reasons discussed above and because the evidence is strong that other organic inhibitors of carbonic anhydrase such as carboxylic acids[26] and aromatic thiols[27] react with the enzyme in their anionic form, we favor the hypothesis that sulfonamide–carbonic anhydrase formation occurs as illustrated by eq 9.

As suggested earlier,[8] the mechanism of carbonic anhydrase–sulfonamide complex formation appears to be similar to that for the binding of anionic ligands to Zn(II) in inorganic complexes.[28] According to this hypothesis, rapid pre-equilibrium formation of an outer-sphere complex between the sulfonamide anion and the active site zinc ion is followed by the loss of zinc-bound water in the rate-determining step and finally the entry of the sulfonamide anion into the inner sphere of the metal ion. The variation in the association rate constants for sulfonamide–carbonic anhydrase complex generation with structural changes in the inhibitor might seem at first to be somewhat greater than would be expected if this mechanism holds. However, the ligand geometry of the metal ion in the enzyme is different than it usually is in inorganic complexes,[4] and this may affect the observed rate behavior. Also, the outer-sphere association constants may be more variable than those for inorganic anion–Zn(II) complex formation because of substantial variations in the hydrophobic interactions of the enzymatic binding site and the aromatic or heteroaromatic rings present in the sulfonamide inhibitors.[22]

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Acknowledgments. This research was partially supported by a grant and a Postdoctoral Fellowship (S. F. B.) from the National Institute of General Medical Sciences. Support by an Alfred P. Sloan Foundation Fellowship (E. T. K.) is gratefully acknowledged.

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

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Abstract: Two different haptens have been constructed to resemble the Schiff base intermediate of pyridoxal phosphate dependent enzymes which utilize the substrate tyrosine. The synthesis of *N*-(5-phosphopyridoxyl)-3′-amino-L-tyrosine (II) and its cyclized derivative **III** was accomplished, and certain aspects of their conformation have been elucidated by spectroscopic means. Compound II is a flexible molecule which, in aqueous solution, behaves as though it is in an extended form. The detection of coupling interactions between its aromatic rings indicates that the molecule spends some time in a folded or stacked arrangement. Analog III is covalently locked into a compact configuration, with its tyrosyl and pyridoxyl rings in perpendicular apposition. These conformationally distinct haptens each evoked an antibody response specific for both the phosphopyridoxyl and tyrosine regions. Antibody directed against the unhindered form II reacted tenfold more effectively with it than with its cyclic counterpart III. Antibody specific for the cyclic molecule bound it 10 times more tightly than the extended compound II. The antibody obtained in response to these specifically designed haptens may provide valuable insight by simulating the effects of enzyme active sites.

The reversible formation of specific antibody–hapten complexes has long provoked analogy to the enzyme–substrate intermediate required for enzyme catalysis. Indeed, certain similarities are seen to exist when both of these ligand–protein interactions are examined at the level of their fundamental rate constants of complex formation and dissociation.[1,2] Most haptens used to elicit antibody response, however, bear little resemblance to enzyme substrates as they might appear while undergoing catalysis. Comparisons between the resulting antihapten antibodies and enzymes are thereby severely restricted. The nature of an antibody combining site is determined by the chemical and steric features of the hapten employed to initiate a given response.[3–5] We decided to specifically design a hapten for the purpose of eliciting antibody sites with binding properties characteristic of enzyme active sites. An attempt to select and synthesize such a molecule was therefore undertaken.

Schiff bases I formed by the condensation of amino

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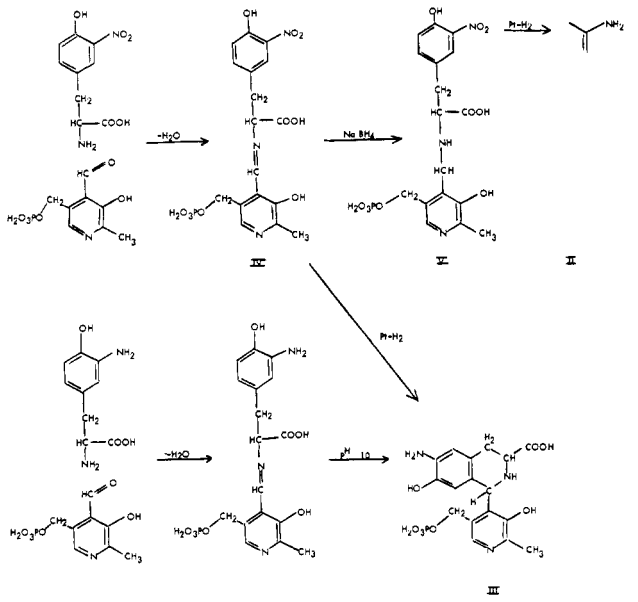
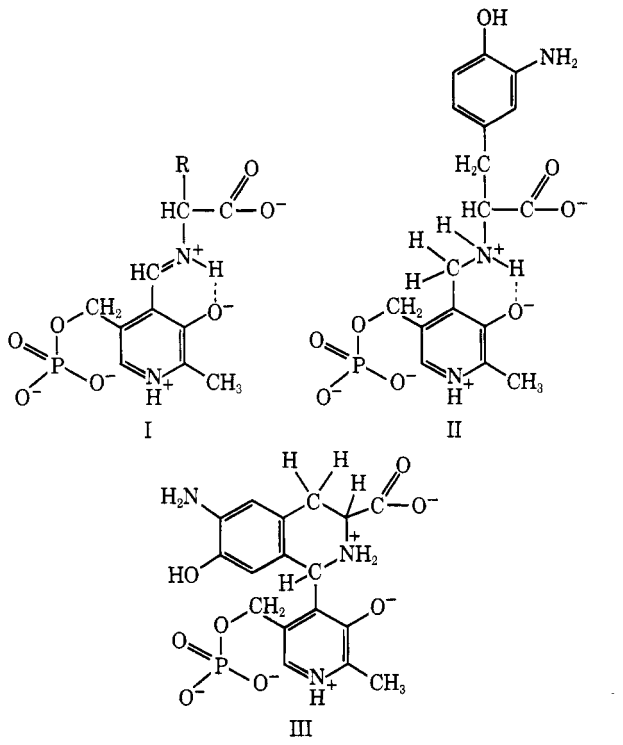


Figure 1. Scheme for the synthesis of *N*-(5-phosphopyridoxyl)-3′-amino-L-tyrosine (II), and its cyclized analog III.



acids and pyridoxal 5-phosphate (PLP) are known to occur as covalent reaction intermediates during catalysis by PLP-dependent enzymes.[6-9] Simple reduction of these imines provides a stable analog of the various coenzyme–substrate complexes. The fact that each resultant *N*-(5-phosphopyridoxyl)amino acid is a good inhibitor of its respective enzyme suggests that they conform to and fill both the coenzyme and specific amino acid binding sites.[10-13]

N-(5-Phosphopyridoxyl)-3′-amino-L-tyrosine (II) belongs to this class of compounds and forms the basic structure of our hapten. Inhibition experiments with various tyrosine-utilizing PLP enzymes indicate that both the phosphopyridoxyl portion as well as the tyrosyl half of the molecule contribute to its overall effectiveness.[13] Attachment to a macromolecule is a prerequisite for small compounds to be effective in stimulating production of antibodies. A strategically placed 3′ amino group was incorporated into the molecule to facilitate its linkage to a protein carrier. Reduction of the imine and the introduction of a 3′ amino function to the tyrosine ring would be expected to decrease similarity to the PLP–tyrosine Schiff base. However, the resemblance is close enough so that our analog should induce antibody which would bind and specifically interact with the tyrosine Schiff base. In addition to a molecule with freely moving portions, II, a restrained cyclic version, III, was also synthesized. This molecule, and the antibody specific for it, are of particular interest in view of recent speculations concerning the role of strain in enzyme catalyses.[14]

In this article, we present the synthesis and the chemical and conformational characterizations of the two haptens. Through extrapolation of these properties, one can hypothesize about the spatial orientation and chemical nature of the complementary binding site. It is equally beneficial to explore various means of studying the analogs so that each may be monitored while bound to and interacting with its specific antibody. The report also includes a preliminary description of the specificity of the two distinct types of antibody induced by the different analogs. A more detailed analysis of these antibodies and a comparison with previously described antibodies to pyridoxal and PLP[15] will be presented elsewhere.

Results and Discussion

Synthetic Design of the Hapten. The synthesis of various *N*-(5-phosphopyridoxyl)-L-amino acids, including the tyrosine derivative, has been described by Ikawa.[16] Attachment of this hapten to a protein carrier should ideally leave the entire coenzyme portion and the amino acid side chain region as well as most of the tyrosine ring structure unaffected and freely exposed. Addition of an amino group at the distal 3′ position was chosen to avoid disturbing the conformation and charge density of critical regions in the molecule. Eventual covalent attachment to protein could then be accomplished by diazo coupling of the amine or by forming a carbodiimide facilitated amide linkage.

In order to synthesize II, a two-step sequential method of reduction was employed (Figure 1). An alkaline aqueous solution containing equimolar quantities of 3-nitrotyrosine and PLP forms Schiff base IV. Subsequent addition of $NaBH_4$ reduces the imine but leaves

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(11) J. H. Fellman and E. S. Roth, *Biochemistry*, **10**, 408 (1971).
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(14) D. C. Phillips, *Proc. Nat. Acad. Sci. U. S.*, **57**, 484 (1967).
(15) (a) F. Córdoba, C. González, and P. Rivera, *Biochim. Biophys. Acta*, **127**, 151 (1966); (b) H. Ungar-Warron and M. Sela, *ibid.*, **124**, 147 (1966).
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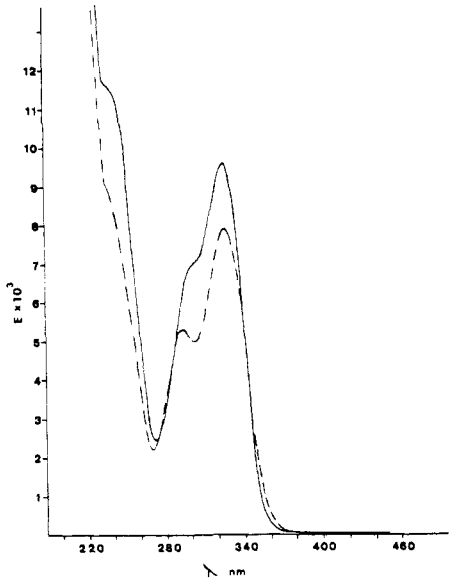


Figure 2. Ultraviolet absorption spectra of compound II (-----) and compound III (——).

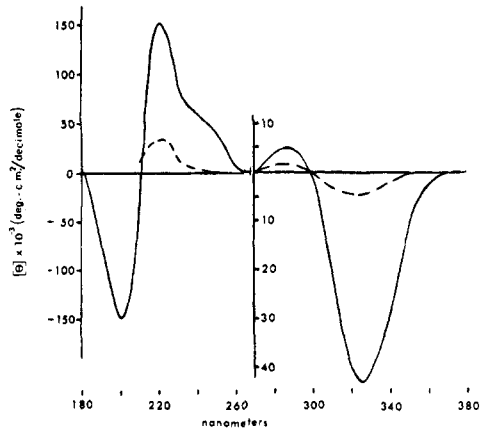


Figure 3. Circular dichroism spectra of compound II (-----) and compound III (——).

the NO_2 group unaffected. Purification of V followed by reduction of its NO_2 group with H_2 and Pt yields the desired aromatic amine II.

Another approach to synthesize II also involved preliminary condensation of 3-nitrotyrosine and PLP (final pH at ~8) to form Schiff base IV. Upon direct hydrogenation with platinum catalyst, sequential reduction first of the imine and then of the NO_2 group was anticipated. Less than theoretical hydrogen uptake as well as a low hydrogen analysis of the crystallized amine raised questions regarding the nature of the product. The possibility of intramolecular condensation was advanced in direct analogy to the known spontaneous cyclization of 3-hydroxytyrosine with PLP.[11,17] Thus, if hydrogenation first effected reduction of the 3-NO_2 → 3-NH_2, the alkaline conditions would activate the 6′ position and facilitate cyclization. To demonstrate that this was feasible and to confirm the identity of the cyclic derivative, an unambiguous method of synthesis was employed. A mixture of 3-aminotyrosine and PLP under alkaline conditions was found to spontaneously lose its 420-nm Schiff base absorbance and gain a 325-nm peak characteristic of the saturated or substituted imine (Figure 1). Upon crystallization this product was shown to be identical, in every respect, with the single-step reduction product and distinct from the two-step compound II.

Ultraviolet Absorption Analysis. Figure 2 shows a comparison of the uv spectra of compound II and its closely related cyclic analog III. The former molecule displays a spectrum characteristic of its two unsaturated ring systems, a 325-nm maximum due to the pyridoxyl portion and a 295-nm peak resulting from the amino substituted tyrosine (λ_{max} 288). A shoulder in the 240-nm region is attributable to the 250- and 230-nm absorbance of coenzyme and aminotyrosine, respectively.

Compound II is a composite molecule and while there are no wavelength shifts, the intensities of the observed absorptions differ from a computed composite spectrum of pyridoxamine phosphate (PMP) and 3-aminotyrosine. A small decrease in the extinction coefficient at 325 nm and a concomitant increase in the extinction coefficient at 288 indicates some interaction between the transition dipoles of the two chromophore halves.

The absorption spectrum of the cyclic compound III deviates from a simple cumulative description. Ring closure provides an additional substitution and effects a red shift for the aminotyrosine chromophore. This displaced absorbance appears as a 300–305-nm shoulder on the 325-nm pyridoxyl peak. The molar extinction at 325 nm is significantly greater than that for PMP and the linear analog II.

Circular Dichroism. It is often possible to obtain useful correlations between the structure and intrinsic optical activity of different molecules. Thus, to augment the uv data, an examination of the circular dichroism of the linear and cyclic compounds and their constituent halves, PMP and 3-amino-L-tyrosine, was undertaken.

Within the limits of the instrumentation, no optical activity could be assigned to free PMP. 3-Amino-L-tyrosine, containing the α assymetric center, displayed optically active transitions in the 285- and 215-nm regions.

Molecule II possesses similar positive 285- and 220-nm bands; however, their magnitude is three–four times greater than those of the free amino acid (Figure 3). The L configuration of the amino acid moiety seems to have been retained in the new compound. In addition, there is a strong negative 322-nm peak attributable to its pyridoxyl region. Thus, covalent combination has bestowed an increased dissymetry to both regions of the composite molecule. Such increases are not deemed inordinate for this type of connection, but may represent transient intramolecular hydrogen bonding as depicted in II.

Indication of imposed conformational restraint and assymmetry is provided by the overall five- or tenfold greater optical activity of the cyclic molecule III compared with its unhindered counterpart II (Figure 3). In addition to previously denoted bands, a large negative peak is observed at 200 nm and a positive shoulder appears in the 240-nm region.

Inspection of a Corey–Pauling–Koltun (CPK) molecular model reveals compound II as having many possible modes of intramolecular movement. The two

(17) H. F. Schott and W. G. Clark, *J. Biol. Chem.*, **196**, 449 (1952).

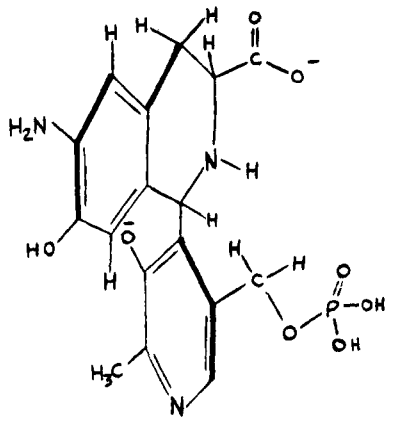


Figure 4. Schematic representation of the conformation of the cyclized molecule III.

rings can be oriented in a number of positions varying from a close contact stacked arrangement to an elongated form similar to the structure depicted in II.

Upon constructing a molecular model of the cyclized compound IV, one readily detects steric restrictions of its conformation. Rotation about the 4 position is prevented by adjacent 3-OH and 5-CH_2–R substituents. The ring systems are thus constrained into perpendicular apposition as pictured in Figure 4.

Proton Magnetic Resonance Spectroscopy. A comprehensive nmr study of *N*-(5-phosphopyridoxyl)-3′-aminotyrosine and its cyclic counterpart was undertaken. Correlations were made with the spectra of molecules corresponding to the constituent halves of both II and III.

The first spectrum in Figure 5 shows the proton resonances of 3-amino-L-tyrosine at a slightly basic pH in D_2O. Aromatic ring hydrogens, as well as those of the amino acid side chain, are clearly discernible.[18,19] Spectra of PMP in neutral aqueous solution have been described[20] (Figure 5b). Comparisons show that the signals arising from II (Figure 5c) are a composite of those from its coenzyme and aminotyrosine halves. Slight displacements were noted ($\leq$0.1 ppm) but these seem attributable, in part, to the small variance in pH for the different samples. Free PMP displays a 4.27-ppm singlet as a result of its two equivalent 4-CH_2 protons.[20] In compound II, however, they appear as a doublet centering at 4.21 and 4.27 ppm. Hindered internal rotation or more likely proximity to the asymmetric L-amino acid center has rendered them nonequivalent. Resonance from the 5-CH_2 position is masked by the HDO peak.[20]

A closer examination was performed on the signals derived from the three ring hydrogens of the aminotyrosine moiety. The multiplet of the composite molecule appears 0.1 ppm upfield compared with free 3-aminotyrosine, but the peak distribution of both multiplets is identical. The fact that this complex arrangement was not perturbed in the composite molecule is a good indication that the tyrosyl half is outside the vicinity of the coenzyme portion of the molecule.

(18) "Varian High Resolution NMR Spectra Catalog," Vol. 2, Varian Associates, Palo Alto, Calif., 1963, p 534.
(19) E. H. Abbott and A. E. Martell, *J. Amer. Chem. Soc.*, **92**, 1754 (1970).
(20) W. Korytnyk and R. P. Singh, *ibid.*, **85**, 2813 (1963).

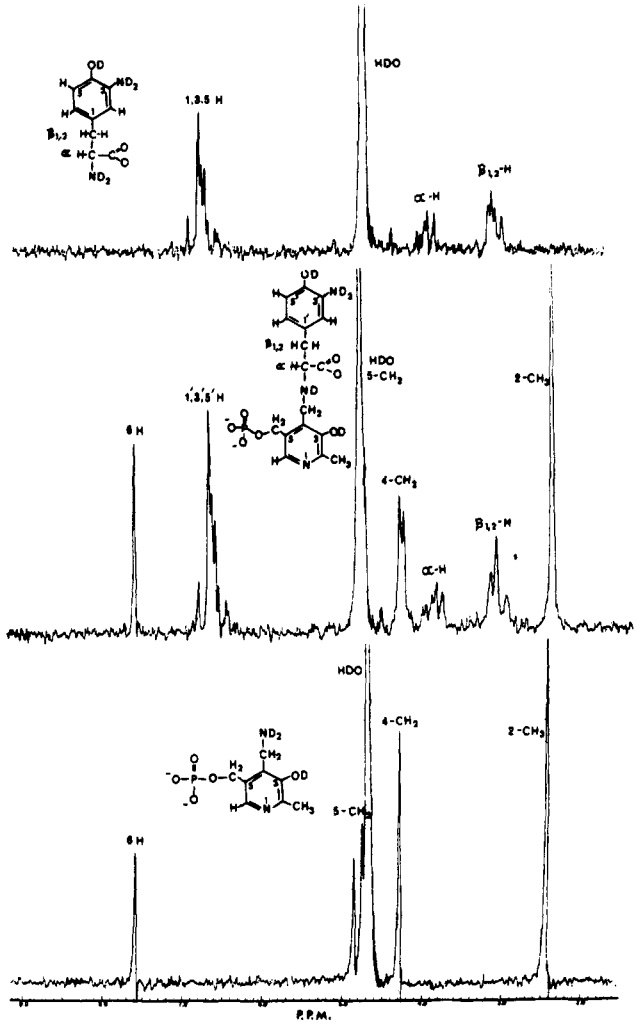
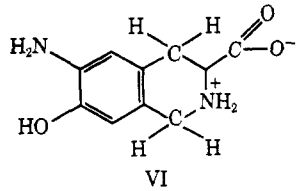


Figure 5. Nmr spectra of (a) 3-aminotyrosine, (b) *N*-(5-phosphopyridoxyl)-3′-amino-L-tyrosine, and (c) pyridoxamine phosphate. All samples in D_2O at pH 7.0–7.5

Two predictions were advanced concerning an expected nmr spectrum of the cyclic compound. The cyclization eliminates one of the aminotyrosine ring hydrogens and, of the remaining two, one sits directly over the pyridoxyl ring (Figure 4). Thus, the molecular model predicts that this 5′ hydrogen will be shielded and its signal displaced from the 6.6-ppm region assigned to aminotyrosine aromatic protons. Figure 6a shows that there are only two ring hydrogens and indicates that a shielding effect is present, but is not of great magnitude. The validity of assigning this upfield shift to diamagnetic shielding by the pyridoxyl ring was verified by analysis of VI. Formaldehyde



VI

cyclized readily with aminotyrosine in direct analogy to the spontaneous PLP reaction seen in Figure 1.[21,22] The absence of the pyridoxyl portion in this cyclic

(21) D. Heyl, S. A. Harris, and K. Folkers, *ibid.*, **70**, 3429 (1948).
(22) Wellisch, *Biochem. Z.*, **49**, 173 (1913).

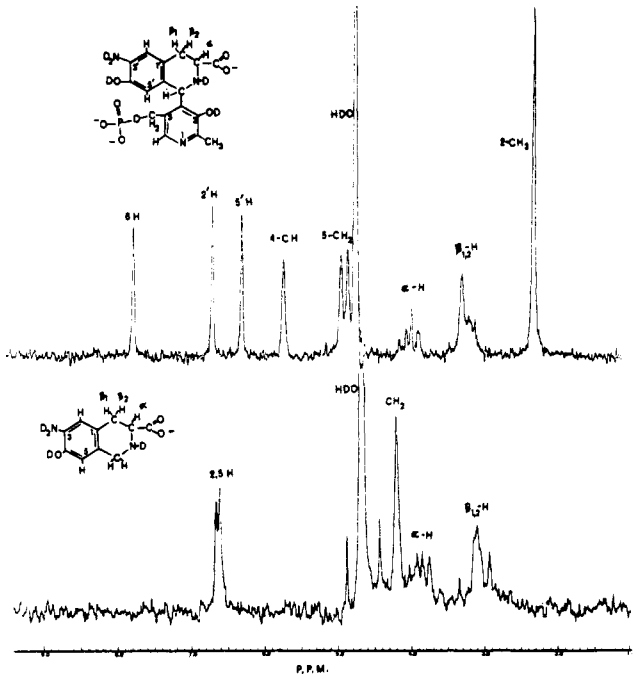


Figure 6. Nmr spectra of (a) the cyclic analog III and (b) the formaldehyde product VI. Both compounds in D_2O at pH 7.0–7.5.

molecule restores the two aminotyrosine protons to their more equivalent status (Figure 6b).

Spectrofluorimetry. The previous methods of exploring the nature of molecules II and III gave information concerning normal ground-state interactions. Fluorescence analysis monitors involvements with an excited state entity which usually acquires chemical properties quite different than those of the ground state from which it derives.

Fluorescence exitation and emission spectra of PMP, the extended compound II, and the cyclic derivative III are qualitatively similar. Aminotyrosine itself is a fluorescent compound but its specific fluorescence was not detected in either the extended or cyclic molecules.[23] The luminescence of these composite forms seems solely due to the pyridoxyl component[24] and is characterized by excitation and emission maxima at 325 and 395 nm, respectively. Favorable spectral overlap makes energy transfer from the aminotyrosine half to the coenzyme portion feasible; however, excitation curves show no sign of this occurring.

The quantitative nature of the pyridoxyl fluorescence of compounds II and III is dependent on pH (Figure 7) while the emissions of PMP, of the *N*-(5-phosphopyridoxyl)-L-tyrosine analog (containing no 3′-NH_2 group), and of a 3′ N-acetylated derivative of II remain relatively constant in the pH 3–8 range.[24,25] At its maximum, the fluorescence intensity of the extended molecule is comparable with that of equimolar PMP, but decreases with a rise in pH. The cyclic analog follows a similar pH dependence but is only one-tenth as fluorescent (Figure 7). This reversible decrease in fluorescence intensity with increasing pH is most assuredly due to the production of free 3′-amine.

(23) R. W. Cowgill, *Photochem. Photobiol.*, **13**, 183 (1971).
(24) Y. V. Morozov, N. P. Bazhulina, and M. Y. Karpeisky, *Pyridoxal Catal. Enzymes Model Syst., Proc. Int. Symp., 2nd, M.*, **35**, 53 (1968).
(25) Unpublished observations from this laboratory.

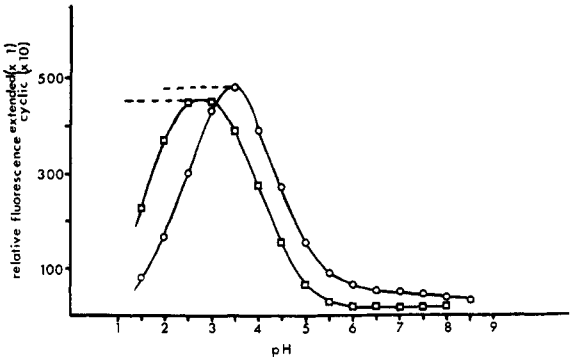


Figure 7. Relative emission of the extended molecule II (○) and its cyclic analog III (□) as a function of pH. Excitation was at 325 nm and emission was monitored at 395 nm. The values for the cyclic compound have been multiplied by 10.

Indeed, one can utilize the graph in Figure 7 to calculate a pK_a of 4.5 for the transition of the extended molecule and one of 4.1 for that of the cyclic amine. This agrees with the spectrophotometrically determined pK_a value of free 3-aminotyrosine of 4.4.[23] It is important to stress that the fluorescence of the pyridoxyl chromophore is being monitored. Its intensity, however, is modulated by the extent of ionization of the distal 3′-NH_2 moiety on the tyrosyl ring. Thus, the ground-state, electron-rich, aromatic amine region may form a charge complex with the excited state pyridoxyl ring and cause quenching. It is curious that the inherent luminescence of free 3-aminotyrosine shows a similar dependence with pH.[23]

Electrophoretic Mobility. The apparent difference in pK_a of the extended *vs.* the cyclic compound prompted an attempt to distinguish them by electrophoretic means. At pH 4.6, the extended amine II having a pK_a of 4.5 would be more in its protonated form and therefore less negative than its cyclic counterpart with a pK_a of 4.1. An artificial mixture of the two was readily separated on cellulose acetate strips at pH 4.6. At pH 4.6 both compounds moved toward the positive electrode and, as predicted, the fluorescent extended amine lagged behind the uv absorbing cyclic amine. When run at pH values of 6.5 and higher, the bands almost completely overlapped. Partial separation was still noted at pH 3.5 and 2.2 so that it is possible that other ionizations differ in that region of acidity. Electrophoretic analysis provided a means of demonstrating the purity of II and III and at no time was more than one band observed.

Immunochemistry. Protein was multisubstituted with either compound II or compound III and these antigens were subsequently injected into separate groups of rabbits. Evidence showing that hapten attachment was *via* the 3′-amino group of these molecules was obtained and will be presented in conjunction with additional immunochemical studies in a subsequent article. Antibody produced by each group was pooled and then purified by affinity chromatography utilizing the appropriate analog attached to cellulose. The two types of hapten-specific antibody thus isolated were characterized using the hapten inhibition of precipitation assay.

Divalent antibody combines with multivalent antigen to form large insoluble complexes. The ability of a molecule to inhibit this precipitation is directly related

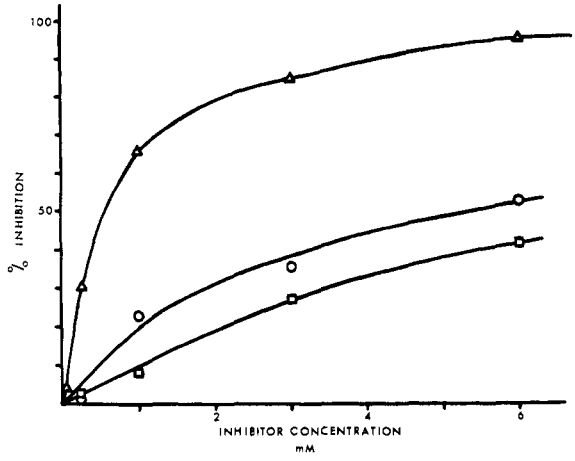


Figure 8. Inhibition of precipitation using specifically purified antibody directed against the extended hapten II. The inhibitors employed are pyridoxamine phosphate (○), the extended compound II (△), and the cyclic analog III (□).

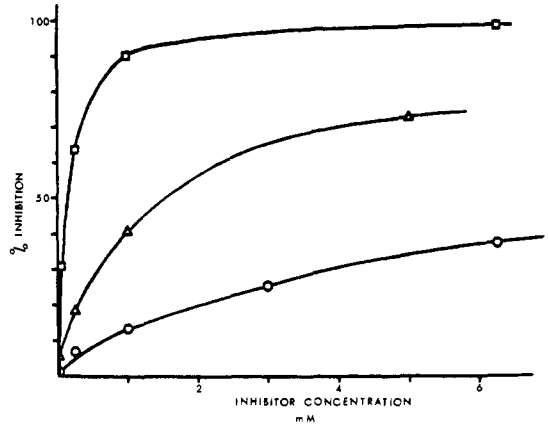


Figure 9. Inhibition of precipitation using specifically purified antibody directed against the cyclic hapten III. The inhibitors employed are pyridoxamine phosphate (○), the extended compound II (△), and the cyclic analog III (□).

to its strength of binding to the antibody sites.[26] Thus, the inhibition plot in Figure 8 shows the relative binding specificities of antibody directed against the extended hapten II. It reacts most strongly with the injected extended form and only one-tenth as well with the cyclic analog. Comparison with the inhibition curve of PMP proves useful in assessing the role of the conformationally dissimilar tyrosine regions. As expected, a substantial increase in binding over PMP is attained by the addition of the unhindered tyrosine moiety of analog II. Compound III inhibits less than PMP, indicating that the rigid perpendicular ring configuration actually reduces the binding effectiveness of its phosphopyridoxyl region.

As can be seen from Figure 9, antibody directed against the cyclic hapten III has quite different characteristics. It binds the cyclized molecule 10 times as well as it binds the extended form and approximately 70 times more strongly than PMP. Thus, in this case, the compact cyclic tyrosine region seems to contribute a greater proportion to the overall strength of binding of compound III. The fact that compound II inhibits more efficiently than PMP indicates that the flexible tyrosine portion of the extended molecule can also be utilized to increase upon the basal binding ability of its phosphopyridoxyl region. The antibody may actually strain the tyrosine region of II into a quasi-cyclic configuration to facilitate useful contact with its site and thus produce tighter binding.

Conclusion

This study of *N*-(5-phosphopyridoxyl)-3′-amino-L-tyrosine and its cyclized analog has demonstrated that some major conformational differences exist between these two otherwise very similar molecules.

All of the data accumulated for the cyclic compound are consistent with a compact structure, the two ring systems being rotationally restrained and mutually perpendicular. The molecule exhibits an anomalous ultraviolet absorption spectrum and its optical activity is greatly exaggerated. Two adjacent CD bands of opposite sign and equal rotational strength are centered at 210 nm. These provide a good indication of coupling between the two chromophore halves of the compound. The proximity of the tyrosyl and pyridoxyl moieties was further verified by nmr spectroscopy. Ring currents from the coenzyme region produce a shielding effect which displaces the 5′-tyrosyl proton. This result implies that the hydrogen must lie directly over the coenzyme region as depicted in Figure 4. Thus, the conformation anticipated from examination of the CPK model of compound III is borne out.

The conformation of the flexible molecule II is more difficult to define and in solution it probably assumes a variety of shapes. Its nmr spectrum is best interpreted as showing little or no intramolecular stacking interaction. If the tyrosyl region were stacked or otherwise in the vicinity of the pyridoxyl ring currents, its three aromatic protons would be disproportionately shifted. This type of perturbation was not detected.

The circular dichroism exhibited by compound II corresponds to its absorption bands and can be most simply construed as arising from the asymmetric α-amino acid center. Hindered internal rotation, perhaps due to hydrogen bonding, imparts increased rotational strength to both chromophore halves.

The ultraviolet absorption spectrum of compound II shows a definite coupling between the transition dipoles of its pyridoxyl and tyrosyl regions. Although this suggests a certain degree of proximity between the two chromophore halves, the nature and extent of interaction cannot be ascertained from the spectrum. Perhaps a folded or stacked configuration occurs in solution, but is not of considerable duration and is an insubstantially populated state at room temperature.

The fluorescence of the pyridoxyl portion of both the extended and cyclic molecules is modulated by the extent of ionization of the 3′-NH_2 group on their tyrosyl halves. Each is maximally fluorescent when the amine is fully protonated and the intensity diminishes as the free amino form accumulates. Since this pH-dependent quenching occurs in both the covalently fixed cyclic molecule as well as in the flexible compound, the quenching must not be dependent on a particular alignment of donor and acceptor regions.

At its optimal pH, the fluorescence intensity of the flexible molecule is comparable with free pyridoxamine

(26) L. Pauling, D. Pressman, and A. L. Grossberg, *J. Amer. Chem. Soc.*, 66, 784 (1944).

phosphate while that of the cyclic molecule attains no more than 10% of this value. Thus, in addition to the pH-dependent quenching, cyclization has in some way irreversibly limited the inherent luminescence of the pyridoxyl region of this molecule.

The antibodies elicited in response to these conformationally distinct molecules promise to be an interesting system for the study of protein–ligand interactions. It has been shown that the two types of antibodies preferentially react with the injected hapten form, but display a cross-reactivity with the related heterologous analog.

The cyclic compound has its tyrosine moiety pulled close to the pyridoxyl ring and, therefore, presents itself as a compact structure with its tyrosyl region easily accessible. It shows a 70-fold increase in reactivity with its antibody compared with free PMP. The extended molecule provides only a tenfold increase over PMP when binding to its homologous antibody. This less pronounced specificity for the tyrosyl region may be a reflection of the increased length of the extended molecule.

It will be of interest to further ascertain how the geometry of the haptens employed for immunization has influenced the topography of the antibody combining sites. The fluorescent and circular dichroic properties of compounds II and III make them suitable for utilization as site specific probes. Exploration of the sites by these means, as well as through comparative binding studies, can help define the fundamental similarities as well as the distinctions which must exist between the two types of induced antibodies.

A certain formal correlation exists between pyridoxal phosphate enzymes and these antibodies. Both bind coenzyme and show specificity for an amino acid, in this case tyrosine. Further parallels will hopefully prevail between enzymes and these antibodies and they will be tested to see if they possess any catalytic properties.

Experimental Section

General Procedures. Ultraviolet absorption measurements were taken with a Cary Model 14 spectrophotometer and circular dichroism spectra were obtained using a Jasco J-20 recording spectropolarimeter. Sodium phosphate buffer, 0.01 *M*, at pH 7.0 was employed as the medium for these measurements.

A Hitachi Perkin-Elmer R-20B 60-MHz nmr instrument was used to record ^1H spectra at 35°. $(CH_3)_3SiCD_2CD_2CO_2Na$, 0.1 *M* in D_2O, served as an internal standard. Samples were added to this solution and the pH was adjusted to 7.0–7.5 with 10 *N* NaOH. The phosphorylated derivatives were employed at concentrations of 50 mg/ml while the others, being less soluble at neutral pH, were examined at 20 mg/ml. The sample in D_2O solution was subsequently freeze-dried to remove hydrogen which had exchanged and then D_2O, 99.8%, was added to restore the original volume.

Excitation and emission spectra (uncorrected) were recorded with an Aminco Bowman spectrofluorometer and recorder. Fluorometric titrations of the various analogs were performed at a concentration of 1×10^{-5} *M* in 0.005 *M* phosphate–0.050 *M* NaCl, adjusted to the indicated values of pH, with hydrochloric acid. Similar results were obtained within a pH 2.5–7.0 range when a 0.1 *M* sodium acetate–acetic acid buffer system was utilized.

Thin layer chromatography was carried out on Eastman cellulose sheets with a fluorescent indicator. The developing solvents employed were 1-butanol–H_2O–concentrated HCl (3:2:1), and 1-butanol–H_2O–acetic acid (3:2:1). Sample migration was followed by its inherent fluorescence upon short-wave illumination. Plates were also analyzed with an aromatic amine reagent (dimethylaminobenzaldehyde, 0.5%, in isopropyl alcohol),[27] a molybdate reagent for organic and free phosphates,[28] ninhydrin, and by Gibbs reagent which detects the pyridoxyl moiety.[29] The two solvent systems and the various reagents described were employed to help assess the purity of all newly synthesized compounds.

Electrophoresis was performed on cellulose acetate strips (Shandon Scientific Co., SAE 2663) using 0.05 *M* sodium acetate–acetic acid buffer at pH 3.5, 4.6, and 6.5, glycine-HCl, 0.05 *M*, at pH 2.2, and 0.01 *M* sodium phosphate at pH 7.0 and 7.8. Samples were dissolved in the running buffer and adjusted to the proper pH before application. Voltage and running time varied for each pH value. Migration of each compound could be followed by most of the detection methods employed for chromatography.

Elemental analyses were performed by Alfred Bernhardt Mikroanaltisches Laboratorium, West Germany. The values reported for oxygen were obtained by difference.

Chemicals. Pyridoxal 5′-phosphate, pyridoxamine 5′-phosphate, and L-tyrosine were obtained from Sigma Chemical Co. 3-Nitro- and 3-aminotyrosine was purchased from K & K Laboratories or synthesized as described by Greenstein and Winitz.[30] Carboxylic cation exchange resins, Amberlite XE-64 and CG-50 (200–400 mesh), were products of Rohm and Haass Co., Philadelphia, Pa. *N*-(5-Phosphopyridoxyl)-L-tyrosine was prepared according to Ikawa.[13]

Preparative Methods. ***N*-(5-Phosphopyridoxyl)-3′-nitro-L-tyrosine (V).** Ten millimoles of 3-nitro-L-tyrosine (2.26 g) was dissolved in 15 ml of H_2O with the addition of 4 ml of 10 *N* KOH. Upon mixture of 10 mmol of pyridoxal phosphate (2.47 g) with this solution, the pH dropped to a final value of 8.0–8.5. After 10 min at room temperature, 5 mmol of $NaBH_4$ (0.19 g) was added with stirring. Reduction was allowed to proceed for 30 min and then the solution was titrated with concentrated hydrochloric acid to pH 5.5. The mixture was immediately applied to an XE-64 column (85 cm × 4.3 cm) and eluted with H_2O. Fractions (10 ml) were collected and small aliquots were removed, diluted into 0.1 *M* phosphate buffer (pH 7), and monitored at 325 and 420 nm. Those samples with an absorbance ratio (325/420 nm) of 3.5–3.9 were pooled and flash evaporated to a final volume of 5 ml. Addition of 50 ml of absolute ethanol, followed by cooling on ice for a few hours, facilitated good yield of the NO_2 compound. The product was collected by filtration and dried at reduced pressure in the presence of P_2O_5. Typical yields of V ranged from 70 to 80% and at pH 7 it displayed uv absorbance peaks at 420 nm (ϵ 2300), 325 (8300), and 285 (6100). The compound moved as a single component when analyzed by various chromatographic and electrophoretic systems.

***N*-(5-Phosphopyridoxyl)-3′-amino-L-tyrosine (II).** Five millimoles of V (2.28 g) was dissolved in 50 ml of H_2O by adjusting the pH to 7.4 with NaOH solution. One gram of PtO_2 was added and hydrogenation (at room temperature and atmospheric pressure) was carried out in a closed apparatus after flushing with H_2. Vigorous magnetic stirring was employed and hydrogen uptake ceased after the theoretical amount was incorporated. A near colorless reaction mixture is observed when the aggregated catalyst settles, but upon exposure to air, it gradually turns brown. The reaction mixture was not filtered, but after dilution with 50 ml of H_2O, the solution was merely pipetted from the settled Pt and loaded onto an Amberlite CG-50 column (37 cm × 2.3 cm). The catalyst was washed with some additional H_2O and this was also applied to the resin. Flushing the flask and column with N_2 as well as accelerating the application of the reaction mixture with 3 psi N_2 pressure helped prevent some of the coloration. Upon elution with H_2O, those fractions containing II were identified using both a spot test for the pyridoxyl region and one for the aromatic amino group.[27,29] Those samples which were positive in both tests were pooled and flash evaporated to a volume of about 15 ml. The flask was then heated on a steam bath and hot H_2O was gradually added until the product redissolved. This step should not be prolonged since heating darkens the solution. During crystallization, however, the colored contaminant remains soluble and readily washes from the product. After slow cooling at room temperature and a few hours on ice, the product was filtered on a medium porosity sintered glass filter. The white product was gently washed with small volumes of cold H_2O

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until the filtrate was colorless. Drying was achieved at reduced pressure in a desiccator with P_2O_5. Final yields of II were usually about 50%. The molecule displays ultraviolet absorption peaks at 325 nm (ε 7900), 294 (5300), and a shoulder at 240 (8500).

Anal. Calcd for $C_{17}H_{22}N_3O_8P \cdot H_2O$: C, 45.84; H, 5.39; N, 9.44; O, 32.36; P, 6.97. Found: C, 45.93; H, 5.62; N, 9.32; O, 32.17; P, 6.96.

The pmr and circular dichroic spectra of II are shown in Figures 3 and 5b. Electrophoretic and chromatographic analyses show the product to be a single migrating species.

"Cyclic" *N*-(5-Phosphopyridoxyl)-3′-amino-L-tyrosine (III). Ten millimoles of PLP (2.47 g) and 10 mmol of 3-nitro-L-tyrosine (2.26 g) were dissolved in 100 ml of H_2O with the addition of 4 ml of 10 *N* KOH. Upon addition of 2 g of PtO_2, H_2 reduction was carried out as described for compound II. The reaction stopped after theoretical H_2 uptake, and upon removal from the reduction apparatus N_2 was used to retard coloration. An Amberlite CG-50 column, 67 cm × 2.7 cm, was employed and elution was carried out with H_2O. After detection of the proper fractions,[27,29] and flash evaporation of these to 5–10 ml, cooling on ice gave crystals suitable for collection. These were retained on a sintered glass filter and washed with small volumes of cold H_2O until the filtrate was colorless. Yields of 80–85% were obtained after drying over P_2O_5. Compound III shows a uv absorption maximum at 325 nm (ε 9600) with shoulders at 305 (7000) and 240 (11,400).

Anal. Calcd for $C_{17}H_{20}N_3O_8P$: C, 48.10; H, 4.72; N, 9.90; O, 30.02; P, 7.30. Found: C, 47.70; H, 4.75; N, 10.03; O, 29.80; P, 7.72.

Figures 3 and 6a show the pmr and circular dichroic spectra of compound III.

Spontaneous Synthesis of III. A mixture of pyridoxal phosphate, 0.25 mmol (61.8 mg), and 0.25 mmol of 3-amino-L-tyrosine (49 mg) was formed in 1.5 ml of H_2O. Upon the addition of 0.1 ml of 10 *N* KOH the pH rose to 10.5. Analysis of the reaction mixture showed a uv spectrum corresponding to the cyclic compound III as described previously. The material was loaded onto an Amberlite XE-64 column, 70 cm × 2.5 cm, eluted with H_2O, and collected in 10-ml fractions. Fractions absorbing at 325 nm eluted as a major peak. These fractions were pooled and crystals were obtained upon reduction to a small volume. This product was identical with that obtained in the previous synthesis as judged by uv, CD, and pmr spectra, as well as its behavior in the various chromatographic and electrophoretic systems. The two cyclic preparations also acted in an indistinguishable manner when employed in the immunological studies.

Compound VI. Two millimoles of 3-amino-L-tyrosine, 392 mg, was added to 2 ml of H_2O and addition of 1–2 small drops of 10 *N* KOH brought the pH of the stirred solution to approximately 10. Two millimoles of formaldehyde (0.165 ml of 37% solution in H_2O) was added and the pH was maintained at 9–10 with KOH solution. The reaction was permitted to proceed for 15 min after which concentrated HCl was added to lower the pH to 5–6. The precipitate was left to sit on ice for 1 hr and was then collected and washed with cold H_2O. Even after reprecipitation, chromatography showed the preparation to contain some minor contaminants.

The compound displayed a uv peak at 293 nm (ε 4000) and a shoulder at 235 (6600). Upon chromatography, the major component demonstrated an ability to react with aromatic amine reagents. It did not react with ninhydrin to give the blue color typical of an amino acid, but gave a yellow spot. This result is indicative of α-imino acids such as proline and is consistent with structure VI. The pmr analysis shown in Figure 6b confirms the product.

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Nucleosides. LXXIX. Facile Base-Catalyzed Hydrogen Isotope Labeling at Position 6 of Pyrimidine Nucleosides[1]

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Abstract: Quantitative or nearly quantitative incorporation of deuterium at C-6 in all common pyrimidine nucleosides, including ara-C, has been achieved. The method involves the treatment of such nucleosides with a mixture of deuterated base and DMSO-d_6. In those cases where the sugar hydroxyls can easily participate in the saturation of the 5,6-double bond, quantitative incorporation of deuterium at C-5 was also obtained. In these latter cases, 6-deuterated nucleosides were obtained from 5,6-dideuterated derivatives by treating them under conditions in which only the 5 position exchanges. The mechanism of H-6 exchange is thought to involve the direct abstraction of H-6 by base. Experimental evidence supporting this mechanism is given. The method for isotope labeling at C-6 of nucleosides described herein should be eminently adaptable for the incorporation of tritium label into pyrimidine nucleosides for use in biochemical studies.

One of the important aspects of the chemistry of pyrimidines and their nucleosides is the susceptibility of the 5,6-double bond to 1,4-nucleophilic addition reactions. A manifestation of this reactivity is the observed hydrogen isotope exchange at the 5 position when reversible addition reactions are performed in deuterated media. Some of the most prominent examples of H-5 exchange are: of cytidine in acidic buffers,[2] of certain pentofuranosyluracils in basic media,[3-6] and of uridine with catalysis by glutathione.[7] On the other hand, a facile base-catalyzed exchange at

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