5′-phosphate (Figure 2, curve 1); this may be ascribed to transamination of enzyme-bound pyridoxamine 5′-phosphate with pyruvate to yield enzyme-bound pyridoxal 5′-phosphate (Novogrodsky and Meister, 1964a,b). The binding of pyridoxamine 5′-phosphate to the 19S apoenzyme (prepared by resolution of the holoenzyme at pH 6 (Tate and Meister, 1968)) was studied at pH 6 by equilibrium dialysis using pyridoxamine-^{32}P 5′-phosphate as described under Methods. Figure 4 illustrates that the binding of pyridoxamine 5′-phosphate follows a simple Langmuir isotherm. The Scatchard (1949) plot of the data yields a straight line which extrapolates to a value of 1 mole of pyridoxamine 5′-phosphate/50,000 g of

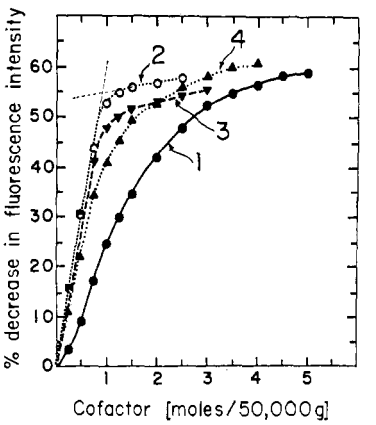


FIGURE 3: Fluorimetric titrations of the apoenzyme with pyridoxamine 5′-phosphate in the absence (curve 1) and presence (curve 2) of 0.2 mM sodium pyruvate, 4′-deoxypyridoxine 5′-phosphate (curve 3), and pyridoxine 5′-phosphate (curve 4); the conditions were as given in Figure 2.

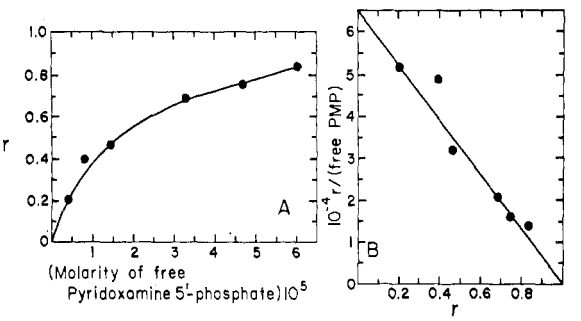


FIGURE 4: Binding of pyridoxamine 5′-phosphate to the apoenzyme. The apoenzyme (1.4 mg in 0.8 ml of 0.1 M sodium acetate buffer (pH 6)) was dialyzed at 25° as described in the text against 0.8 ml of the same buffer containing various amounts of pyridoxamine-^{32}P 5′-phosphate for 20 hr. (A) Plot of *r* (average number of moles of pyridoxamine 5′-phosphate bound per 50,000 g of enzyme) against concentration of free pyridoxamine 5′-phosphate. (B) Scatchard plot.

enzyme. A dissociation constant of 1.56×10^{-5} M can be calculated from this plot. It is of interest that at higher salt concentration the affinity of the apoenzyme for pyridoxamine 5′-phosphate is reduced; thus, the dissociation constant determined in 0.6 M sodium acetate (pH 6) was found to be 4.9×10^{-5} M.

Binding of N-Methylpyridoxal 5′-Phosphate to the Enzyme. Although all of the vitamin B_6 5′-phosphate derivatives bind relatively tightly to the enzyme, it is evident that such binding cannot be associated with Schiff base formation (between enzyme and vitamin B_6 derivative) in the cases of pyridoxine 5′-phosphate, 4′-deoxypyridoxine 5′-phosphate, or pyridoxamine 5′-phosphate. We have made the interesting observation that *N*-methylpyridoxal 5′-phosphate also does not form a Schiff base with the enzyme. This conclusion is based on several experimental approaches: (a) analysis of the fluorimetric data, (b) absorbance studies, (c)

TABLE III: Effect of Sodium Borohydride Treatment on the Desulfinase Activities of Various Forms of L-Aspartate β-Decarboxylase.

Enzyme Derivative[a]	Desulfinase Activity (units/mg)[b]		
	Untreated Form	$NaBH_4$-Treated Form[c]	$NaBH_4$-Treated Form after Preincubn with Pyridoxal 5′-Phosphate
Apoenzyme	1.2	0	40.8
Holoenzyme (pyridoxal 5′-phosphate form)	50.5	0.6	1.9
2-Nor-2-ethylpyridoxal 5′-phosphate derivative	9.8	0	0.8
3-*O*-Methylpyridoxal 5′-phosphate derivative	2.2	0	0.6
N-Methylpyridoxal 5′-phosphate derivative	2.3	0	38.0

[a] The enzyme derivatives were prepared by incubating apoenzyme (1.1 mg in 1 ml of 0.05 M sodium acetate buffer, pH 6) with 22 mμmoles of the vitamin B_6 5′-phosphate derivative for 15 min at 25°. [b] Desulfinase activity was measured as described (Tate and Meister, 1968) either in the presence of 10 mM L-cysteinesulfinate and 1 mM α-ketoglutarate (columns 2 and 3) or after preincubation with 0.5 mM pyridoxal 5′-phosphate and 1 mM α-ketoglutarate for 10 min followed by the addition of L-cysteinesulfinate (column 4). [c] The enzyme derivative (1.1 mg/ml in sodium acetate buffer) was cooled at 0° and about 5 mg of sodium borohydride was rapidly stirred into the solution, which was immediately centrifuged at about 5000 g for 20 min. The enzyme solution was then dialyzed against 500 volumes of 0.05 M sodium acetate buffer (pH 6) for 18 hr.

studies in which $NaBH_4$ reduction was carried out, and (d) gel filtration.

The fluorimetric studies with *N*-methylpyridoxal 5′-phosphate (curve 4, Figure 2) indicate relatively low affinity as compared with the vitamin B_6 5′-phosphate derivatives that possess an aldehyde at C-4 of the pyridine ring; furthermore, the titration data with the *N*-methyl derivative are similar to those found with pyridoxamine 5′-phosphate and pyridoxine 5′-phosphate (Figure 3).

As reported earlier (Novogrodsky and Meister, 1964a) the maximum absorbance of free pyridoxal 5′-phosphate is shifted to about 358 mμ when this cofactor becomes bound to the enzyme. Virtually identical absorbance changes are obtained with 2-norpyridoxal 5′-phosphate and 2-nor-2-ethylpyridoxal phosphate (Figure 5, curve 1) and a shift of maximum to 370 mμ is observed with enzyme-bound 2-nor-2-butylpyridoxal 5′-phosphate (Figure 5, curve 2). A similar result has been noted with the 2-nor-2-butylpyridoxal 5′-phosphate derivative of pig heart glutamate–aspartate transaminase (Bocharov *et al.*, 1968). A marked shift in absorbance maxima also occurs with 3-*O*-methylpyridoxal 5′-phosphate. Thus, as shown in Figure 6 the free 3-*O*-methyl derivative exhibits maxima at 279 and 313 mμ, while a maximum is observed at 299 mμ for the enzyme-bound form. In contrast, no shift in absorbance maxima was observed when *N*-methylpyridoxal 5′-phosphate was bound to the enzyme (Figure 7).

When the *N*-methylpyridoxal 5′-phosphate enzyme was treated with $NaBH_4$ (Fischer *et al.*, 1958) and then dialyzed and treated with pyridoxal 5′-phosphate, no appreciable loss of desulfinase activity was observed

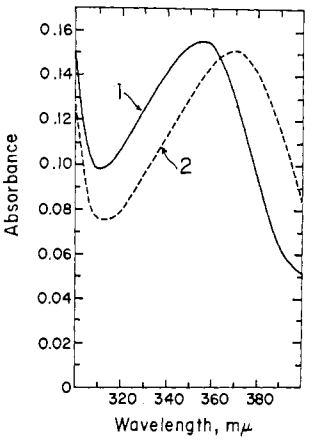


FIGURE 5: Absorption spectra of the pyridoxal 5′-phosphate, 2-norpyridoxal 5′-phosphate, 2-nor-2-ethylpyridoxal 5′-phosphate (curve 1), and 2-nor-2-butylpyridoxal 5′-phosphate (curve 2) forms of the enzyme. The spectra were taken with enzyme (1 mg/ml) in 0.05 M sodium acetate buffer (pH 6).

(Table III). A similar result was obtained with the apoenzyme, while such treatment of the pyridoxal 5′-phosphate–enzyme, the 2-nor-2-ethylpyridoxal 5′-phosphate–enzyme, and the 3-*O*-methylpyridoxal 5′-phosphate–enzyme resulted, as expected, in loss of desulfinase activity.

Additional evidence of the very weak binding of *N*-methylpyridoxal 5′-phosphate was obtained in gel filtration studies. While pyridoxal 5′-phosphate and the other vitamin B_6 5′-phosphate derivatives remained attached to the enzyme during passage through columns

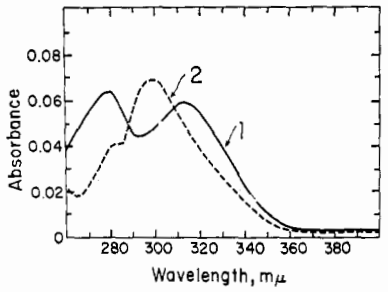


FIGURE 6: Absorption spectra of free (curve 1) and enzyme-bound (curve 2) 3-*O*-methylpyridoxal 5′-phosphate. The apoenzyme (1.1 mg/ml in 0.05 M sodium acetate buffer (pH 6)) was incubated with 22 mμmoles of 3-*O*-methylpyridoxal 5′-phosphate for 15 min. The spectrum of the bound vitamin B_6 derivative was obtained by measuring the absorbance of this solution against one containing apoenzyme alone. Curve 1 gives the absorbance of the vitamin B_6 derivative alone (22×10^{-6} M) in the same buffer.

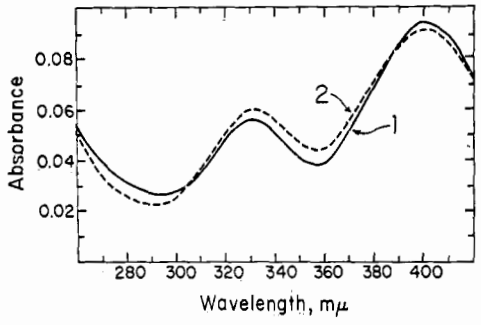


FIGURE 7: Absorption spectra of free (curve 1) and enzyme-bound (curve 2) *N*-methylpyridoxal 5′-phosphate; conditions as given in Figure 5; 24×10^{-6} M.

of Sephadex G-25, *N*-methylpyridoxal 5′-phosphate dissociated completely from the enzyme.

Effect of Vitamin B_6 5′-Phosphate Derivatives on Enzyme Activity. Values for L-aspartate β-decarboxylase, L-cysteinesulfinate desulfinase, and L-aspartate–pyruvate transaminase activities of the various forms of the enzyme are summarized in Table II. In general, the values for decarboxylase and desulfinase parallel each other; replacement of the methyl group at C-2 of the pyridine ring by a hydrogen atom, ethyl group, or butyl group leads to progressive loss of activity. It is of note, however, that the K_m value for cysteine sulfinate is not substantially affected by replacement of the C_2-methyl group by a hydrogen atom or ethyl group. The decarboxylase and desulfinase activities of the 3-*O*-methyl- and *N*-methylpyridoxal 5′-phosphate forms of the enzyme are also relatively low. In contrast, the transaminase activities of the several forms of the enzyme are at least 65% of that of the pyridoxal 5′-phosphate enzyme, and the 2-norpyridoxal 5′-phosphate and 2-nor-2-ethylpyridoxal 5′-phosphate–enzymes are considerably more active than the natural holoenzyme.

Activity of the N-Methylpyridoxal 5′-Phosphate–

TABLE IV: D-Aspartate–Pyruvate Transaminase Activity of the *N*-Methylpyridoxal 5′-Phosphate (*N*-Methylpyridoxal 5′-Phosphate)–Enzyme.[a]

Enzyme Derivative	Transaminase Act. (μμmoles of alanine-^{14}C)
None (*N*-methylpyridoxal 5′-phosphate present)	40[b]
Apoenzyme	80[b]
N-Methylpyridoxal 5′-phosphate	540
N-Methylpyridoxal 5′-phosphate	840[b]
Pyridoxal 5′-phosphate	80
2-Nor-2-ethylpyridoxal 5′-phosphate	30
2-Nor-2-butylpyridoxal 5′-phosphate	80
3-*O*-Methylpyridoxal 5′-phosphate	50

[a] Transamination between D-aspartate and pyruvate-^{14}C was measured in reaction mixtures consisting of 0.2 M sodium acetate buffer (pH 5.5), 10 mM D-aspartate, 2 mM sodium pyruvate-^{14}C, 5×10^{-5} M vitamin B_6 5′-phosphate derivative, and apoenzyme (120 μg/ml); volume, 1 ml. The apoenzyme was incubated for 10 min at 37° with the vitamin B_6 5′-phosphate derivative and pyruvate-^{14}C after which the reaction was initiated by addition of D-aspartate. After 30 min, an aliquot of the reaction mixture was removed and analyzed, after chromatography on a column of Dowex 1-acetate, for alanine-^{14}C as described (Tate and Meister, 1968). [b] Incubated for 60 min instead of 30 min.

TABLE V: Decarboxylation of D-Aspartate by the *N*-Methylpyridoxal 5′-Phosphate–Enzyme.[a]

Enzyme Derivative	Alanine-^{14}C Formed (mμmoles/hr)
None (*N*-methylpyridoxal 5′-phosphate present)	4.5
None (pyridoxal 5′-phosphate present)	3.9
Apoenzyme	1.5
Pyridoxal 5′-phosphate	2.3
2-Nor-2-ethylpyridoxal 5′-phosphate	2.7
2-Nor-2-butylpyridoxal 5′-phosphate	2.0
3-*O*-Methylpyridoxal 5′-phosphate	1.9
N-Methylpyridoxal 5′-phosphate	44.0

[a] The apoenzyme (0.2 mg) was incubated at 37° for 5 min with 50 mμmoles of vitamin B_6 5′-phosphate derivative and 1 μmole of sodium α-ketoglutarate in 1 ml of 0.1 M sodium acetate buffer (pH 5.5). D-Aspartate-1-^{14}C (5 μmoles; 0.05 ml) was added and incubation was continued at 37° for 1 hr. Aliquots were removed for alanine-^{14}C determination by Dowex 1-acetate chromatography.

TABLE VI: Evidence That L-Alanine Is the Product of the Decarboxylation of D-Aspartate by *N*-Methylpyridoxal 5′-Phosphate–Aspartate β-Decarboxylase.[a]

Reaction Mixtures	Alanine-^{14}C (mμmoles)
Alanine-^{14}C (isolated) + D-amino acid oxidase; control	35.2
Alanine-^{14}C (isolated) + D-amino acid oxidase	36.7
DL-Alanine-^{14}C + D-amino acid oxidase; control	40.0
DL-Alanine-^{14}C + D-amino acid oxidase	18.5

[a] Decarboxylation of D-aspartate-1-^{14}C was carried out with the *N*-methylpyridoxal 5′-phosphate form of the enzyme as described in Table V. The eluate from the Dowex 1-acetate column containing alanine-^{14}C was evaporated to dryness *in vacuo* and the residue was dissolved in 0.25 ml of 0.01 M sodium pyrophosphate buffer (pH 8.3). An aliquot (0.2 ml) was incubated with D-amino acid oxidase (35 μg) and flavin–adenine dinucleotide (2 μg) in a final volume of 0.25 ml for 1 hr at 37°. In a parallel experiment, the D-amino acid oxidase was inactivated by heating at 100° for 4 min prior to incubation (D-amino acid oxidase; control). As an additional control DL-alanine-1-^{14}C (40 mμmoles) was incubated in the same way with active and with inactivated D-amino acid oxidase. After incubation, samples of the reaction mixtures were applied to strips (2 × 56 cm) of Whatman No. 3MM paper and subjected to electrophoresis in a sodium acetate buffer (0.04 M; pH 4.0; containing 0.5 mM EDTA) for 1 hr at 35 V/cm. The distances moved from the origin by alanine, β-alanine, aspartate, and pyruvate, were, respectively, −0.5, −2.5 to −3.5, +9 to +11, and +16 to +18 cm. The ^{14}C on the strips was determined with a Nuclear-Chicago Actigraph III, Model 1002 strip scanner.

Enzyme toward D-*Aspartate.* Previous studies have indicated that the pyridoxal 5′-phosphate–enzyme exhibits virtually absolute L specificity toward aspartate and other amino acids. We have found that the various enzyme forms studied here in which there is evidence of a Schiff-base linkage between enzyme and vitamin B_6 5′-phosphate derivative also exhibit L specificity. However, the *N*-methylpyridoxal 5′-phosphate–enzyme, which does not appear to possess such a linkage, exhibits small but significant enzymatic activity toward D-aspartic acid. As indicated in Table IV, a value of 840 μμmoles of alanine-^{14}C was obtained in the D-aspartate-^{14}C-pyruvate transamination reaction with the *N*-methylpyridoxal 5′-phosphate–enzyme as compared to 80 μμmoles (or less) with the other enzyme derivatives and the controls (apoenzyme; free *N*-methylpyridoxal 5′-phosphate). Although the *N*-methylpyridoxal 5′-phosphate enzyme catalyzes D-aspartate–pyruvate transamination at about only 0.3% of the rate for L-aspartate–pyruvate transamination, the data indicate this form of the enzyme does indeed interact to a significant extent with D-aspartate.

The *N*-methylpyridoxal 5′-phosphate–enzyme also catalyzes the decarboxylation of D-aspartate (Table V). The rate of decarboxylation of D-aspartate, although low (about 0.2% of the rate of L-aspartate decarboxylation by this form of the enzyme), is substantially greater than the controls and the results obtained with the other forms of the enzyme. It is of significance that the alanine formed in the decarboxylation of D-aspartate is predominantly of the L configuration. Thus, the alanine formed in the decarboxylation of D-aspartate was not destroyed by incubation with D-amino acid oxidase; under the same conditions, about 50% of racemic alanine disappeared (Table VI).

Discussion

The present studies have shown that a number of vitamin B_6 5′-phosphate derivatives can promote association of the 6S apoenzyme to yield a 19S form of the enzyme. This striking structural change, observed previously with pyridoxal 5′-phosphate (Tate and Meister, 1968), does not occur in the presence of free pyridoxal or pyridoxal 5′-sulfate. The ability of pyridoxine 5′-phosphate, pyridoxamine 5′-phosphate, and 4′-deoxypyridoxine 5′-phosphate to induce such association indicates that this phenomenon does not require the formation of a covalent bond between the cofactor and the enzyme. Furthermore, *N*-methylpyridoxal 5′-phosphate which, as shown here does not form a Schiff base with the enzyme, also promotes association. Morino and Snell (1967a) reported that pyridoxal 5′-phosphate promotes the conversion of dimeric apotryptophanase into a tetrameric holoenzyme, and that at higher molar ratios of coenzyme to enzyme, pyridoxine 5′-phosphate, pyridoxamine 5′-phosphate, and 5′-phosphopyridoxaltryptophan also promote conversion of the dimer to tetramer. As suggested previously (Tate and Meister, 1968), it seems possible that such coenzyme-influenced subunit interactions may be a general property of vitamin B_6–enzymes.

It is notable that all of the vitamin B_6 5′-phosphate derivatives studied here bind to aspartate β-decarboxylase to about the same extent as pyridoxal 5′-phosphate. In general, the vitamin B_6 5′-phosphate derivatives which form a Schiff base with the enzyme exhibit higher affinity than the others; however, 4′-deoxypyridoxine 5′-phosphate is an exception to this statement. The fluorimetric titration data (Figure 3) indicate that the affinity of the enzyme for 4′-deoxypyridoxine 5′-phosphate is almost identical with that for pyridoxal 5′-phosphate. Pyridoxine 5′-phosphate is bound somewhat less tightly than 4′-deoxypyridoxine 5′-phosphate; however, the affinity for both of these derivatives is greater than that for pyridoxamine 5′-phosphate. These observations seem to be of interest in relation to the previous findings that 4′-deoxypyridoxine 5′-phosphate and pyridoxine 5′-phosphate exert a protective effect on aspartate β-decarboxylase (in the presence of aspartate

and α-keto acid) (Novogrodsky and Meister, 1964b). It was proposed that the binding of 4′-deoxypyridoxine 5′-phosphate (and of pyridoxine 5′-phosphate) to some of the active sites of the enzyme exerts a stabilizing effect on the binding of pyridoxamine 5′-phosphate to other sites. The relatively weak binding of pyridoxamine 5′-phosphate may possibly be ascribed to its dipolar nature. The mechanism by which its binding is affected by the presence of 4′-deoxypyridoxine 5′-phosphate is not yet clear. It is of interest that the curve obtained in the fluorimetric titration of the apoenzyme with pyridoxamine 5′-phosphate has a sigmoidal shape (Figure 3, curve 1). Although an explanation for this result is not evident, the studies in which the binding of pyridoxamine-^{32}P 5′-phosphate to the 19S apoenzyme was examined failed to reveal evidence of cooperative homotropic interactions (Figure 4). The finding of a somewhat higher dissociation constant for enzyme–pyridoxamine 5′-phosphate in the presence of 0.6 M sodium acetate buffer (as compared with 0.1 M buffer) is in accord with earlier observations on the activity of the enzyme in buffers of low and high acetate concentration (Novogrodsky and Meister, 1964a).

Vitamin B_6 5′-phosphate derivatives in which the methyl group at C-2 of the pyridine ring is replaced by a hydrogen atom, ethyl group, or butyl group serve much less effectively than pyridoxal 5′-phosphate in the decarboxylase and desulfinase reactions (Table II). The 3-*O*-methylpyridoxal 5′-phosphate–enzyme and the *N*-methylpyridoxal 5′-phosphate–enzyme also exhibit very low decarboxylase and desulfinase activities. In contrast, the L-aspartate–pyruvate transaminase activity of the enzyme with 2-norpyridoxal 5′-phosphate is more than twice that found with pyridoxal 5′-phosphate, and the 2-nor-2-ethylpyridoxal 5′-phosphate is also more effective than pyridoxal 5′-phosphate. The 2-nor-2-butylpyridoxal 5′-phosphate, 3-*O*-methylpyridoxal 5′-phosphate, and *N*-methylpyridoxal 5′-phosphate derivatives of the enzyme all exhibited substantial transaminase activity, *i.e.*, 65–76% of the value obtained with pyridoxal 5′-phosphate. Morino and Snell (1967b) found that 2-norpyridoxal 5′-phosphate is more effective than pyridoxal 5′-phosphate as a coenzyme for pig heart glutamate–aspartate transaminase and for *Escherichia coli* arginine decarboxylase. Studies on tryptophanase, glutamate–aspartate transaminase, arginine decarboxylase, and D-serine dehydrase showed that the efficiency of replacement of pyridoxal 5′-phosphate with 2-norpyridoxal 5′-phosphate or 2-nor-2-ethylpyridoxal 5′-phosphate varies both with the enzyme and with the criterion of activity chosen for comparison (Morino and Snell, 1967b). In the cited studies there appears to be no general pattern with respect to affinity of apoenzyme for coenzyme, affinity of the reconstituted holoenzyme for substrates, or maximal velocity of the reaction catalyzed. The observation that different enzymes behave differently when pyridoxal 5′-phosphate is replaced by another vitamin B_6 5′-phosphate derivative probably reflects differences in the manner in which coenzyme is bound by different enzymes. However, in the present studies on aspartate β-decarboxylase, it is notable that replacement of pyridoxal 5′-phosphate by various vitamin B_6 5′-phosphate derivatives causes substantial decrease of decarboxylase and desulfinase activities while the transaminase activity may be greatly increased or only moderately decreased. This dissociation of effects on activities catalyzed by the same enzyme seems to be related to differences in the mechanisms of the reactions catalyzed. It was previously observed that the effects of pH on aspartate decarboxylation and transamination are different (Novogrodsky and Meister, 1964a). Furthermore, although the decarboxylase and desulfinase activities of the enzyme may be increased or decreased when *p*-mercuribenzoate moieties are introduced into the enzyme, the transaminase activity is not affected (Tate and Meister, 1968). It seems probable that the orientation of the several vitamin B_6 5′-phosphate derivatives on the enzyme must be different than that between the pyridoxal 5′-phosphate and the enzyme. Such differences might be expected to affect the rates of the decarboxylation and desulfination reactions if, as seems likely, these reactions take place in close contact with a specific group on the enzyme. On the other hand, in the transamination reaction, hydrolysis of the ketimine Schiff base may be brought about by a different group of the enzyme (*e.g.*, a site that binds water), which might be differently affected by the spatial relationship between coenzyme and enzyme.

The present findings indicate that *N*-methylpyridoxal 5′-phosphate does not form a Schiff-base linkage with the enzyme. Thus, there is no characteristic spectral shift when this vitamin B_6 5′-phosphate derivative interacts with the enzyme, and sodium borohydride treatment of the *N*-methylpyridoxal 5′-phosphate enzyme does not result in loss of enzymatic activity. Since the *N*-methylpyridoxal 5′-phosphate–enzyme exhibits about 70% as much transaminase activity as the natural pyridoxal 5′-phosphate–holoenzyme, it is evident that azomethine formation between coenzyme and enzyme is not required for a substantial rate of transamination. At least in the case of the transaminase activity of aspartate β-decarboxylase, it would seem that relatively little catalytic advantage can be ascribed to a trans-Schiffization mechanism. The data indicate that while the aldehyde group of *N*-methylpyridoxal 5′-phosphate does not interact with an ϵ-amino group of the enzyme, it can nevertheless form a Schiff base with L-aspartate and L-cysteinesulfinate. The failure of *N*-methylpyridoxal phosphate to form a covalent link with the enzyme suggested to us that in this form of the enzyme the substrate binding site might be less restricted than with pyridoxal 5′-phosphate. The studies with D-aspartate were carried out in order to test this possibility. As documented above, the *N*-methylpyridoxal 5′-phosphate–enzyme can catalyze both the decarboxylation of D-aspartate and D-aspartate–pyruvate transamination. Although the rates of these reactions are much lower than the corresponding reactions with L-aspartate, they are nevertheless significantly higher than the values for the appropriate controls and those for the other vitamin B_6 5′-phosphate–enzymes. The findings suggest that the strict L-amino acid specificity of the pyridoxal 5′-phosphate–enzyme is related at

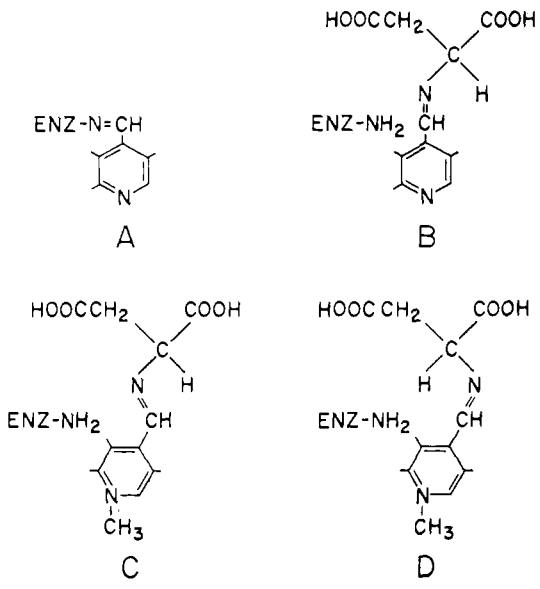


FIGURE 8: Postulated relationships between *N*-methylpyridoxal 5′-phosphate, substrate, and enzyme.

least in part to Schiff base formation between coenzyme and enzyme. Study of a molecular model of pyridoxal 5′-phosphate indicates that the 4′-aldehyde carbon atom is part of a rigid planar structure which includes the pyridine ring. Displacement of the pyridine ring on the enzyme by introduction of an *N*-methyl group would be expected to result in corresponding displacement of the 4′-aldehyde group. As indicated diagrammatically in Figure 8, the 4′-aldehyde group of pyridoxal 5′-phosphate can form a Schiff base with either the enzyme (A) or with L-aspartate (B). Displacement of the *N*-methylpyridoxal 5′-phosphate derivative on the enzyme could serve to move the $C_{4'}$ away from the amino group of the enzyme to a site that is close to the positions of the amino groups of L-aspartate (C) and D-aspartate (D). It is notable that the product of the β-decarboxylation of D-aspartate is L-alanine rather than D-alanine. Since asymmetry about the α-carbon atom of aspartate is lost on formation of the ketimine Schiff base, it is evident that the same ketimine is formed from D- and L-aspartate and therefore that the pathways of the reactions with both isomers of aspartate would lead to L-alanine. According to this interpretation, the substrate binding site of the *N*-methylpyridoxal 5′-phosphate–enzyme is altered, as compared with the pyridoxal 5′-phosphate–enzyme, so as to allow D-aspartate to be bound; however, the remainder of the catalytic process seems to be the same (and L specific) for both forms of the enzyme. It seems possible that other structural modifications of vitamin B_6 might also affect the specificity of this and other vitamin B_6–enzymes. Such experiments, perhaps in conjunction with the interesting lines suggested by Dunathan (1966), might offer a fruitful approach to study of the mechanisms involved in the stereospecificity of vitamin B_6–enzymes.

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