In Vitro Reaction of L-Tryptophan and Vitamin B_6. Synthesis of the Corresponding β-Tetrahydrocarbolines†

Chris J. Argoudelis

Department of Food Science, 580 Bevier Hall, University of Illinois, 905 South Goodwin Avenue, Urbana, Illinois 61801

The L-tryptophan load test is used to study vitamin B_6 deficiency in animals. This test might suffer from an inherent defect due to the possible nonenzymatic formation of cyclic compounds between L-tryptophan and pyridoxal or pyridoxal 5′-phosphate. In this paper the synthesis, under physiological conditions (pH 7.0 and 37 °C), of the cyclic compounds (β-tetrahydrocarbolines) formed from L-tryptophan and pyridoxal or pyridoxal 5′-phosphate is reported. The *in vitro* formation of these compounds has been implied for many years through evidence from biological experiments and ultraviolet absorption spectra. The synthesized compounds will be valuable to investigators studying their possible formation in animals, their toxicity, and their possible inhibition of certain pyridoxal 5′-phosphate requiring enzymes.

Keywords: *Tryptophan; vitamin B_6; β-tetrahydrocarbolines; pyridoxal 5′-phosphate*

INTRODUCTION

One of the most widely accepted tests for detection of vitamin B_6 deficiency in animals, including man, is the L-tryptophan (TRP) load test (Sauberlich, 1981; Brown, 1981), although concerns about the test have been raised at times (Hughes and Raine, 1966; Schaeffer et al., 1991). This test assumes that determination of TRP metabolites in the urine, after an oral TRP load, provides an indirect measure of the vitamin B_6 nutritional status of the animal due to the fact that quite a few enzymes require pyridoxal 5′-phosphate (PLP) as their cofactor for the metabolism of TRP. However, the TRP load test might suffer from an inherent defect due to the possible nonenzymatic formation of a cyclic compound from TRP and pyridoxal (PL) [3-carboxy-1-pyridoxyl-1,2,3,4-tetrahydro-β-carboline (**1**)] or TRP and PLP [3-carboxy-1-pyridoxyl-1,2,3,4-tetrahydro-β-carboline 5′-phosphate (**2**)] (Figure 1). Thus, when excess quantities of TRP are given to animals, including man, the cofactor might be removed from some enzymes by TRP to form the cyclic compound, which in turn might inhibit certain PLP-requiring enzymes, metabolize differently from TRP and PLP, or be excreted as such in the urine of animals; in other words, the test may be interfering with the situation it was designed to investigate. There are reports in the literature for the presence of similar cyclic compounds formed from L-Dopa and PLP or PL in the liver and blood of rats (Bringmann and Schneider, 1986) and from histamine and PLP or PL in the tissues and urine of mice (Kierska et al., 1981).

The formation of a cyclic compound from TRP and PL or PLP by Pictet–Spengler condensation has been implied through evidence from biological experiments (Snell, 1945, 1981) and spectrophotometric data (Schott and Clark, 1952) quite a few years ago. However, to our knowledge, the synthesis of these compounds has not been reported, although there is a report (Bringmann et al., 1987) of a high-performance liquid chromatographic (HPLC) method for the determination of pyridoxyl alkaloids, among them compound **1**.

† A preliminary report of this work was presented at the 202nd National Meeting of the American Chemical Society, New York, NY, Aug 25–30, 1991; Abstract BIOL 80.

In this paper, the *in vitro* synthesis of compounds **1** and **2** under physiological conditions, pH 7.0 and 37 °C, is presented as well as their ultraviolet absorption spectra and their HPLC separation. This information will be helpful to researchers investigating the possible presence of these compounds in biological fluids, their possible toxicity, or inhibition of certain PLP-requiring enzymes.

MATERIALS AND METHODS

Chemicals. L-Tryptophan, pyridoxal hydrochloride, and pyridoxal 5′-phosphate were obtained from Sigma Chemical Co. All other chemicals used were certified ACS grade.

Instruments. Ultraviolet absorption spectra were taken with a Hitachi Model U-2000 spectrophotometer. The HPLC system and experimental conditions have been reported (Argoudelis, 1988). For this work the fluorescence detector was connected in series with an ISCO Model V^4 variable-wavelength detector. High-resolution mass spectra (FAB) were taken with a 70-SE-4F mass spectrometer (VG Instruments Inc.) in 3% ammonium hydroxide solution.

Synthesis of 3-Carboxy-1-pyridoxyl-1,2,3,4-tetrahydro-β-carboline (1). TRP (0.5040 g, 2.468 mmol) was dissolved with some heating in 100 mL of 0.1 M potassium phosphate buffer, pH 7.0, and cooled to room temperature. To this solution was added 0.5032 g (2.472 mmol) of PL hydrochloride, stirred and placed at 37 °C. Precipitation of fluffy white needles was observed within 45 min. The suspension was left overnight. The precipitate was filtered under vacuum, washed a few times with water, and dried at room temperature. The weight of the dried precipitate was 0.4540 g (47.3% yield). The filtrate was acidified to pH 5.8 and left at room temperature overnight. The next day a second crop of precipitate was collected by filtration under vacuum, washed a few times with water, and air-dried, wt 0.1621 g (16.9% yield). No more precipitate was formed when the yellow filtrate was acidified to pH 2.9. The cyclic compound was purified by dissolving it in 0.1 N NaOH and reprecipitating it with 1 N HCl: mp 261 °C dec; $C_{19}H_{19}N_3O_4 \cdot 2H_2O$ Calcd: C, 58.60; H, 5.95; N, 10.79. Found: C, 58.43; H, 5.91; N, 10.76. High-resolution fast atom bombardment mass spectrometry (FAB MS) gave a mass of 354.1445 for $[M + H]^+$ consistent with the structural formula.

Synthesis of 3-Carboxy-1-pyridoxyl-1,2,3,4-tetrahydro-β-carboline 5′-Phosphate (2). TRP (0.5189 g, 2.541 mmol) was dissolved, with some heating, in 100 mL of 0.1 M potassium phosphate buffer, pH 7.0, and cooled to room temperature. PLP·H_2O (0.6718 g, 2.533 mmol) was dissolved with some heating in 100 mL of the same buffer and cooled to room temperature. The two solutions were mixed and placed

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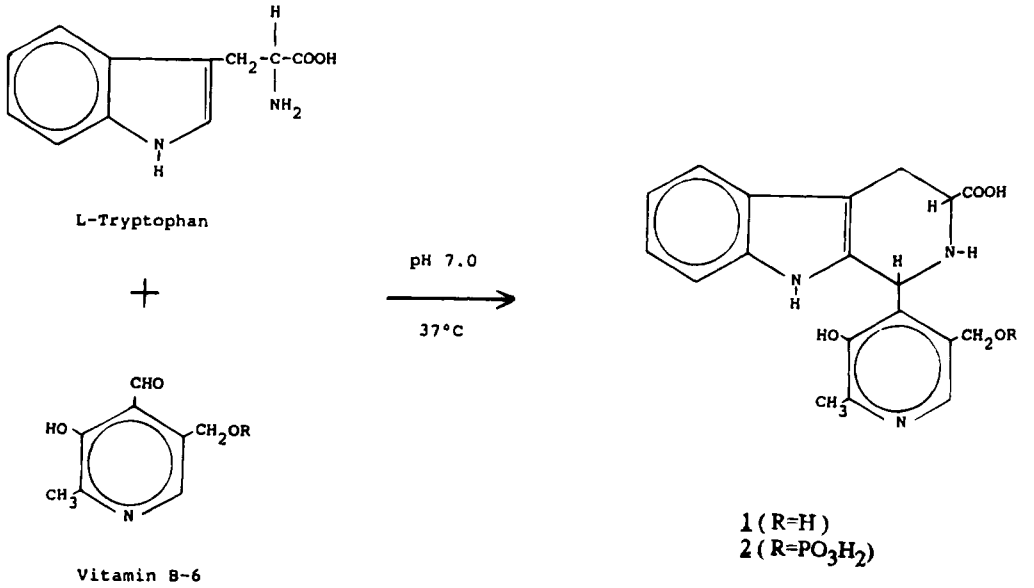

Figure 1. Synthesis and structure of cyclic compounds from TRP and PL (R = H) or PLP (R = PO_3H_2).

at 37 °C overnight. The next day, an aliquot of the solution showed almost no absorption at 390 nm in 0.1 N NaOH. A white precipitate appeared when 200 mL of water was added to the solution and the pH made to 2.0 by adding, dropwise, concentrated hydrochloric acid. The suspension was left for 2 h at room temperature and the precipitate filtered under vacuum, washed a few times with water, and dried at room temperature. The weight of the dried precipitate was 1.0273 g (88.2% yield). The cyclic compound was purified by dissolving it in 0.1 N NaOH and reprecipitating it with 1 N HCl. Finally, it was recrystallized from water: mp 215 °C dec.; $C_{19}H_{20}N_3O_7P{\cdot}1.5H_2O$ Calcd: C, 49.57; H, 5.04; N, 9.13. Found: C, 49.37; H, 5.10; N, 9.09. FAB MS gave a mass of 434.1117 for $[M + H]^+$, consistent with the structural formula.

All experiments of this study were carried out under conditions of subdued light.

RESULTS AND DISCUSSION

The synthesis of compounds **1** (R = H) and **2** (R = PO_3H_2) is presented in Figure 1. The Pictet–Spengler reaction, as with other uncatalyzed bimolecular reactions, requires that the reactants, or at least one of them, be present in high concentrations for the reaction to proceed at a significant rate (Whaley and Govindachari, 1951). In these experiments, when the concentrations of TRP and PLP in 0.1 M phosphate buffer and at room temperature were both 0.1 mM, the rate of cyclization to form compound **2** was extremely slow. However, when the concentration of TRP was increased to 2 mM under the same conditions, the reaction was fairly fast, with half of PLP forming the cyclic product in about 4 h. The reaction was still faster, as expected, when the concentrations of both TRP and PLP were 12.5 mM under the conditions reported under Materials and Methods; half of PLP was converted to the cyclic compound in less than 1 h. Compound **2** was formed even when PLP was preincubated with twice its concentration with L-lysine (25 mM) before the addition of TRP under the same conditions reported under Materials and Methods. However, in that case the rate of cyclization was approximately half of that without the L-lysine. This observation means that PLP could be displaced from the ϵ-amino group of lysine where it is attached through a Schiff base to the proteins (or enzymes) and form the cyclic compound if TRP is present in larger than usual concentrations as during the TRP load test.

Table 1. Ultraviolet Absorption Maxima and Molar Absorptivities [nm (ϵ)]

compd	0.1 N HCl	pH 7.0	0.1 N NaOH
1	281 (9020)	251 (7820)	282 (7630)
	287 (9390)	272 (6920)	289 (7860)
		279 (7070)	310 (9250)
		323 (6230)	
2	281 (8160)	251 (7620)	291 (7710)
	287 (8240)	278 (6280)	304 (7760)
		325 (7010)	

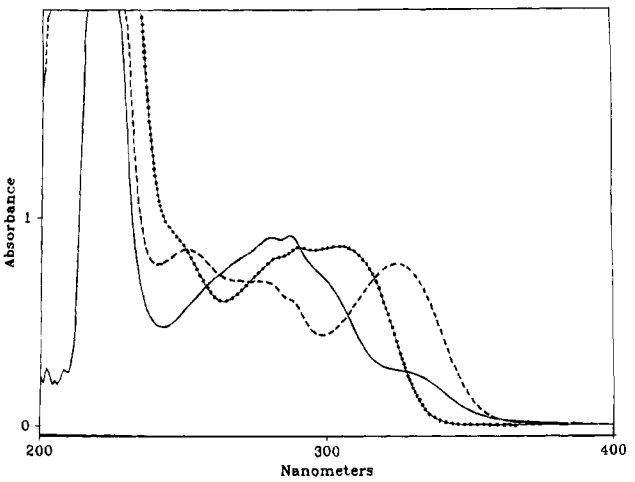

Figure 2. Ultraviolet absorption spectra of compound **2** in 0.1 N HCl (continuous line), 0.1 M phosphate buffer, pH 7.0 (dashed line), or in 0.1 N NaOH (dotted line).

The lower yield in the synthesis of **1** compared to that of **2** could be explained as being due to the fact that near pH 7.0, PL is present almost entirely in its hemiacetal form (Heyl et al., 1951), while PLP exists in a free aldehyde form which can form easily a Schiff base with TRP and then cyclize. In Table 1 are reported the absorption maxima and the molar absorptivities of compounds **1** and **2**, while in Figure 2 are presented the ultraviolet absorption spectra of compound **2**; those of compound **1** were very similar (see Table 1).

The progress of the cyclization reaction could be followed either spectrophotometrically by measuring the reduction of absorbance at 389 nm (disappearance of PLP) of an aliquot of the reaction mixture in 0.1 M NaOH or by HPLC. For HPLC, the fluorescence detec-

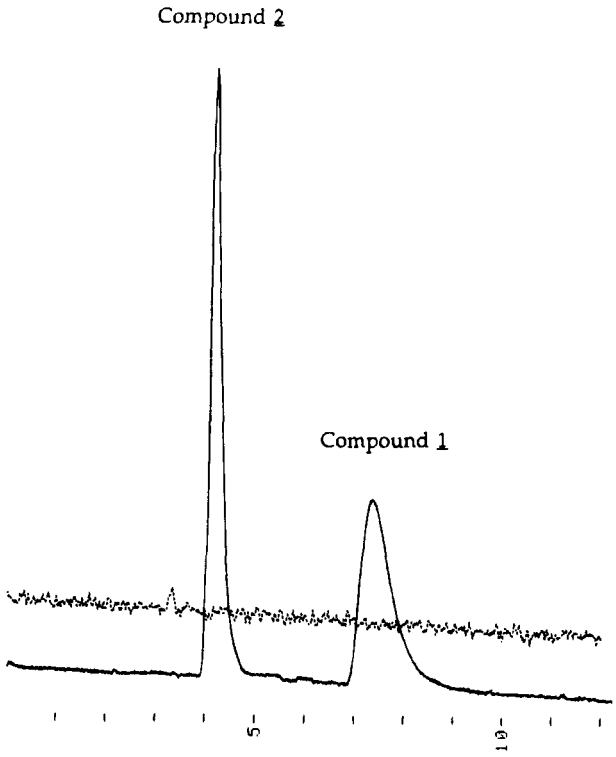

Figure 3. Chromatographic tracing of compound **1** (second peak) and compound **2** (first peak): ultraviolet detector at 326 nm (solid line); fluorescence detector, excitation 270, 290, or 326 nm, emission 389 nm, long pass cutoff filter (dotted line). The column was 250 × 4.6 mm i.d. Partisil-10SCX and mobile phase 0.1 M $NH_4H_2PO_4$ at a flow rate of 1 mL/min.

tor was set at 290 nm excitation wavelength and emission was measured using a 389 nm long pass cutoff filter (measurement mainly of TRP and to much lesser extent of PLP) while the ultraviolet detector was set at 326 nm (measurement of cyclic compound and PLP). As the cyclization proceeded, the fluorescence peak of TRP became smaller while a new peak, with a retention time slightly longer than that of PLP, appeared in the ultraviolet HPLC tracing. There is chromatographic evidence that two cyclic isomers (cis and trans) were formed from TRP and PLP, one in very small quantity. When an aliquot from the filtrate of the purification step of compound **2** was injected into HPLC (ultraviolet detector set at 326 mm), in addition to the peak for compound **2** another, much smaller, peak with a little longer retention time appeared. Since this small peak and, of course, the peak for compound **2** were not present in the fluorescence tracing (exc 290 nm), it is believed that the peak is an isomer (cis/trans) of compound **2**. If the small peak were TRP, PLP, or an acyclic derivative of them it should have been present in the fluorescence tracing. No attempt was made to characterize the stereochemical form of the isolated compound **2**. Under the conditions used for HPLC, neither of the cyclic compounds fluoresces (Figure 3) when the excitation wavelength is set at 270, 290, or 326 nm absorption maxima for TRP, PLP, or cyclic compounds, respectively. This observation seemed to be unusual since both TRP and PL fluoresce and PLP fluoresces but with much less intensity under the same HPLC conditions. The presence of the carbonyl group in PLP reduces its fluorescence intensity. However, the cyclic compounds **1** and **2** do not have a carbonyl group, and it was thought that they should fluoresce. To verify the absence of fluorescence of the synthesized cyclic compounds, the cyclic compound from TRP and acetaldehyde was made following the method reported in the literature (Brossi et al., 1973). This cyclic compound, however, showed a strong fluorescence peak (Figure 4) when the excitation wavelength was set at 290 nm. It has been reported (Raso and Stollar, 1973) that the fluorescence intensity of the cyclic compound formed from 3-aminotyrosine and PLP was only $^1/_{10}$ that of the noncyclized analog. The fluorescence of compound **1** or **2**, if they fluoresce, might depend on the pH, as was the case of the cyclic compound from 3-aminotyrosine and PLP.

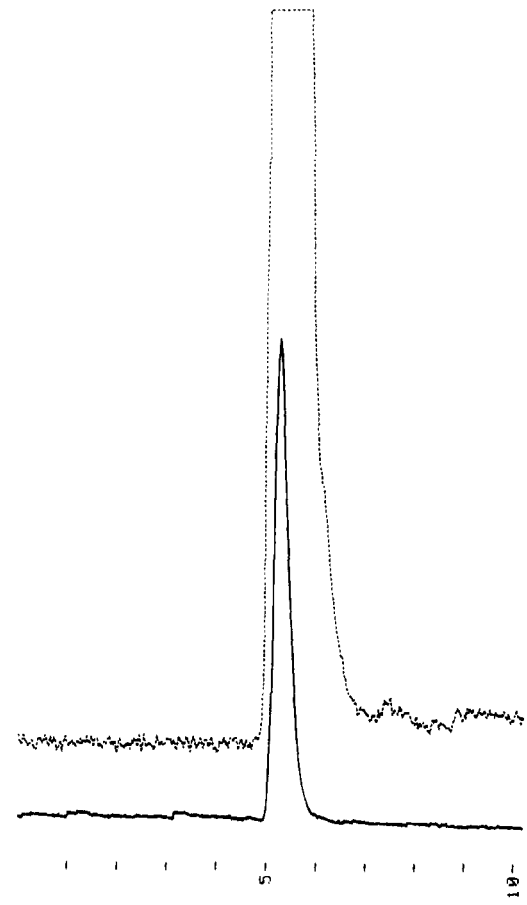

Figure 4. Chromatographic tracing of the cyclic compound made from TRP and acetaldehyde. Conditions were the same as in Figure 3 except that the ultraviolet detector was set at 290 nm (solid line) and the fluorescence detector was set at an excitation wavelength of 290 nm (dotted line). Both detectors were set at the same sensitivity.

The synthesized cyclic compounds **1** and **2** were stable for 2 days in 0.1 M NaOH, 0.1 M potassium phosphate, pH 7.0, and 0.1 M HCl (no changes in their ultraviolet absorption spectra); even after 2 months their ultraviolet absorption spectra did not change appreciably. The stability of a similar cyclic compound formed from histamine and PL was recently reported (Mathews and Manohar, 1992).

Very recently, it was shown (Schaeffer et al., 1991) that the TRP load test was not useful in detecting marginal vitamin B_6 intake in rats. Previously, it was reported (Schaeffer, 1988) that repeated TRP load test, in food-restricted rats, affected growth and feed efficiency, and the possibility was raised that TRP loading actually aggravated vitamin B_6 deficiency. Undesirable side effects, such as drowsiness, nausea, and a reduction in food intake, to a dose of TRP have been reported in humans (Babcock et al., 1960; Smith and Prockop, 1962; Hrboticky et al., 1985). Could all of these results be

due to the formation of cyclic compounds from TRP (or its metabolites) and PLP or PL? The *in vivo* formation, in animals, of similar cyclic compounds has been reported from PLP and L-Dopa (Bringmann and Schneider, 1986) or PLP and histamine (Kierska et al., 1981) when large quantities of L-Dopa or histamine were given to the animals. In both cases, the *in vitro* rate of formation of the cyclic compounds was approximately 20 times faster than that of TRP and PLP when equimolar quantities of the two reactants were used (Schott and Clark, 1952). In human plasma, under normal conditions, the concentration of PLP is very low (about 50 nM); however, that of TRP is about 3 orders of magnitude higher [e.g., Kang-Yoon and Kirksey (1992)] and during the TRP load test it will be much higher. Therefore, the possibility for the Pictet–Spengler reaction (cyclization of the Schiff base formed from TRP and PLP) to proceed *in vivo* increases since one of the reactants (TRP) will be present in fairly high concentrations. Also, the concentration of the other reactant (PLP) increases in blood when excess vitamin B_6 is given to animals. The possibility also exists that in certain animal tissues the concentration of TRP (or some of its metabolites) as well as of PLP might be higher than that in blood. Unfortunately, the fact that the cyclic compound **1** or **2** does not fluoresce under the HPLC conditions reported here makes their detection, with an ultraviolet detector, difficult (lower limit of detection of about 4 ng at a 3:1 signal to noise ratio). Development of more sensitive methods for the detection of these compounds might be necessary before one excludes the possibility of their formation *in vivo*.

It should be mentioned that the contaminant in a commercial batch of TRP believed to have caused eosinophilia-myalgia syndrome (EMS) in quite a few persons, a few years ago, was the condensation product resulting from two TRP molecules and one acetaldehyde molecule, with the methine bridge coupling the two TRP molecules across the indole nitrogens (Mayeno et al., 1990).

ABBREVIATIONS USED

Pyridoxal, PL; pyridoxal 5′-phosphate, PLP; L-tryptophan, TRP; high-performance liquid chromatography, HPLC.

ACKNOWLEDGMENT

This study was a part of Project 50-0330 of the Agricultural Experiment Station, College of Agriculture, University of Illinois at Urbana–Champaign. High-resolution (FAB) mass spectra were obtained in the Mass Spectrometry Laboratory, School of Chemical Sciences, University of Illinois. The 70-SE-4F mass spectrometer was purchased in part with funds from the National Institute of General Medical Sciences (GM 27029).

LITERATURE CITED

Argoudelis, C. J. Simple isocratic high-performance liquid chromatographic method for the separation of the six vitamers of vitamin B_6. *J. Chromatogr.* **1988**, *424*, 315–323.

Babcock, M. J.; Brush, M.; Sostman, E. Evaluation of vitamin B_6 nutrition. *J. Nutr.* **1960**, *70*, 369–376.

Bringmann, G.; Schneider, S. The first evidence of endogenous pyridoxal isoquinoline alkaloids in mammalian organisms. *Angew. Chem., Int. Ed. Engl.* **1986**, *25*, 177–178.

Bringmann, G.; Schneider, S.; Richter, K. D. HPLC Analysis for highly polar pyridoxyl Alkaloids in mammalian blood: endogenous alkaloids in humans. *Fresenius' Z. Anal. Chem.* **1987**, *329*, 480–484.

Brossi, A.; Focella, A.; Teitel, S. Alkaloids in mammalian tissues. 3. Condensation of L-tryptophan and L-5-hydroxytryptophan with formaldehyde and acetaldehyde. *J. Med. Chem.* **1973**, *16*, 418–420.

Brown, R. R. The tryptophan load test as an index of vitamin B_6 nutrition. In *Methods in Vitamin B_6 Nutrition: Analysis and Assessment*; Leklem, J. E., Reynolds, R. D., Eds.; Plenum Press: New York, 1981.

Heyl, D.; Luz, E.; Harris, S. A.; Folkers, K. Phosphates of the Vitamin B_6 Group. I. The Structure of Codecarboxylase *J. Am. Chem. Soc.* **1951**, *73*, 3430–3433.

Hrboticky, N.; Leiter, L. A.; Anderson, G. H. Effects of L-tryptophan on short term food intake in lean men. *Nutr. Res.* **1985**, *5*, 595–607.

Hughes, P. A. M.; Raine, D. N. In vivo formation of pyridoxal phosphate Schiff's base-An inherent defect in the tryptophan load test. *Clin. Chim. Acta* **1966**, *14*, 399–402.

Kang-Yoon, S. A.; Kirksey, A. Relation of short-term pyridoxine-HCl supplementation to plasma vitamin B_6 vitamers and amino acid concentrations in young women. *Am. J. Clin. Nutr.* **1992**, *55*, 865–872.

Kierska, D.; Sasiak, K.; Maslinski, C. In vivo formation of histamine phosphopyridoxal cyclic compounds. *Agents Actions* **1981**, *11*, 28–32.

Mathews, I. I.; Manohar, H. Non-enzymatic reactions involving vitamin B_6: Spectroscopic investigations on the effect of pH and metal ions on the decyclization reactions of the pyridoxal-histamine cyclized Schiff base. *J. Inorg. Biochem.* **1992**, *46*, 259–265.

Mayeno, A. N.; Lin, F.; Foote, C. S.; Loegering, D. A.; Ames, M. M.; Hedberg, C. W.; Gleich, G. J. Characterization of "Peak E" a novel amino acid associated with Eosinophilia-Myalgia Syndrome. *Science* **1990**, *250*, 1707–1708.

Raso, V.; Stollar, B. D. Antibodies specific for conformationally distinct coenzyme-substrate transition state analogs. A fluorescence, nuclear magnetic resonance, circular dichroism, and antibody study of N-(5-phosphopyridoxyl)-3′-amino-L-tyrosine. *J. Am. Chem. Soc.* **1973**, *95*, 1621–1628.

Sauberlich, H. E. Vitamin B_6 status assessment: Past and present. In *Methods in Vitamin B_6 Nutrition: Analysis and Assessment*; Leklem, J. E., Reynolds, R. D., Eds.; Plenum Press: New York, 1981.

Schaeffer, M. C. Detrimental effects of repeated administration of the tryptophan-load test for vitamin B_6 status on deficient and pair-fed control rats. *Nutr. Res.* **1988**, *8*, 1307–1318.

Schaeffer, M. C.; Sampson, D. A.; Skala, J. H.; O'Connor, D. K.; Gretz, D. Insensitivity of the tryptophan-load test to marginal vitamin B_6 intake in rats. *J. Nutr.* **1991**, *121*, 1627–1634.

Schott, H. F.; Clark, W. G. Dopa decarboxylase inhibition through the interaction of coenzyme and substrate. *J. Biol. Chem.* **1952**, *196*, 449–461.

Smith, B.; Prockop, D. J. Central-nervous-system effects of ingestion of L-tryptophan by normal subjects. *New Engl. J. Med.* **1962**, *267*, 1338–1341.

Snell, E. E. The vitamin B_6 group V. The reversible interconversion of pyridoxal and pyridoxamine by transamination reactions. *J. Am. Chem. Soc.* **1945**, *67*, 194–197.

Snell, E. E. Vitamin B_6 analysis: Some historical aspects. In *Methods in Vitamin B_6 Nutrition: Analysis and Assessment*; Leklem, J. E., Reynolds, R. D., Eds.; Plenum Press: New York, 1981.

Whaley, W. M.; Govindachari, T. R. The Pictet-Spengler synthesis of tetrahydroisoquinolines and related compounds. *Org. React.* **1951**, *6*, 151–190.

Received for review April 21, 1994. Accepted August 29, 1994.[⊗]

[⊗] Abstract published in *Advance ACS Abstracts,* October 15, 1994.