additional 483 mg. of DL-lysine-1-C^{14} was obtained, making a total average yield of 77.5% for the two Schmidt reactions. The over-all yield of pure DL-lysine-1-C^{14} from $KC^{14}N$ was 66.5%.

All of the portions of DL-lysine-1-C^{14} monohydrochloride were chemically and radioactively pure as shown by paper chromatography and radioautography; m.p. 263-264° dec.

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[CONTRIBUTION FROM THE DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY, UNIVERSITY OF CALIFORNIA SCHOOL OF MEDICINE]

Formation of Schiff Bases of Pyridoxal Phosphate. Reaction with Metal Ions[1]

BY YOSHIHIKO MATSUO

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From a spectrophotometric study of the reactions of pyridoxal phosphate (PLP) with amino acids and amines evidence was secured for the formation in neutral aqueous solution of Schiff bases between PLP and various amino compounds. The stability constants of the Schiff bases were evaluated by an optical method. Spectrophotometric evidence was obtained of the chelation of the PLP Schiff bases with certain metal ions. It was found that chelating metal ions can be removed from some metal–PLP–amino acid complexes by suitable concentrations of ethylenediaminetetraacetic acid or 2,3-dimercaptopropanol. The significance of the findings is discussed in connection with the mechanism of reactions of vitamin B_6 enzymes.

During investigation of homoserine deaminase of rat liver, the activity of which is significantly increased by the addition of pyridoxal phosphate (PLP), it was noted that addition of homoserine to a dilute neutral solution of PLP (10^{-5} *M*) caused a rapid intensification of the yellow color of the mixture. This was observed with many other amino acids and also with several primary amines. However, N-substituted amino acids, such as sarcosine, did not cause the color change. From this it was inferred that the intensification of the yellow color of PLP on addition of amino compounds is due to the formation of Schiff base. It is known that Schiff base is formed readily between pyridoxal and amino acids,[2,3] or pyridoxal and amines,[4,5] both in an alcoholic medium[2–4] and in aqueous solution.[5] Roberts[6] has observed a change in the absorption spectrum of PLP in the presence of glutamic acid or alanine, and suggested that this shift in the spectrum might be due to the formation of Schiff base. More recently, while the present work was in progress, Blakley[7] reported the ultraviolet spectra of such bases formed between PLP, and glycine and serine, respectively. He also showed that the presence in the reaction mixture of bisulfite, which blocks the formyl group of PLP, prevents such change in spectrum on addition of the amino acids. Since the formation of Schiff base is generally believed to be involved in the mechanism of reaction catalyzed by vitamin B_6 enzymes, a more detailed study was made on the chemistry of this reaction between PLP and amino acids and the results are reported in this paper. Interaction of metal ions and the Schiff base was also studied to a limited extent.

(1) Aided by research grants from the National Cancer Institute (No. 327 and 2327), the American Cancer Society (Met-45A), and the Cancer Research Funds of the University of California.

(2) D. Heyl, S. A. Harris and K. Folkers, THIS JOURNAL, **70**, 3429 (1948).

(3) H. Brandenberger and P. P. Cohen, *Helv. Chim. Acta*, **36**, 549 (1953).

(4) D. Heyl, E. Luz, S. A. Harris and K. Folkers, THIS JOURNAL, **70**, 3669 (1948).

(5) V. R. Williams and J. B. Neilands, *Arch. Biochem. Biophys.*, **53**, 56 (1954).

(6) E. Roberts, *J. Biol. Chem.*, **198**, 495 (1952).

(7) R. L. Blakley, *Biochem. J.*, **61**, 315 (1955).

(1) Reaction of Pyridoxal Phosphate with Amino Acids and Amines.—Addition of amino acids or primary amines to a solution of PLP prepared in a potassium phosphate buffer, *p*H 7.5, caused a rapid and marked intensification of the yellow color of the solution. Upon measurement of the ultraviolet absorption spectra of PLP in the presence and absence of amino acids or amines it was observed, as previously reported by other workers,[6,7] that the spectrum is significantly altered by the addition of amino acids or amines. This is illustrated in Fig. 1. PLP has two absorption maxima; one at 330 mμ and the other at 388 mμ. In the presence of various amino compounds, a new maximum appeared at 278 mμ, and the 388 mμ peak moved to a higher wave length (400 to 415 mμ), while the maximum at 330 mμ decreased in intensity and was reduced to an inflection point. Spectral changes of this type were observed on addition of the following compounds to the solution of

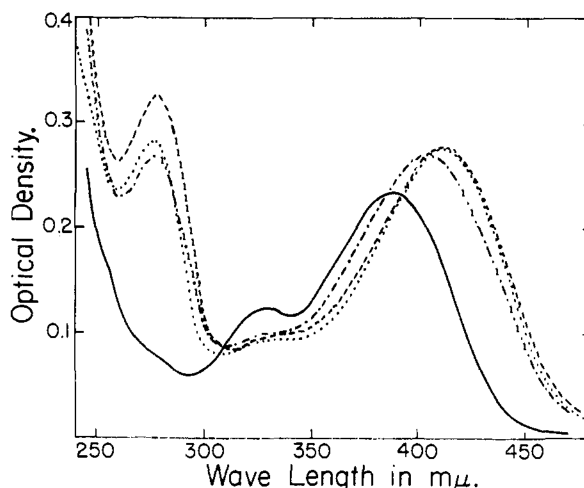

Fig. 1.—Spectrum of pyridoxal phosphate (4.30×10^{-5} *M*) in 0.17 *M* potassium phosphate buffer, *p*H 7.5: —, alone; ----, plus 8×10^{-2} *M* α-amino-*n*-butyric acid; ...., plus 8×10^{-2} *M* ethanolamine; -·-·-, plus 8×10^{-2} *M* α-methyl-α-amino-*n*-butyric acid. All the amino compounds neutralized to *p*H 7.5 before addition.

PLP: glycine, alanine, α-aminoisobutyric acid, β-alanine, α-amino-*n*-butyric acid, β-amino-*n*-butyric acid, γ-aminobutyric acid, α-methyl-α-amino-*n*-butyric acid, valine, norvaline, leucine, norleucine, isoleucine, glutamic acid, α-methylglutamic acid, aspartic acid, threonine, *allo*-threonine, serine, homoserine, ethanolamine, *n*-amylamine and tris-(hydroxymethyl)-aminoethane.

It was observed that addition of cystathionine, tryptophan, arginine, lysine and histidine also intensifies the yellow color of PLP.

From these results it is very likely that any compounds that possess an unsubstituted amino group can react with PLP to form Schiff base.

(2) **Reaction of PLP with Amino Acids with Sulfhydryl Group.**—When cysteine or homocysteine was added to a solution of PLP, it resulted in the disappearance of the faint yellow color of the solution. The spectrum of the PLP–cysteine complex is shown in Fig. 2. The spectrum, which is entirely different from that of PLP or that of Schiff bases formed with other amino compounds, very probably represents the formation of a thiazolidine ring by reaction of the formyl group of PLP with the amino and sulfhydryl groups of cysteine. That 2-(2-methyl-3-hydroxy-5-hydroxymethyl-4-pyridyl)-4-thiazolidinecarboxylic acid is formed by reaction of pyridoxal with cysteine in alcohol has been reported previously.[2] By an analogous reaction homocysteine will form a thiazine ring when it reacts with PLP.

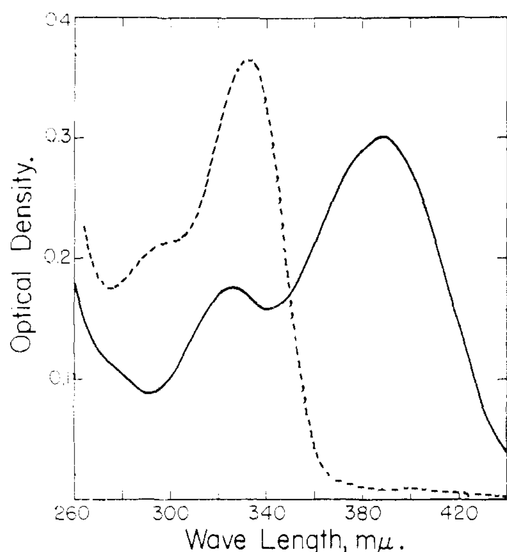

Fig. 2.—Spectrum of pyridoxal phosphate (5.47×10^{-5} *M*) in 0.067 *M* potassium phosphate buffer. *p*H 7.5: —, alone; – – – –, in the presence of 0.0067 *M* cysteine (adjusted to *p*H 7.5). Spectrum measured 20 min. after the mixing of reagents, against blanks containing everything but pyridoxal phosphate.

(3) **Stability of the Schiff Bases of PLP in Neutral Aqueous Solution.**—Stability constants,[8] *K*, of Schiff bases formed between PLP and amino compounds were measured spectrophotometrically in a neutral aqueous solution. Table I lists the values of *K* defined as

$$K = \frac{[\text{Schiff base}][\text{Water}]}{[\text{PLP}][\text{A}]} \qquad (1)$$

where A stands for the amino compound which reacts with PLP. In the calculations, 55.5 *M* was used as the concentration of water in the aqueous medium. The water term is contained in the equation, because the formation of the Schiff base produces a molecule of water.

TABLE I

STABILITY CONSTANT OF THE SCHIFF BASES OF PYRIDOXAL PHOSPHATE

Reactant	$K \times 10^{-4}$[a]	Reactant	$K \times 10^{-4}$[a]
Glycine	0.54 ± 0.01	Glutamate	0.42 ± .01
Alanine	0.32 ± .01	α-Methylglutamate	0.03 ± .01
β-Alanine	0.61 ± .01	Aspartate	0.26 ± .01
α-Aminoisobutyrate	0.07 ± .01	Methionine	2.06 ± .04
α-Amino-*n*-butyrate	1.16 ± .02	Ethionine	2.8 ± .3
β-Amino-*n*-butyrate	0.22 ± .00	Serine	1.46 ± .03
γ-Aminobutyrate	.57 ± .03	Threonine	2.3 ± .3
α-Methyl-α-amino-*n*-butyrate	.09 ± .00	*allo*-Threonine	4.3 ± .2
Valine	4.5 ± .2	Homoserine	1.08 ± .01
Norvaline	1.2 ± .1	Ethanolamine	1.38 ± .01
Leucine	1.0 ± .1	*n*-Amylamine	0.96 ± .02
Norleucine	1.1 ± .1	Tris-(hydroxymethyl)-aminomethane	0.67 ± .01
Isoleucine	3.0 ± .2		
allo-Isoleucine	2.6 ± .2		

[a] Average of two determinations at different concentrations, ± the mean deviation. See text for method of determination.

Measurements of the stability constant of the complexes formed between PLP and sulfhydryl amino acids were not attempted. It was observed, however, that the spectrum of a mixture containing 0.2 μmole of PLP, 100 μmoles of α-amino-*n*-butyric acid and 20 μmoles of cysteine was essentially the same with that of the same mixture minus α-amino-*n*-butyrate. This indicates the much greater stability of the PLP–cysteine complex than that of the ordinary PLP–Schiff base.

(4) **Reaction of PLP–Schiff Base with Metal Ions.**—Addition of small amounts of metal ions,[9] such as Cu^{++} and Al^{+++}, rapidly bleached the yellow color of the Schiff base of PLP. Spectrophotometric investigation revealed that addition of metal ions shifts the absorption maxima to shorter wave lengths. The resulting spectra were found to be not those of the complex formed between the metal and amino acid or amine in question. Effects of the addition of metal ions to the solution of PLP–glycine complex are given in Fig. 3 and Table II. It is seen from these that the relative effectiveness of metal ions, at the concentrations of the reagents tested, to shift the two absorption maxima of PLP–glycine Schiff base is $Co^{++} \geqq Al^{+++} > Cu^{++} > Zn^{++} > Ni^{++} > Mn^{++}$. Cr^{+++}, Hg^{++} and Cd^{++} were without appreciable effect under the conditions of the experiment. The spectrum of PLP–glycine measured in the presence of Fe^{++} and Fe^{+++} showed no resemblance to that measured in the presence of other metal ions tested. This is probably due to the light absorption of iron–glycine chelates.

(8) A. E. Martell and M. Calvin, "Chemistry of the Metal Chelate Compounds," Prentice-Hall, Inc., New York, N. Y., 1953, p. 76.

(9) Metals were added to the reaction mixture as chlorides, except for Al^{+++}, Zn^{++} and Fe^{++}, which were sulfates.

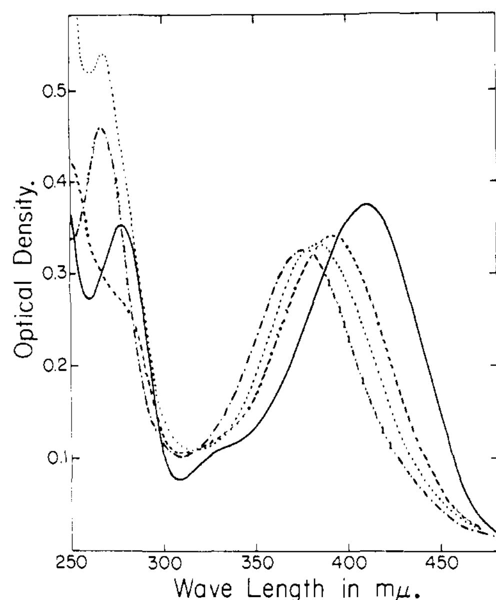

Fig. 3.—Spectrum of pyridoxal phosphate (6.7×10^{-5} *M*) in $KHCO_3$ buffer (0.03 *M*, equilibrated with $N_2 + CO_2$ (95:5), *p*H 7.2), containing 1.6×10^{-5} *M* glycine: —, no addition; - - - - -, plus 6.7×10^{-5} *M* Ni^{++}; . . . . , plus 6.7×10^{-5} *M* Cu^{++}; -·-·-·, plus 6.7×10^{-5} *M* Al^{+++}. Metal ions added 20 min. after the mixing of other reagents, and spectrum measured 10 min. later, against a blank containing glycine and the buffer as above.

No evidence of complex formation between PLP and the metal ions mentioned above was obtained by the optical means. The spectrum of a mixture containing PLP and metal ions merely represented the sum of the component spectra.

TABLE II

ABSORPTION MAXIMA OF PYRIDOXAL PHOSPHATE-GLYCINE SCHIFF BASE IN THE PRESENCE OF METAL IONS

Each mixture contained $KHCO_3$ buffer (0.03 *M* equilibrated with $N_2 + CO_2$ (95:5), *p*H 7.2), glycine (0.016 *M*), pyridoxal phosphate (6.7×10^{-5} *M*) and 6.7×10^{-5} *M* metal ions as indicated. Blank contained glycine and the buffer.

	Absorption maximum			
	A[a]		B[a]	
Metals	Wave length	OD	Wave length	OD
None	408	0.375	278	0.355
Co^{++}	375	.316	[b]	. . .
Al^{+++}	376	.327	266	0.460
Cu^{++}	383	.332	268	.540
Zn^{++}	389	.344	272	.327
Ni^{++}	392	.342	[b]	. . .
Mn^{++}	403	.341	276	0.341
Cr^{+++}	408	.380	278	.353
Cd^{++}	408	.365	278	.349
Hg^{++}	408	.373	278	.350

[a] A, in 400 mμ region; B in 270 mμ region. [b] No maximum present, but an inflection point instead.

The change in the spectrum of PLP–glycine Schiff base in the presence of metal ions strongly suggests the formation of chelates. Formation of metal chelates of pyridoxal–amino acid Schiff bases has been studied previously.[10]

(10) G. L. Eichhorn and J. W. Dawes, THIS JOURNAL, **76**, 5663 (1954).

The fact that the spectrum of PLP–glycine–Cu is very similar to that of PLP–ethanolamine–Cu suggests that the carboxyl group of the amino acid does not participate in chelate formation, though it does not altogether exclude the possibility.

Figure 4 shows the effect of varying concentrations of Mn^{++} on the spectrum of the PLP–glycine complex. It is seen that as the concentration of

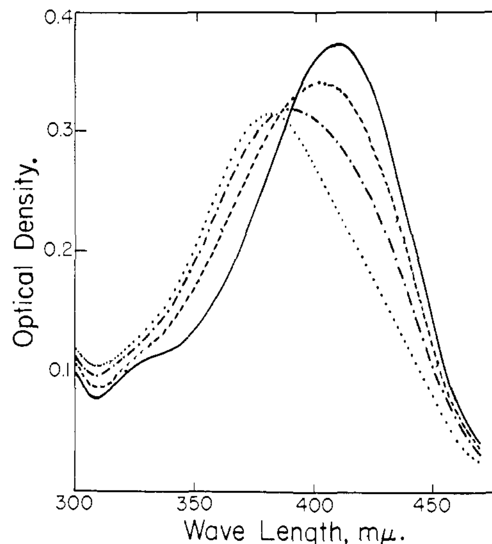

Fig. 4.—Spectrum of pyridoxal phosphate (6.7×10^{-5} *M*) in $KHCO_3$ buffer (0.03 *M*, equilibrated with $N_2 + CO_2$ (95:5), *p*H 7.2), containing glycine (1.6×10^{-2} *M*): —, no addition; - - - -, plus 6.7×10^{-5} *M* Mn^{++}; -·-·-· , plus 1.34×10^{-4} *M* Mn^{++}; . . . . , plus 2.68×10^{-4} Mn^{++}. Mn^{++} added 20 min. after the mixing of other reagents and spectrum measured 10 min. later, against a blank containing the buffer and glycine as above.

Mn^{++} increases the shift of the absorption maximum is exaggerated. At the higher concentration of Mn^{++} the spectrum is similar to that obtained in the presence of Cu^{++} (*cf.* Fig. 3). This indicates that the degree of spectral change is probably determined by the equilibrium of the two reactions which compete for the metal under the conditions employed: *i.e.*, chelation of metal by the Schiff base on the one hand and by glycine on the other. And it also indicates that various metal chelates of the Schiff base have essentially the same "color," as is the case in other chelate compounds.[11]

(5) Effect of the Metal-binding Reagents on the Metal Chelates of PLP Schiff Base.—When a suitable amount of ethylenediaminetetraacetic acid (EDTA) is added to a solution of a metal chelate of the Schiff base of PLP, which is colorless, the yellow color characteristic to the Schiff base appears after a period of time. Figure 5 shows that the EDTA restores the spectrum of the free Schiff bases when added to metal–PLP–glycine complex. The effect of 6.7×10^{-5} *M* Cu^{++} added to the mixture of glycine (1.6×10^{-2} *M*) and PLP (6.7×10^{-5} *M*) is nearly completely reversed by the addition of 10^{-4} *M* EDTA, as judged from the wave length of the absorption maxima. In the case of Al^{+++} addition of 10^{-4} *M* EDTA, other conditions being the same as above, did not produce any no-

(11) B. P. Geyer and G. M. Smith, *ibid.*, **64**, 1649 (1942).

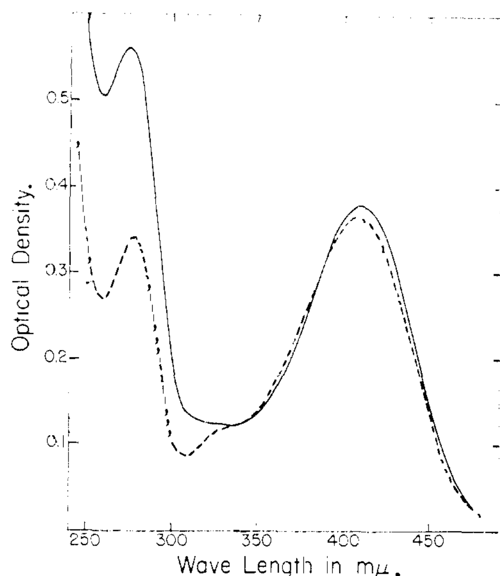

Fig. 5.—Spectrum of metal–pyridoxal phosphate–glycine complex in the presence of EDTA. Each reaction mixture contained glycine (1.6×10^{-2} *M*), $KHCO_3$ buffer (0.03 *M*, equilibrated with $N_2 + CO_2$ (95:5). *p*H 7.2), and the following: —, 6.7×10^{-5} *M* Cu^{++} and 10^{-4} *M* EDTA; - - - - -, 6.7×10^{-5} *M* Al^{+++} and 1.6×10^{-3} *M* EDTA. Spectrum measured 20 min. after the addition of EDTA, against blanks containing everything but the metals and pyridoxal phosphate.

ticeable effect in the spectrum. However, complete restoration of the spectrum of the PLP–glycine Schiff base was obtained at EDTA concentration of 1.6×10^{-3} *M* (Fig. 5). EDTA was also found to reverse the effect of Zn^{++}, Co^{++}, Mn^{++} and Ni^{++} on the spectrum of the PLP–glycine complex.

2,3-Dimercaptopropanol (BAL) was able also to reverse the chelation of homoserine–PLP by Cu^{++}. However, the Cu–BAL complex was insoluble in the buffer solution and had to be removed from the reaction mixture before the spectrum could be measured.

Experimental

Chemicals.—Pyridoxal phosphate monohydrate was obtained from the California Foundation for Biochemical Research, Los Angeles. The extinction coefficient of this preparation, measured at 388 mμ in 0.1 *M* potassium phosphate buffer, *p*H 7.0, is 5100 ± 150. This compares favorably with the value reported for a pure compound.[12] Amino acids and amines are from commercial sources. Amino acids are chromatographically pure and their elementary analyses agree satisfactorily with the theoretical values. Some amino acids had to be recrystallized, because of their unusually high absorption of ultraviolet light. Throughout the experiment deionized water was used to minimize metal contamination.[13]

Solutions of PLP were not stored for more than one day. A quartz fiber torsion balance was used to weigh a few mg. of PLP with satisfactory accuracy.

Measurements of the Ultraviolet Spectrum.—Ultraviolet absorption spectra were measured at room temperature with a Beckman model DU spectrophotometer. Silica cells of 1 cm. length were used. Concentrations of the reagents in the specific experiments are given in the legends of the figures.

(12) E. A. Peterson and H. A. Sober, THIS JOURNAL, **76**, 169 (1954).

(13) Distilled water was passed through a column of mixed ion exchanger resins, supplied by the Cotroe Laboratory, Chicago, under the trade name of "Quickpure."

(a) **Formation of the Schiff Bases.**—To the solution of various amino compounds prepared in potassium phosphate buffer, *p*H 7.5, was added an appropriate quantity of PLP. The spectrum was measured 20 min. after the mixing of PLP. The $O.D._{278}$ of the solutions increased rapidly after the PLP addition, and reached a steady level in approximately 10 min. at room temperature.

(b) **The Metal Chelates of PLP Schiff Base.**—A potassium bicarbonate–CO_2 buffer, *p*H 7.2, was used to avoid the precipitation of metals. The Schiff base of PLP was prepared as in (a), and to this was added the metal salt under study, and the spectrum was measured 10 min. after the mixing.

(c) **Effects of EDTA and BAL on Metal Chelates of the Schiff Base.**—A solution of metal–PLP-glycine or metal–PLP–homoserine in the bicarbonate buffer was prepared as in (b). Addition of EDTA or BAL to this solution caused the development of a yellow color over a time period, the length of which varied with the kind of metal involved, for a given concentration of the metal-binding reagent. The spectrum was measured, therefore, 20 min. after the addition of the metal-complexing reagents.

Estimation of the Stability Constants of the PLP Schiff Base.—For the calculation of stability constants it was assumed that one molecule of amino compound reacts with one molecule of PLP and forms one molecule each of a Schiff base and water, and that further reaction of the formed Schiff base with the second molecule of the amino compound does not occur. It was also assumed that all the reactants obey Beer's law. With these assumptions the constants were measured by an optical method. Formation of the Schiff base was followed spectrophotometrically at 278 mμ. At this wave length the Schiff bases of PLP have molar extinction coefficient (E_{278}) of approximately 5,000, while E_{278} of PLP is 1,800, and the amino compounds tested have still lower values. To obtain the molar extinction coefficient of a Schiff base, 0.1 μmole of PLP and 240 μmoles[14] of the amino compound in question were mixed in a quartz absorption cell together with 500 μmoles of potassium phosphate buffer, *p*H 7.5, the total volume was adjusted to 3.0 ml. by addition of water, and the optical density of this mixture was determined at 278 mμ. This value was corrected for the absorption of the amino compound added to the mixture and was used to calculate preliminary values of E_{278} of the Schiff base. It was thought that the great excess of amino compound over PLP in this mixture (2,400:1) would push the reaction toward completion, despite the presence of a large concentration of water.

Mixtures of known concentrations of PLP and the reactant were then prepared, and the optical densities of these solutions were determined at 278 mμ. Since E_{278} and the concentrations are known for all the other reagents present in these mixtures, and the E_{278} of the Schiff base has been obtained as described above, the concentration of the Schiff base in the test mixtures can now be calculated by direct application of Beer's law, and thence a first approximation of *K* may be calculated from eq. 1. In an actual experiment two mixtures of 3.0 ml. total volume each were prepared; one contained, besides the quantity of buffer mentioned above, 0.1 μmole of PLP and 10.0 μmoles of the amino compound; the other contained 0.2 μmole of PLP and 20.0 μmoles of the reactant. The preliminary *K* values calculated from these two mixtures agreed satisfactorily.

In the next step the *K* values obtained as described above were used to determine the degree of completion of the reaction at the concentration of the reagent, which was used to obtain the preliminary values of E_{278} of Schiff bases. It was found that a substantial amount (>10%) of PLP remained unreacted under such condition, when the stability constant of a Schiff base is smaller than 6.2×10^3. Therefore, the preliminary values of the E_{278} of Schiff bases were corrected for the unreacted PLP, and the corrected values were used to recalculate the *K* values. Stability constants of various Schiff bases of PLP obtained in this manner are listed in Table I.

The *K* values determined on D-alanine and L-alanine were the same. In most cases the racemic compound was used for determining the stability constant.

Biochemical Significance

In many cases the D-isomer of an amino acid acts as inhibitor in an enzyme system which utilizes its

(14) For leucine and norleucine 120 μmoles, instead of 240 μmoles, were used because of their relative insolubility.

L-isomer as the substrate.[15-19] The findings described in the preceding sections that many amino acids and amines can form Schiff bases with PLP in neutral aqueous solution suggests that any amino compound capable of forming a Schiff base with PLP may behave as a competitive inhibitor of enzyme systems, which involve PLP as an activator or as the prosthetic group.[20] The relative effectiveness of various amino compounds may be predicted, at least to a considerable extent, from the stability constant of their Schiff base, because the degree of stability of such compounds should significantly contribute to the over-all affinity of the reactant to an enzyme. However, no absolute parallelism of the stability constant of an amino compound and its effectiveness as an inhibitor is to be expected, especially in systems where the function of PLP is that of a prosthetic group, being bound to an apoenzyme. Under such circumstances, the affinity of an amino compound for the active site of the enzyme involves, besides its affinity to pyridoxal phosphate, such other factors as the attractive and repulsive forces which may be created among the functional groups of the reactant and the apoenzyme. Steric effects will also offer a complication. Aside from these, if PLP is bound to an apoenzyme through a phosphate bridge, as is commonly believed, then the masking of the negatively charged group may also create a significant difference.

To obtain concrete illustrations of these factors from an actual enzyme system, several amino acids were tested for their ability to inhibit the homoserine deaminase system, which requires PLP for full activity. Dialyzed enzyme was used without the addition of PLP. The results are given in Table III. An excellent parallelism between the inhibitory effect of an amino acid and the stability of its Schiff base is found in some cases, especially between alanine, α-amino-*n*-butyrate and glutamate, and their α-methyl derivatives. Here, the substitution of the α-hydrogen with a methyl group does not cause a change in the relative position or number of ionizable groups, nor does it cause a significant change in the size or shape of the molecules. Therefore, the observed parallelism should be expected. On the contrary, the parallelism no longer holds if glutamate is compared with alanine, where the two are vastly different molecules.[21]

TABLE III

INHIBITION OF HOMOSERINE DEAMINASE BY AMINO ACIDS

Reaction mixtures contained 0.08 *M* DL-homoserine and 0.1 *M* potassium phosphate buffer, *p*H 7.5, with or without 0.08 *M* amino acid tested as indicated. Approximately 7 μg. of the dialyzed enzyme, purified 450-fold from rat liver, was added to each mixture. No PLP was added. Total volume 1.0 ml. Incubation at 37° for 30 min. α-Ketobutyrate formed was measured by a modification of the method of Friedemann and Haugen.[23]

Amino acid added	Inhibition %	$K^a \times 10^{-4}$
Glycine	49	0.54
DL-Alanine	37	0.32
DL-α-Aminoisobutyrate	0	0.07
DL-α-Amino-*n*-butyrate	61	1.16
DL-α-Methyl-α-amino-*n*-butyrate	6	0.09
L-Glutamate	17	0.42
DL-α-Methylglutamate	0	0.03
L-Cysteine	100	[b]

[a] Stability constant of pyridoxal phosphate–amino acid Schiff base (see text). [b] See Section 3 of the text.

Attention should be paid to the fact that tris-(hydroxymethyl)-aminomethane can form Schiff base with PLP. No activation of the homoserine deaminase by PLP could be observed in tris buffer (0.05 *M*, *p*H 7.5), whereas significant activation was obtained in phosphate buffer (0.05 *M*, *p*H 7.5).[22]

Evidence is provided in this paper for the formation of chelates of the Schiff bases of PLP, and the feasibility of removing metals from such chelates by some of the metal binding reagents. On the other hand, there have been several reports[7,23] which showed that EDTA does not inhibit enzyme systems which require PLP for their activity. If the findings do not indicate that a metal has no part in the activity of these enzyme systems, it is still possible that the stability of the chelates of the Schiff bases in question is greatly increased by the presence of the apoenzyme. It is known that the stability of some metal chelate compounds is increased when additional rings are introduced into the structure.[24] But, it is not known whether the addition of a second ring would increase the stability of the original chelate ring.[25]

Acknowledgment.—The author wishes to thank Professor D. M. Greenberg for his continued interest in this work and for his help in preparing the manuscript.

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(15) I. C. Gunsalus, C. C. Galeener and J. R. Stamer, *Methods in Enzymology*, **2**, 238 (1955).

(16) F. Binkley and D. Okeson, *J. Biol. Chem.*, **182**, 273 (1950).

(17) H. J. Strecker, *Arch. Biochem. Biophys.*, **46**, 128 (1953).

(18) C. Yanofsky, *J. Biol. Chem.*, **198**, 343 (1952).

(19) F. W. Sayre, Ph.D. Thesis, University of California, 1955; *cf.* pp. 50 and 52.

(20) A similar view has been expressed by other workers. *Cf.* W. B. Jakoby and D. M. Bonner, *J. Biol. Chem.*, **205**, 709 (1953).

(21) It is suggested that a systematic comparison of the affinity of various amino compounds for free PLP and the coenzyme which is bound to the apoenzyme may yield valuable information concerning the structure on the active site of a Vitamin B_6 enzyme.

(22) Y. Matsuo and D. M. Greenberg, unpublished data.

(23) F. W. Sayre and D. M. Greenberg *J. Biol. Chem.*, **220**, 787 (1956).

(24) M. Calvin and R. H. Bailes, THIS JOURNAL, **68**, 949 (1946).

(25) Klotz and Loh-Ming (*ibid.*, **76**, 805 (1954)) found that the stability constant of an aromatic dye–Zn^{++} complex increased (from 2.29×10^2 to 4×10^3) in the presence of pepsin, but under the same conditions the constant of the dye–Cu^{++} complex decreased (from 1.3×10^5 to 6×10^3).