...itamin B_6 ...urobiology

...ed by M. Ebadi and E. Costa

Advances in Biochem...al Psychopharmacology

Volume 4

Series Editors: E. Costa and P. Greengard

ROLE OF VITAMIN B_6 IN NEUROBIOLOGY

Advances in Biochemical Psychopharmacology Volume 4

EDITORS

Manuchair S. Ebadi, Ph. D.
Chairman, Department of Pharmacology
University of Nebraska
College of Medicine
Omaha, Nebraska

Erminio Costa, M. D.
Chief, Laboratory of Preclinical Pharmacology
National Institute of Mental Health
Washington, D.C.

Raven Press · New York · 1972

Distributed in the Eastern Hemisphere by
North-Holland Publishing Company · Amsterdam

International Standard Book Number 911216–18–9
Library of Congress catalog card number 73–84113

TO PROFESSOR ESMOND E. SNELL

Preface

As a coenzyme, vitamin B_6 participates in the biotransformation of many amino acids and in the metabolism of proteins, lipids, carbohydrates, purines, pyrimidines, and all compounds implicated as neurotransmitters. It is also involved in the synthesis of five other coenzymes, namely, pantothenic acid, nicotinamide, riboflavin, nicotinamide adenine dinucleotide, and coenzyme A. Vitamin B_6 deficiency is characterized by anemia, growth retardation, and alteration in neuronal function, including neuropathies, hyperirritability, hyperexcitability, and convulsions. The importance of vitamin B_6 in the study of brain function assumes still greater significance when one considers the effects of nutritional deficiencies on the growth and development of the brain and mental processes, as well as the involvement of vitamin B_6 in some inborn errors of metabolism which result in mental retardation.

This volume was organized (a) to review and elaborate on the role of pyridoxal phosphate in the regulation of the metabolism of biogenic amines, (b) to discuss the nature and the mechanism of pyridoxine-responsive seizures, (c) to emphasize the diversity of biochemical reactions involving this coenzyme, and (d) especially to stimulate research on vitamin B_6 metabolism as applied to neurobiological aspects of psychopharmacology. The first three objectives have been fulfilled. It is hoped that this book will herald the apperance of greater interest in research on this coenzyme among students of neurobiology.

Despite the fact that various classes of psychoactive agents alter the level of pyridoxal phosphate, as well as the activities of the B_6-related enzymes and the B_6-dependent enzymes in the brain, no theoretical or experimental explanation of the mechanism of these phenomena has been presented. Although various stimulants of the central nervous system reduce the level of pyridoxal phosphate within the CNS and concomitantly cause hyperexcitability and convulsions, the role that vitamin B_6 plays in the maintenance of the integrity of neuronal excitability is obscure. More research on these and similar effects of vitamin B_6 seems indicated.

Nutritionists have established the various effects and complications of B_6 deficiency, and biochemists have delineated many complex mechanisms of pyridoxal phosphate involvement in B_6-dependent reactions. However, there remains a wealth of knowledge about this coenzyme yet to be discovered

by neurophysiologists and neuropathologists who are involved in studies dealing with various neurobiological parameters in health and disease; and by neuropsychopharmacologists who are anxious to study and delineate the pharmacodynamics of various psychoactive agents. It is anticipated that the study of vitamin B_6 in neurobiology will prove most revealing and rewarding.

We are pleased to dedicate this book to Professor Esmond E. Snell, who has defined and described various catalytically active forms of B_6 and has made many outstanding contributions to this field. The generous support of Eaton Laboratories, Hoffmann-La Roche, and Smith, Kline and French is gratefully acknowledged.

December 20, 1971 M. Ebadi and E. Costa

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Advances in Biochemical Psychopharmacology, Vol. 4.
Raven Press, New York © 1972.

Relation of Chemical Structure to Metabolic Activity of Vitamin B_6

Esmond E. Snell

Department of Biochemistry, University of California, Berkeley, California 94720

The relationship of structure to function of physiologically important compounds is a problem central to much of biochemistry and pharmacology, and usually is only poorly understood. This problem arose very early in the history of vitamin B_6, for the observations (a) that pyridoxine was relatively inactive in supporting growth of lactic acid bacteria, but became highly active after appropriate chemical treatments (Snell, 1942, 1944*a*), and (b) that feeding pyridoxine to animals was followed by excretion of compounds much more active than pyridoxine itself in promoting growth of these bacteria (Snell, Guirard, and Williams, 1942) led directly to our discovery of pyridoxal and pyridoxamine (Snell, 1944*a,b*). All three of these compounds are widely distributed in nature and are approximately equally effective in promoting growth of animals on deficient diets. They are now known to function as precursors of pyridoxal 5′-phosphate and pyridoxamine 5′-phosphate which serve as essential coenzymes for a large number of reactions concerned with degradation and biosynthesis of amino acids *in vivo*. Species for which pyridoxine or pyridoxamine show little or no activity fail to convert these compounds to the coenzyme efficiently (for reviews, see Snell, 1958, 1963, and Braunstein, 1960).

We may consider the relationship of structure to activity of these compounds at three levels of increasing specificity: (a) what chemical features of pyridoxal or its phosphate are directly involved in catalysis; (b) what additional structural features are required to permit the coenzyme to interact with its various conjugate apoenzymes to form active holoenzymes; and (c) finally, and most restrictive, what structural features of vitamin B_6 can be varied and still retain vitamin activity in microorganisms or animals? Early experiments in these areas have been reviewed elsewhere (Snell, 1958); here I shall limit the discussion to only a few aspects of this topic, and draw

heavily from results published from my own laboratory (with which I am most familiar) for illustrative material.

I. CHEMICAL FEATURES OF PYRIDOXAL REQUIRED FOR CATALYSIS

Delineation of the chemical features of pyridoxal necessary for catalysis was made possible by discovery that this vitamin catalyzed nonenzymatically in dilute aqueous solutions several reactions of amino acids that also occur *in vivo*, where they are catalyzed by enzymes that require pyridoxal phosphate as a coenzyme. Examples of such reactions include transamination between pyridoxal and any of several amino acids to yield pyridoxamine and the corresponding keto acids, as shown in Equations 1 and 2.

$$\text{pyridoxal} + \text{alanine} \rightleftharpoons \text{pyridoxamine} + \text{pyruvate} \quad (1)$$
$$\alpha\text{-ketoglutarate} + \text{pyridoxamine} \rightleftharpoons \text{pyridoxal} + \text{glutamate} \quad (2)$$

$$\textit{Sum:}\ \text{alanine} + \alpha\text{-ketoglutarate} \rightleftharpoons \text{pyruvate} + \text{glutamate} \quad (3)$$

Since these reactions are fully reversible, complete non-enzymatic transamination reactions analogous to that catalyzed, for example, by alanine aminotransferase (Equation 3), will occur on the addition of an appropriate amino acid-keto acid pair; pyridoxal and pyridoxamine appear only as catalysts in such reactions. Similarly, pyridoxal (PL) catalyzes the conversion of many β-substituted amino acids to pyruvate and ammonia, with liberation of the β-substituent, as shown for serine in Equation 4. This reaction is fully analogous to that catalyzed by serine dehydratase *in vivo*.

$$\underset{\displaystyle |\atop \displaystyle NH_2}{HOCH_2CHCOO^-} \xrightarrow[PL]{-H_2O} [\underset{\displaystyle |\atop \displaystyle NH_2}{CH_2 = CCOO^-}] \xrightarrow{+H_2O} CH_3COCOO^- + NH_3 \quad (4)$$

The illustrative data in Table 1 show that serine is completely stable for 10 min at 100° and pH 5, either alone or in the presence of metal (Al^{3+}) ions. On addition of pyridoxal, pyruvate and ammonia are formed slowly in equimolar amounts, and this reaction is greatly speeded (about 20-fold) by the addition of Al^{3+}. For convenience, most non-enzymatic reactions of pyridoxal have been studied at 100°; however, they occur much more slowly at 37°, and, by choosing the appropriate substrates, they can occur quite rapidly at such temperatures. Serine-3-phosphate, for example, in the presence

TABLE 1. *Catalysis of keto acid production from serine by pyridoxal and alum*[a]

Reactants (mM)			Products (mM)	
Serine	Pyridoxal	Alum	Pyridoxal	Pyruvate
10	0	0	—	0.00
10	0	1	—	0.00
10	10	0	9.7	0.23
10	10	1	9.2	5.6
10	pyridoxamine 10	1	0.01	0.04

[a] From Metzler and Snell (1952). The samples were buffered at pH 5.0 with acetic acid (0.2 M)-ammonia buffer prepared from redistilled acid and ammonia and heated for 10 min at 100°.

of pyridoxal is rapidly converted at room temperature to pyruvate, ammonia, and inorganic phosphate, the reaction being speeded about 10-fold by appropriate metal ions (Table 2). Other β-substituted amino acids with a strongly electronegative substituent in the β-position, such as carbamyl serine and azaserine, yield similar results.

By replacing pyridoxal in such reactions by structurally modified compounds, it is possible to define the structural characteristics of the vitamin necessary for effective catalysis of these reactions. The results (Fig. 1) show that the 4-formyl group, the phenolic group, and the heterocyclic nitrogen atom of pyridoxal are essential for the reactions. If the phenolic group is covered by a methyl group, as in 3-O-methylpyridoxal, or if the heterocyclic N is missing, as in salicylaldehyde, no reaction is observed. The 2-methyl group and the 5-hydroxymethyl group are not essential: 3-hydroxypyridine-4-aldehyde and the electronically equivalent *ortho* compound, 3-hydroxy-

TABLE 2. *Comparative rates of pyruvate production from serine-3-phosphate, carbamylserine, and azaserine on incubation with pyridoxal and metal ions*[a]

Time	Pyruvate production (mmoles per liter) at 37° from					
	Serine-3-phosphate		Carbamylserine		Azaserine	
(min)	Complete	No Ga(III)	Complete	No Ga(III)	Complete	No Ga(III)
15	2.0	0.2	3.6	0.3	2.7	0.3
30	2.9	0.2	4.6	0.4	4.6	0.5
60	3.7	0.3	4.9	0.6	5.2	0.8
120	4.8	0.4	6.8	0.7	5.8	1.6

[a] From Longenecker and Snell (1957). The complete reaction mixture contained, per liter, 10 mmoles of serine phosphate, carbamylserine, or azaserine, 2 mmoles of pyridoxal, and 1 mmole of $Ga(NO_3)_3$, and was buffered at pH 9.0 with 0.1 M bicarbonate buffer. No pyruvate was produced in any of the reaction mixtures when pyridoxal was omitted.

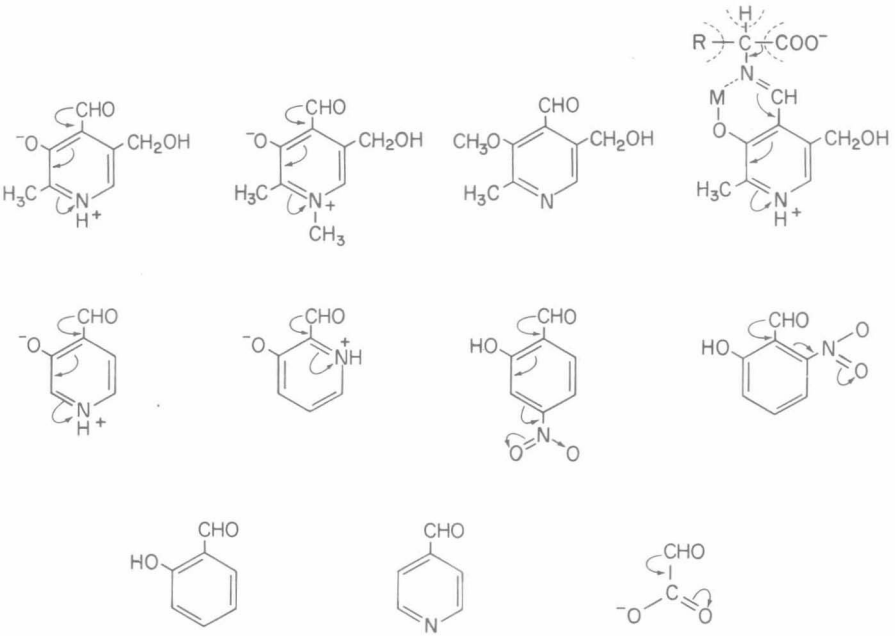

FIG. 1. Structural prerequisites for catalysis of transamination and α, β-elimination reactions of amino acids in aqueous solutions near neutrality. Electron displacements in catalytically active compounds are shown by curved arrows; other compounds are inactive. A metal-ion-stabilized Schiff's base formed between pyridoxal and an amino acid is shown in the upper right. For further discussion, see Snell (1958) and Guirard and Snell (1964).

pyridine-2-aldehyde, are both effective catalysts of such reactions. The role of the pyridine nitrogen as a strongly electronegative center was first suspected from the fact that *p*- and *o*-nitrosalicylaldehyde catalyze somewhat similar reactions of amino acids although much less effectively than pyridoxal.

All of the catalytically active compounds, therefore, contain an acidic hydroxy group *ortho* to the formyl group and a strongly electronegative group so placed as to reduce the electron density about the formyl group. Such compounds react readily with amino acids in aqueous solutions to form Schiff bases which can be stabilized by chelation with a proton or with metal ions, as shown in Fig. 1. In such chelates, the electronegative heterocyclic nitrogen, the azomethine nitrogen, and the chelated metal ion all cooperate to reduce the electron density about the three bonds to the α-carbon atom of the amino acid, thus weakening these bonds and "activating" the amino acid for the several reactions catalyzed by pyridoxal in nonenzymatic reactions.

How such labilization functions to promote dehydration of serine (reaction 4) is clear from Fig. 2. Labilization of the α-hydrogen first occurs followed by elimination of the extra electron pair (present in the first formed

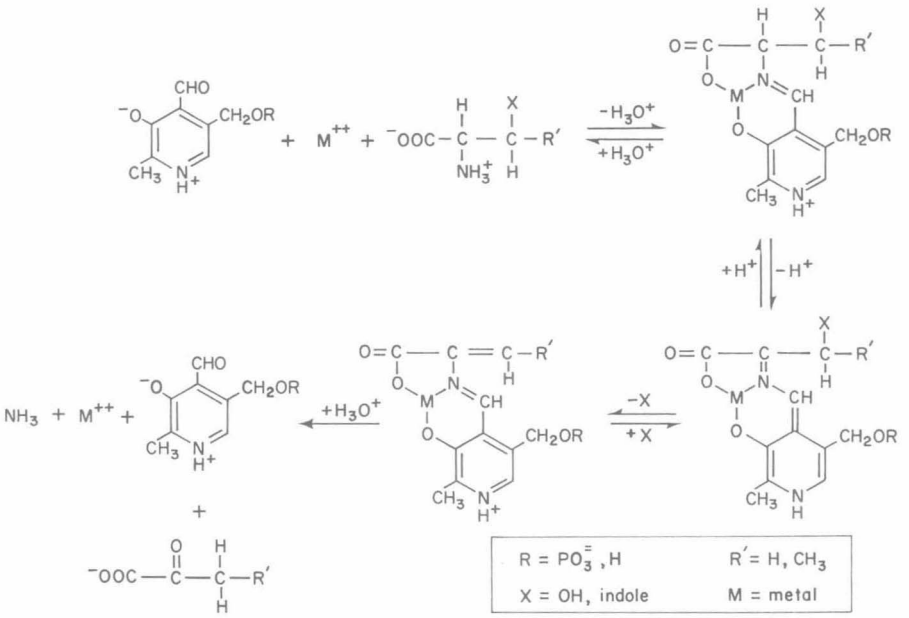

FIG. 2. Mechanism of catalysis of α, β-elimination reactions of serine (R′ = H, X = OH), threonine (R′ = CH_3, X = OH), or tryptophan (R′ = H, X = indolyl residue) by metal ions and pyridoxal (R = H) or pyridoxal-*P* (R = $PO_3^=$).

(quinonoid intermediate) as a hydroxyl ion, with conversion of the quinonoid intermediate to the Schiff's base of α-aminoacrylic acid, which hydrolyzes in aqueous solution to regenerate pyridoxal and metal ions together with free α-aminoacrylic acid. The latter compound is spontaneously unstable in aqueous solution and hydrolyzes to pyruvate and ammonia.

Each of the non-enzymatic reactions catalyzed by pyridoxal can be understood in similar terms, and these reactions appear to be close mechanistic models for the corresponding enzymatic reactions. The subject has been reviewed frequently (e.g., Snell, 1958; Braunstein, 1960; Snell and DiMari, 1970). For our purposes it is important to note that the nonenzymatic reactions can be catalyzed by aldehydes as diverse in structure as pyridoxal and glyoxylate, a common essential feature being the ability to react freely and reversibly with amino acids to form Schiff bases in which appropriately placed electron-attracting groups activate the amino acid by weakening the bonds to its α-carbon atom. We may now compare these chemical requirements for catalysis of non-enzymatic reactions with the structural requirements for coenzyme action.

II. STRUCTURAL FEATURES OF PYRIDOXAL 5′-PHOSPHATE (PLP) NECESSARY FOR PARTICIPATION IN ENZYMATIC REACTIONS

The effect of variations in coenzyme structure on the activity of different PLP-dependent enzymes has been studied recently in several different laboratories; a review is available (Snell, 1970). For our purposes here, a comparison of three enzymes—one that catalyzes a transamination reaction and two that catalyze α, β-elimination reactions—will suffice.

A. Aspartate aminotransferase (AAT).

Catalysis of reaction 5 by this enzyme involves interconversion of pyridoxal-*P* and pyridoxamine-*P* on the enzyme surface as shown in Fig. 3A. Wada and Snell (1962) showed

$$\text{L-aspartate} + \alpha\text{-ketoglutarate} \rightleftharpoons \text{L-glutamate} + \text{oxaloacetate} \qquad (5)$$

that the corresponding apoenzyme could catalyze this same reaction at a very slow rate if free pyridoxal was added, the reaction then proceeding as shown in Fig. 3B. In this latter reaction, pyridoxal obviously occupies the binding site normally occupied by pyridoxal-*P* and is converted to pyridoxamine, just as pyridoxal-*P* in the holoenzyme would be converted to pyridoxamine-*P*. However, the unphosphorylated compounds have comparatively low affinities for the apoenzyme (< 0.001 that of pyridoxal-*P*) and therefore act as diffusible cosubstrates rather than as tightly bound coenzymes. One can safely assume that the 5′-phosphate group, which contributes so

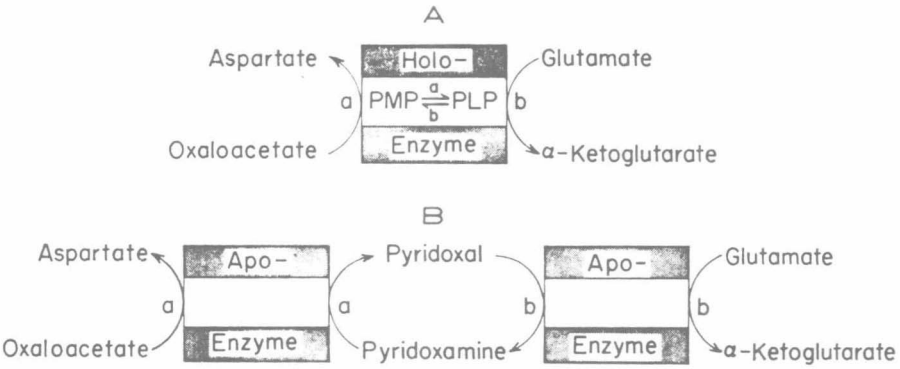

FIG. 3. Schematic representation of reactions catalyzed by (A) aspartate aminotransferase, and (B) the corresponding apoenzyme. Reproduced from Wada and Snell (1962) with consent of the publisher.

greatly to firm binding of the coenzyme, also orients the coenzyme in a position appropriate for further reaction. Both effects promote rapid reaction and are lacking when the unphosphorylated compounds replace the coenzymes. The important point emerges that the 5′-phosphate group of pyridoxal-*P* is required for efficient action of PLP enzymes only because it is required for efficient and conformationally appropriate combination of coenzyme with the apoenzyme; it apparently does not participate in catalysis *per se*.

When pyridoxal-*P* combines with apoAAT, a pronounced spectral band appears at either 430 or 360 nm, depending on the pH (Fig. 4). Similar absorbance changes appear when either 2-nor-PLP, which lacks a methyl group at the 2-position, or 2′-MePLP (ω-MePLP, 2-nor-2-ethyl-PLP), which has an ethyl group at this position, interacts with the apoenzyme. These changes indicate that all three compounds combine in a similar way at the active site of the apoenzyme. The coenzymatic activities of these and related analogues of pyridoxal-*P* are shown in Table 3. Each of the analogues combines with apoAAT as shown both by the spectral changes and the high activity of the resulting complex in catalyzing reaction 5 (*cf.* values for the maximal velocity,

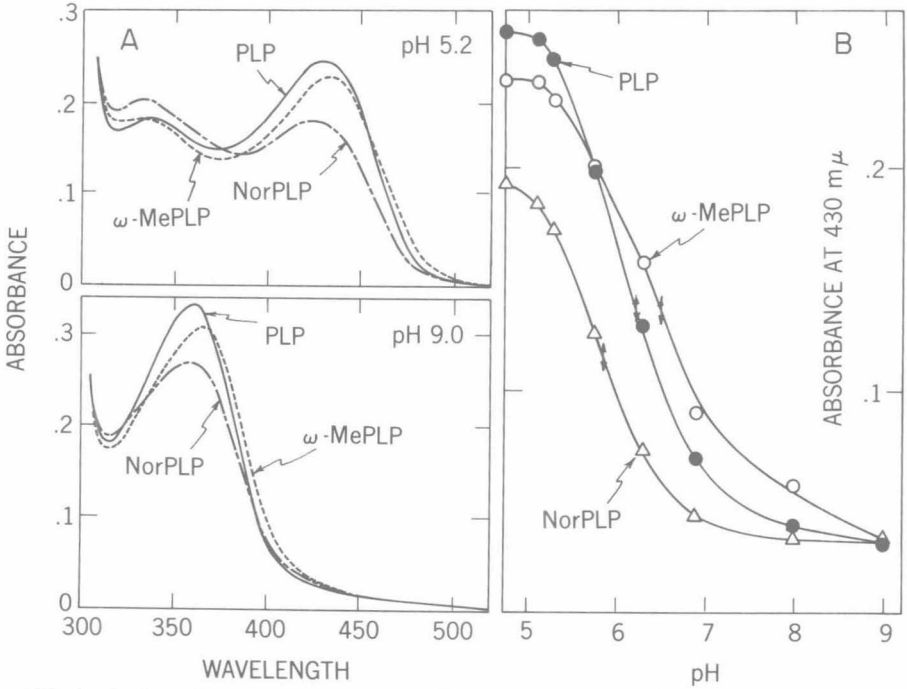

FIG. 4. pH-dependent variation in spectra of aspartate aminotransferase reconstituted from the apoenzyme with PLP, 2-norPLP or 2′-MePLP (ω-MePLP). The arrows (part B) indicate the pK values for the spectral change. For details, see Morino *et al.* (1967). (Reproduced with permission of the publisher.)

TABLE 3. *Some properties of aspartate aminotransferases reconstituted from the apoenzyme with various 6- or 2-substituted analogs of pyridoxal phosphate*[a]

Property measured	Apoenzyme reconstituted with					
	PLP	2-NorPLP	2′-MePLP	2′-Propy1PLP	6-MePLP	2-Nor-6-MePLP
λ_{max}, pH 5.2 (nm)	430	425	435	440	455	455
λ_{max}, pH 8.1 (nm)	360	360	365	370	370	370
pK_a	6.25	5.8	6.5	6.4	6.3	—
V_{max}, relative	1	1.2(1.8)	(0.32)	0.5	0.56	0.47
K_m, α-ketoglutarate (mM)	0.1 (0.16)	0.24(0.5)	(0.05)	0.08	0.14	—
K_m, L-aspartate (mM)	2.0 (3.0)	1.8(5.0)	(0.5)	0.6	1.0	—
K_{co} (μM)[b]	(0.15)	(0.07)	(1.5)	—	—	—

[a] Values in parentheses are from Morino and Snell (1967); all other values are from Bocharov *et al.* (1968).

[b]. Concentration of analog required for half-maximum activity of the reconstituted enzyme under arbitrary nonequilibrium conditions.

V_m, in Table 3). These results demonstrate that neither the 2-methyl group nor the 6-H of pyridoxal-*P* is required for formation of catalytically active complexes with apoAAT: both groups can be replaced either individually (*cf.* 2-nor-PLP, 6-MePLP) or simultaneously (2-nor-6-MePLP) without markedly decreasing activity of the coenzyme. Other studies have shown that pyridoxine-*P* and its 4-deoxy analogue (review, Snell, 1958), as well as 3-O-methylpyridoxal-*P* (Furbish *et al.*, 1969), all of which are inactive in catalyzing non-enzymatic transamination, also do not form catalytically active complexes with apoAAT. N-Methylpyridoxal-*P*, which effectively catalyzes non-enzymatic transamination, is also essentially inactive as a coenzyme for AAT (Furbish, *et al.*, 1969).

We may conclude from these results that activity as a coenzyme requires the same structural features that are required for catalysis of non-enzymatic transamination. However, only some of the compounds that possess these features and catalyze the non-enzymatic reactions fit the coenzyme-binding site of the apoenzyme sufficiently closely to yield catalytically active holoenzyme analogues.

B. Enzymatic α, β-elimination reactions.

The comparative effects of various analogues in replacing pyridoxal-*P* for D-serine dehydratase (which catalyzes reaction 4) and for tryptophanase (which catalyzes the mechanistically similar reaction 6), are summarized in Table 4.

$$\text{L-tryptophan} + H_2O \longrightarrow \text{indole} + \text{pyruvate} + NH_3 \qquad (6)$$

Each active analogue possesses a 4-formyl group, a free 3-phenolic group, and an unsubstituted pyridine nitrogen in the 1-position; i.e., they share the minimum structural features required for catalysis of the corresponding non-enzymatic reactions of Tables 1 and 2. Again, neither the 2- nor the 6-substituent of pyridoxal-*P* is required for activity, although variation in the groups at this position does affect the affinity of the analogue coenzyme for the apoenzyme, as indicated by the K_p and K_{co} values of Table 4, and also affects both the affinity of the resulting analogue holoenzyme for its substrate (as reflected by the K_m values) and the maximal velocity (V_m) of the reaction. It is important to note that these constants vary independently from one enzyme to another. 2′-Hydroxypyridoxal-*P*, for example, combines with aposerine dehydratase almost as well as does pyridoxal-*P*, and the V_m for the analogue holoenzyme is almost twice that of the PLP-apoenzyme complex. By contrast, the affinity of this analogue coenzyme for apotryptophanase is very much less than that of pyridoxal-*P*, and the reconstituted analogue holoenzyme is a less efficient catalyst than is the PLP-enzyme. Even more striking is the result