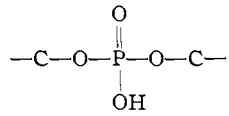
phorus and 13.9% nitrogen, while the preparation which was purified entirely by chemical fractionation contained 4.0% phosphorus and 14.3% nitrogen. These differences in composition appear to be real since the mobilities given in Table IV are greater for the preparation containing the larger amount of phosphorus. Since the preparations were equally homogeneous by electrophoresis it is concluded that they vary slightly in composition. It may be that these peptides vary only in their phosphorus content. A phosphopeptone of the amino acid composition $Asp_1Glu_7Gly_1Val_2Leu_3$-$Ileu_2Ser_4Thr_1Pro_1Arg_2(NH_2)_3(PO_3H)_5$ (as calculated from the results in Table III on the preparations prepared by chromatography using the closest whole number and omitting alanine) would have a phosphorus content of 5.0% and a nitrogen content of 14.9%. This is considered to be in reasonable agreement with the values found of 4.5% phosphorus and 13.9% nitrogen, particularly in view of the possible uncertainties in correcting for the ash content.

While there is no direct evidence for the position of phosphorus or its type of linkage in the phosphopeptone, the composition of the phosphopeptide as given in Table III does not appear to support the conclusion of Perlmann[26] that the phosphorus of β-casein is in the form of a diester. The diester form of phosphorus as postulated by Perlmann involves two hydroxy amino acids for each phosphorus as



The data in Table III show that there are four moles of serine and one mole of threonine and five moles of phosphoric acid in the phosphopeptone; consequently, there are not enough hydroxy-amino acids present in the phosphopeptone for the formation of diester bonds with phosphoric acid. It is possible that trypsin breaks diester bonds in the formation of the phosphopeptone; however, the evidence indicates that trypsin does not break phosphoric acid diesters. Sinsheimer and Koerner,[27] who purified snake venom diesterase, found that it hydrolyzed bis-(*p*-nitrophenyl)-phosphoric acid. The trypsin used in this work was tested for possible action on secondary phosphate bonds by incubating the calcium salt of bis-(*p*-nitrophenyl)-phosphoric acid with a 0.1% solution of Worthington trypsin for 3 hours at 37°. No hydrolysis of the diester occurred. Under similar conditions, the diesterase from calf intestinal mucosa[28] hydrolyzed this diester.

Acknowledgments.—We are indebted to Dr. Clyde Ogg and Mrs. Ruth Kelly who made the phosphorus, ash and nitrogen determinations. Mrs. Betty Harrington made the Van Slyke amino nitrogen determinations.

(26) G. E. Perlmann, *Advances in Protein Chem.*, **X**, 27 (1955).

(27) R. L. Sinsheimer and J. F. Koerner, *J. Biol. Chem.*, **198**, 293 (1952).

(28) C. A. Zittle, *ibid.*, **166**, 491 (1946).

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOLOGICAL CHEMISTRY, UNIVERSITY OF MICHIGAN]

Three Schiff Base Types Formed by Amino Acids, Peptides and Proteins with Pyridoxal and Pyridoxal-5-phosphate[1]

BY HALVOR N. CHRISTENSEN

RECEIVED AUGUST 1, 1957

Amines react with pyridoxal-5-phosphate to form yellow Schiff bases which appear to be hydrogen-chelated, as shown earlier for pyridoxal. An accompanying product with maximal absorption at 278 to 285 mμ probably has the same structure lacking the double bond in conjugation with the ring, possibly because of hydration to the carbinolamine. In addition the yellow Schiff base passes gradually over to a variable extent to a third type of Schiff base in which the H-bonding is believed to be lost so that the 3-hydroxypyridine structure is in the zwitterionic form. In this product the bathochromic effect of the aldimine double bond is small or absent, perhaps also because of conversion to the carbinolamine. Upon protonation spectrophotometric evidence for reversion to the hydrogen-bonded form is obtained. The conversion to the non-hydrogen bonded form is attributed to the electron-attracting action of vicinal groups, especially the carbonyl group of amino acid esters and peptides. Proteins show analogous reactions, bovine serum albumin binding pyridoxal phosphate very tightly mostly as the non-hydrogen-bonded Schiff base.

The yellow Schiff base formed when pyridoxal (pl) reacts with amino acids has its H^+-dissociations oddly displaced from their positions in pyridoxal.[2] A pK' of 10.5 is ascribed by Metzler to the *phenolic* group (4.2 in pyridoxal), H-bonding to the imine-N (formula IIC, Fig. 1) serving to explain both the firmness with which this H^+ is held and the presence of an absorption band at about 415 mμ.[2] An additional consequence is the dissociation of the *pyridinium*-H^+ at about *p*H 5.9 rather than at 8.6 as in pyridoxal. A similar situation now has been found to apply to the Schiff base of pyridoxal-5-phosphate with valine. With pK' values of 5.9 and 10.5 for the pyridine N and the phenolic group of pl in that order very little of the Schiff base would ordinarily be expected to exist as the zwitterion.

A second much less abundant product of the reaction which absorbs maximally at 330 mμ was also noted by Metzler.[2] He proposed an amino-acetal structure (the hemiacetal corresponding to the carbinolamine form of the Schiff base, structure VI Fig. 1), taking into account the reactivity

(1) Supported in part by a Grant (C-2645) from the National Cancer Institute, National Institutes of Health, U. S. Public Health Service.

(2) D. E. Metzler, THIS JOURNAL, **79**, 485 (1957).

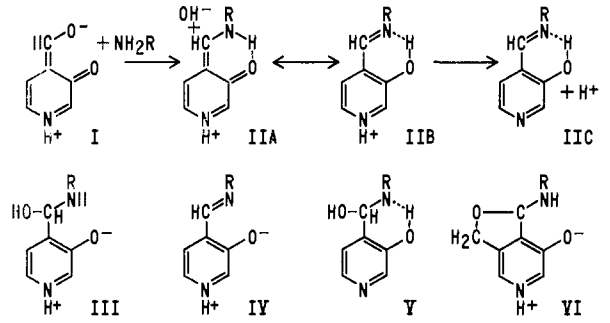

Fig. 1.—A proposal as to nature of reactions. Pl or plp adds to the amine to form the hydrogen-bonded yellow imine IIA-B. Either the chelated proton or the proton on the pyridine N is lost depending upon which is held more firmly, to form either IIC or IV. IV may pass to the carbinolamine form III, or for other reasons assume a form with λ_{max} = 330 to 335 mμ. IIC may likewise hydrate to V to absorb maximally at 278 to 285 mμ. In the case of pyridoxal, III and V presumably would exist as aminoacetals like VI (uninvolved side-chains have been omitted).

of pyridoxal with sarcosine in alkaline solution.[2,3] Four lines of evidence have now led us to reconsider the structure of this product, as well as of a similar one formed by pyridoxal-5-phosphate (plp): (1) Sarcosine and proline react only very slightly if at all with pl or plp at *p*H 7.5; (2) plp forms a very similar product with amines (which often becomes the principal product with peptides, amino acid esters and amides and also with proteins), although the aminoacetal structure is impossible with plp; (3) infrared study supports the presence of an aldimine structure in the crystalline potassium salt of pyridoxylidene-DL-leucylglycylglycine; (4) titration places the *pK*'s of the colorless product at about 5.0 and 9.0, with losses in the 330 absorption characteristic of the zwitterionic structure of pyridoxal upon either acidification or alkalinization; however, appearance of absorption at 400 mμ on acidification suggests that the added H^+ becomes bonded between the phenolic O and imine N.

Evidence for the Character of the Schiff Bases from Ultraviolet Spectra.—The present investigation was stimulated by the observation that bovine serum albumin first reacts in about 2 min. with plp to form a Schiff base absorbing maximally at about 415 mμ, but then this yellow product passes more gradually to a colorless form absorbing maximally at 335 mμ (Fig. 2), the rate being that of a first-order reversible reaction (Fig. 3). Upon acidification the absorbancy at 400 mμ again increases. Taking into account the much less extensive reaction with ovalbumin (which lacks a terminal amino group), the yellow Schiff base only being detected, it is tentatively concluded that this conversion occurred with the Schiff base of the terminal (aspartyl) amino group of serum albumin and not with lysine ε-amino groups. Distal amino groups would be expected from the present results to react less extensively and their Schiff bases to remain in the yellow form. Upon this basis a preliminary

(3) H. N. Christensen and T. R. Riggs, *J. Biol. Chem.*, **220**, 265 (1956).

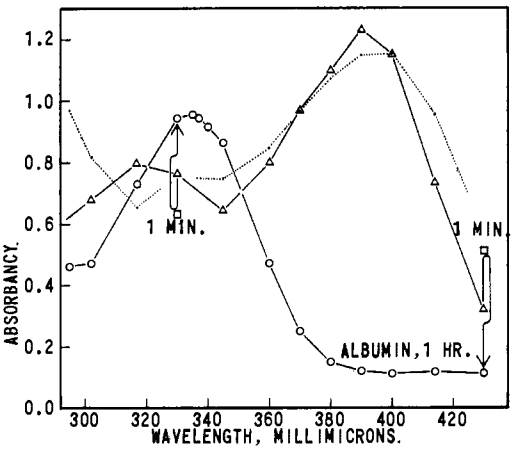

Fig. 2.—Spectral changes when plp reacts with bovine serum albumin at *p*H 7.50: Δ—Δ, spectrum of 0.3 m*M* plp solution. The squares show how the density at 430 mμ rose and that at 330 mμ fell in one minute. After 100 seconds these changes were reversed and at 1 hour the steady state marked by circles was reached. The line ..... shows how the yellow color returned upon subsequent adjustment of the *p*H to 4.46.

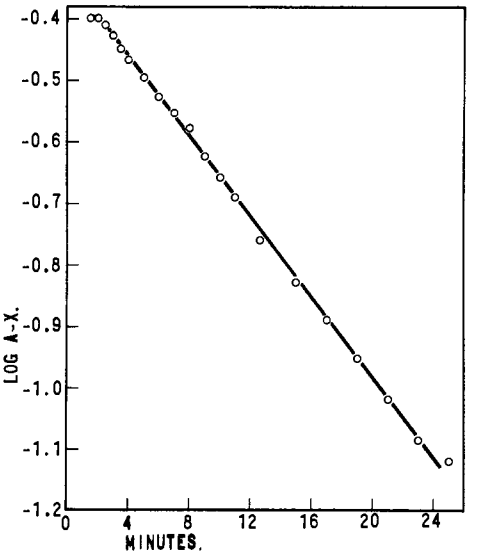

Fig. 3.—Kinetics of the loss of absorbancy at 430 mμ during reaction of plp with bovine serum albumin: $(a - x)$ = the absorbancy at time t, less the absorbancy at a steady state at 2 hours; potassium phosphate buffer, *p*H 7.50, $\Gamma/2$ = 0.20.

estimate places the formation constant [plp-albumin (= plp loss)]/[plp][albumin] near 10^5.

The same kind of behavior of plp was observed with DL-leucylglycylglycine, the absorption at 430 mμ likewise fading with first-order reversible kinetics (Fig. 4). Pyridoxal reacted more slowly with serum albumin, with high absorption appearing at both 330 and 415 mμ.

The behavior of a group of peptides was surveyed at various concentrations at *p*H 7.50 in a potassium phosphate buffer, $\Gamma/2$ = 0.2, pl or plp being present at 0.0002 *M* concentrations. Peptides, proteins and amines in general were found to react much more extensively with plp than with

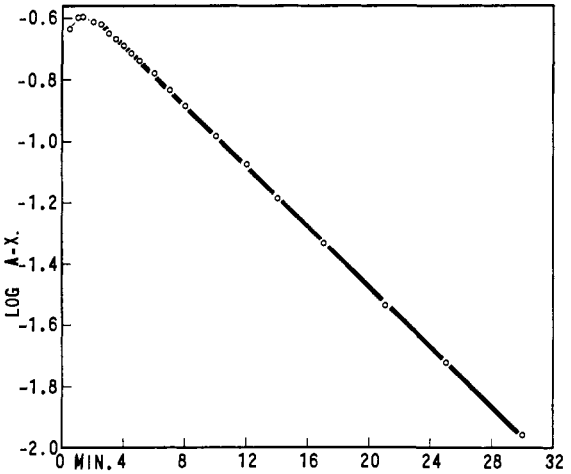

Fig. 4.—Kinetics of the gain and loss of absorbancy of 430 mμ during reaction of plp with DL-Leu·Gly·Gly: the fading measures the conversion of the yellow to the colorless Schiff base; conditions as in Fig. 3.

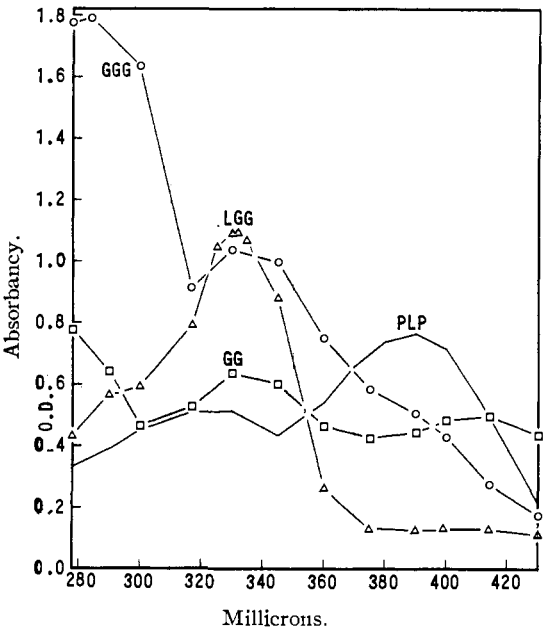

Fig. 5.—Spectra of products of reactions of some peptides with plp: The spectrum of plp (0.2 mM) is shown as a solid line. The curve marked with squares shows the conversion of α-glutamylglutamic acid partially to the yellow Schiff base, partially to the form absorbing at 330 mμ and partially to the form absorbing at 278 mμ, and is characteristic for many peptides. The curve marked with triangles shows the conversion of DL-Leu·Gly·Gly almost entirely to the colorless product. The curve marked with circles shows the unusual absorbancy at 284 mμ produced by Gly.Gly.Gly and plp. Peptide concentrations 0.1 M in a potassium phosphate buffer, pH 7.50, $\Gamma/2$ = 0.20.

pl. The same conclusion may be drawn for amino acids comparing the results of Metzler[1] with those of Matsuo.[4] The peptides (and proteins) usually bound plp much more tightly than did the amino acids.

Figure 5 pictures several of the types of spectra produced with plp. Only the atypical carnosine remained almost entirely in the yellow Schiff base, like an amino acid and with a low stability (the formation constant [plp-peptide]/[plp][peptide] from the loss of plp absorption at 380 mμ was less than 1). At the opposite extreme DL-Ala·Gly·Gly as well as DL-Leu·Gly·Gly went almost entirely over to the form absorbing at about 330 mμ and reacted far more completely with the two aldehydes. The molar extinctions at 330 mμ were estimated at 5450 and 4600 for the plp derivatives and about 6000 for the pl derivatives. Gly·Gly, L-Leu·L-Leu, DL-Ala·Gly, Gly·DL-Ala and L-Ala·L-Leu, all formed roughly as much of the Schiff base absorbing at 330 mμ as of the one absorbing at about 415 mμ.

Matsuo[4] showed that when amines and amino acids[5] react with plp to form the yellow Schiff base, the extent of reaction could be estimated by the increase at a second absorption maximum of unidentified origin at about 278 mμ, this peak having an intensity similar to the one at 415 mμ. With the present group of peptides the density at 415 mμ was always less than that at 278 mμ. α-L-Glu·Glu, Gly·Tyr and Gly·Phe like the preceding group yielded peaks at 330 mμ about as high as at 278 mμ, but the absorption at 415 mμ was conspicuously small. In the cases of Gly·DL-Leu and Gly·Gly·Gly absorption in the two regions 278 and 415 mμ was clearly caused by two different substances, the formation rates and constants being obviously different. The density at 430 mμ rose in the first minute after mixing and then decreased while the density at 285 mμ continued to rise. Furthermore, the yellow Schiff base remained present at equilibrium in 10 to 30 mM Gly·Leu but almost disappeared at a 100 mM level; the density at 278 mμ increased by 64% over the latter concentration interval. Other glycine dipeptides and Gly·Gly·Gly·Gly were more typical in the relationship among the heights of the peaks. The behavior of Gly·Leu and Gly·Gly·Gly is probably only an exaggeration of the tendency of peptides to form imines absorbing more strongly at 280 than at 415 mμ. Comparison with the spectral behavior of "β-pyridoxylserine"[6] does not suggest that derivatives of this character have been formed. Acidification or alkalinization eliminated the 285 peak of Gly·Leu, just as for plp–valine and at similar pH values; coincidentally with Gly·Leu peaks at about 337 and 342 mμ, respectively, appeared.

With pyridoxal, various peptides yielded results parallel to those with plp (Fig. 6). Leu·Gly·Gly, Ala·Gly·Gly and Gly·Gly·Gly reacted strongly; with the first two a maximum at about 330 replaced the 317 mμ maximum of pl, although not quite completely at 0.1 M peptide concentrations. Only slight rises occurred at 278 and 414 mμ. Gly·Gly·Gly gave a peak absorbancy at 286 mμ with successively lower shoulders in the 317–330 and 360–380 regions.

(4) Y. Matsuo, THIS JOURNAL, **79**, 2011 (1957).

(5) The inflection point for plp-glycine seen in Matsuo's curves at 330 mμ probably represents the colorless Schiff base rather than residual plp. Other amino acids show a similar low peak.

(6) D. E. Metzler, J. B. Longenecker and E. E. Snell, THIS JOURNAL, **76**, 639 (1954).

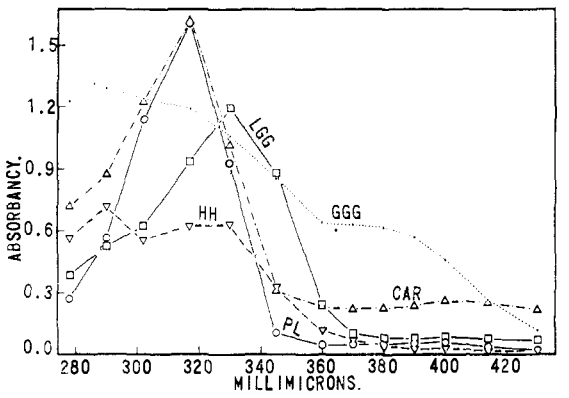

Fig. 6.—Spectra of products of reactions of some peptides with pl: The spectrum of pl (0.2 mM) is marked with circles; △·—·△, carnosine, 0.05 M, showing small formation of yellow Schiff base; ▽---▽, Leu·Gly·Gly, 0.1 M, showing major conversion to the form absorbing at about 330 mμ; ....., Gly·Gly·Gly, 0.1 M; and ▽---▽, His·His, 0.05 M, showing special products of the reactions.

Gly·Phe, Gly·Gly, Gly·Ala, Ala·Gly and Leu·Leu reacted moderately to produce peaks at about 278 and 415 mμ, but also produced more density at 330 mμ than could be caused by the residual free pl. The extent of reaction decreased in the order listed. The reactions of Ala·Leu and α-Glu·Glu were still weaker. Carnosine reacted slightly with increased absorbancy at 278 and 414 mμ but not at 330 mμ. The extent to which pl and plp react with peptides tends to correlate with the preference for the "330" form; probably the yellow Schiff base is an intermediate in the dissociation as well as formation of this colorless product.

Histidylhistidine quickly eliminated the absorption characteristic of pl or plp with the appearance of absorption maxima at 280 and 324 mμ. From the loss of plp absorption at 390 mμ one obtains a formation constant of about 8000 for the sum of reaction products. A crystalline product of the reaction of pl and His·His in methanol gave a spectrum at pH 7.5 very much like that of the aqueous reaction mixture. The similarly formed crystalline product from L-histidine, 4-(2-methyl-3-hydroxy-5-hydroxymethyl-4-pyridyl)-1-imidazo-[C]tetrahydropyridine-6-carboxylic acid,[7,8] showed an absorption maximum at 320 mμ. From the similar modes of preparation and the infrared spectra the two are believed to have similar structures.

Equilibrium Constants.—In the foregoing experiments, when the peptide level was varied in the presence of 0.2 mM plp or pl at pH 7.50, Γ/2 = 0.20, the equilibrium "constants" obtained spectrophotometrically tended to decrease rapidly with rising peptide concentrations. The ionic strength contributed by the zwitterionic peptide had, however, been ignored in these experiments. To establish the equilibrium nature of the reactions, four experiments were run, using the contrasting reactions of Leu·Gly·Gly, Leu·Leu, Gly·Gly·Gly·Gly and Ala·Gly, and adding the relatively inert proline in sufficient quantity to maintain constant zwitterion concentrations of 0.050 or 0.100 M. As Table I illustrates, steady constants were calculated from the loss of plp under these conditions. In the case of Ala·Gly, half-formation of two of the products (one measured at 330 mμ, the other at 278 mμ) occurred at almost identical peptide concentrations.

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

(8) H. N. Christensen, *ibid.*, **79**, 4073 (1957).

TABLE I

FORMATION CONSTANTS FOR ALL REACTION PRODUCTS FROM Plp AND SOME PEPTIDES AT pH 7.50

Phosphate buffer, pH 7.50, Γ/2 = 0.2, containing in addition proline and zwitterionic peptide to total 0.05 M (leucine peptides) or 0.10 M (other peptides); K = [Schiff bases]/[plp][peptide], calculated from the loss of absorbancy at 380 mμ, which served to measure plp loss. The densities corresponding to 100% conversion were obtained with 0.05 and 0.10 M peptide solutions, these values being successively corrected using the tentative K obtained.

Peptide concn., mM/l.	Leu·Gly·Gly	Gly·Gly··Gly·Gly	Leu·Leu	Ala·Gly
20	..	465	..	145
10	..	..	..	142
5	2560	542	439	153
2	2608	508	421	156
1	2600	580	426	154
0.5	2511	..	400	..

Reversibility was also confirmed by showing that pl reached the same distribution when bovine serum albumin was dialyzed against a phosphate buffer, whether the pl was added to the non-protein phase or first permitted to react with the protein.

Infrared Evidence of Structure of the "330" Product.—The crystalline pl-Leu·Gly·Gly potassium salt gave a band at 1585 cm.$^{-1}$ suggesting a double bond in conjugation with the pyridine ring. This was interpreted as an uncomplicated aldimine by Dr. J. L. Johnson of the Upjohn Co., to whom the author is indebted for this result.

Titrimetric Evidence of Structure of the "330" Product.—The colorless plp-Leu·Gly·Gly was sufficiently stable so that it could be titrated at least to pH 10, and, in the presence of 0.1 M Leu·Gly·Gly, to pH 4.0 without so much destabilization as to confuse the localization of the H$^+$-dissociations. Plp itself was first titrated as a standard. At a 0.01 M concentration we obtained the approximate values $pK_2' = 4.0$, $pK_3' = 6.4$, $pK_4' = 8.4$ (Γ/2 = 0.15 − 0.20, Fig. 7). The 330 mμ absorption of plp was lowered by either acidification or alkalinization, in close association with the pK_2' and pK_4' titrations, which are thereby identified with the phenolic –OH and pyridinium groups, respectively. A peak at 294 mμ appeared with acidification in correspondence with pK_2'; meanwhile the absorbancy at 390 mμ was nearly eliminated. The 390 mμ band was further strengthened by alkalinization in association with pK_4'. Undoubtedly this band is associated with the resonance of the aldehyde group.[9] No significant absorption changes occurred in the neighborhood of pK_3, which is not unexpected for the secondary phosphoric dissociation.

The plp-valine Schiff base, in contrast to plp-Leu·Gly·Gly, showed pK's of about 6.3 and 11.5 for the elimination by acid or alkali of the peak at 278 mμ. The latter pK' also was obtained for the

(9) D. E. Metzler and E. E. Snell, *ibid.*, **77**, 2481 (1955).

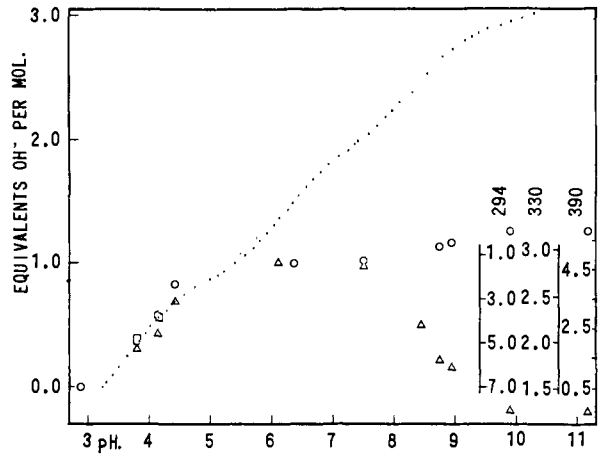

Fig. 7.—Portion of the titration curve of plp: The dots show observations of the *p*H *versus* the quantity of KOH added, left-hand scale, $\Gamma/2 = 0.15$–0.20. The triangles show the change of molar absorbancy $\times 10^{-3}$ at 330 mμ with *p*H, middle scale at the right. The squares show the change of molar absorbancy $\times 10^{-3}$ at 294 mμ with *p*H, first scale at the right. The circles show the change of the molar absorbancy $\times 10^{-3}$ at 390 mμ, scale at extreme right. The ionic strength was 0.10 for the spectral observations.

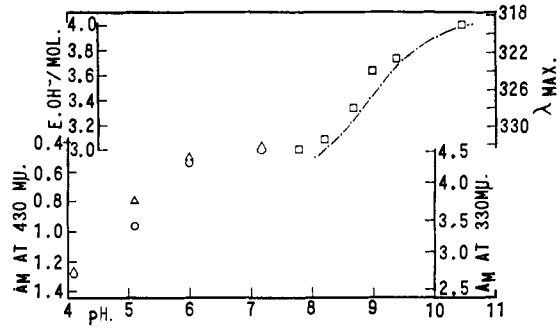

Fig. 8.—Approximate positions attributed to pK'_3 and pK'_5 of plp-Leu·Gly·Gly: The solid line joining the dots shows the *p*H values reached during addition of the fourth equivalent of KOH to an equimolar mixture of plp and Leu·Gly·Gly, after allowing 2 hours for reaction with the first 2 equivalents of KOH. The squares show the shift in the position of the λ_{max} from 331 to 318 mμ during this titration, scale at right, $\Gamma/2 = 0.2$. The triangles show the loss of absorbancy at 330 mμ (scale at right), the circles the gain of absorbancy at 430 mμ (scale at left) upon acidification of the product formed at *p*H 7.5. A pK'_4 attributed to the secondary dissociation of the esterified phosphoric acid presumably lies between the two dissociations shown.

decrease of the density at 415 mμ on adding alkali. Acidification lowered the 415 mμ peak to the extent plp was released. This behavior is interpreted to mean that this Schiff base, like pl-valine,[2] has the order of the two dissociations of 3-hydroxypyridine reversed by H-bonding. The upper *pK* of the plp-valine Schiff base was observed by direct titration as well as by spectrophotometry. To a solution containing 0.05 molar valine and plp was added 2 equivalents of KOH. Two hours later further alkali titration showed a fairly distinct group (the fourth equivalent of alkali added; not pictured) with an approximate *pK* of 10.8. This result is not necessarily inconsistent with the spectrophotometric determination because of ionic strength differences (0.42 for the direct titration, *versus* 0.10). Because tri- and tetravalent ions are involved, a high sensitivity to ionic strength may be anticipated.

For plp-Leu·Gly·Gly, a *pK'* at about 9.0 by direct alkali titration was attributed to the dissociation of the pyridinium ion (Fig. 8). Its titration was associated with a fall in the position of the absorption maximum from 332 to 318 mμ. Such changes have been observed for the colorless form of pl-glycine,[2] and various zwitterionic analogs of pl.[8] The titration with acid produced a distinct yellowing (measured at 430 mμ, although the maximum as for serum albumin was at about 400 mμ) with its midpoint at roughly *p*H 5.1. The tentative conclusion is drawn that the H^+ adding to the phenolic oxygen bonds also to the imine N, the 415 band having been associated by Metzler[2] with the H-chelated Schiff base. The suggested H-bonding thus appears to be sufficiently strong to increase by nearly 10 times the affinity of the phenoxide ion for H^+; but not strong enough to hold this proton more firmly than the one on the pyridine N.

The simplest theory as to the nature of the colorless form of the Schiff base forming with Leu·Gly·Gly and other amines is that it is the uncomplicated aldimine IV, that is, a non-hydrogen-bonded zwitterion. The infrared spectrum agrees with this thesis, and the ultraviolet spectral changes on titration support a zwitterionic structure. If for some reason the imine-N fails to hydrogen-bond strongly enough to cause a proton to be retained on the phenolic oxygen, then the first proton would add to the pyridine N instead, making much more difficult the subsequent addition of a proton to the phenolic oxygen. To put the matter in another way, when pl or plp reacts with an amine at neutral *p*H, if the primarily formed Schiff base (IIA–C Fig. 1) is to survive, the chelated proton must be held firmly enough to displace the other proton from the pyridine N. A similar consideration applies in determining how metals chelate to pyridoxylidene amino acids.[8]

If the colorless Schiff base derivatives, for example, plp-Leu·Gly·Gly, are zwitterionic non-hydrogen-bonded aldimines, the λ_{max} would be expected to be increased from the values of 324 to 330 mμ characteristic of pyridoxal derivatives lacking the double bond in conjugation with the ring.[9] The found values of 331 to 335 mμ seem rather low. The aldimine double bond could be lost by hydration in solution to the carbinolamine form, even though plp has no hydroxymethyl group to stabilize that structure. The less extensive formation with pl than with plp argues somewhat against this view. This effect and also the lack of reactivity of proline and sarcosine could arise from inability to form the intermediate yellow Schiff base. Both of these amino acids form metal-chelated pyridoxylidene derivatives.[3,8]

The double bond probably has not shifted out of conjugation with the ring to form a pyridoxamine derivative (or a cyclized product derived from this[10]) because the pyridoxal can readily be re-

(10) B. Witkop and T. W. Beiler, THIS JOURNAL, **76**, 5589 (1954).

covered upon adding excess ethanolamine to crystallized pl-Leu·Gly·Gly (see below). Other cyclization reactions proposed by Witkop and Beiler also seem unlikely.[10] Neighboring amino groups might permit ring closure to imidazolidine derivatives[11]; hydroxyl groups might react similarly, but this seems to the writer less likely for an adjoining carbonyl group, taking enolization into account. A possibly related, unexplained yellowing upon rubbing of a colorless reaction product of *p*-hydroxybenzaldehyde and *o*-aminobenzyl alcohol was considered not to result from the change of a cyclic form to a yellow Schiff base.[10]

Consideration also needs to be given to the unexplained product absorbing at 278 to 285 mμ, formed in amounts similar to the hydrogen-bonded Schiff base from free amino acids, but more abundant than the yellow form with most peptides. Metzler and Snell[9] have shown that pyridoxal has an absorption maximum at 280 mμ in 60% dioxane and conclude that it passes over to a non-zwitterionic form with increasing alcohol or dioxane concentrations. Similar changes were reported with other B_6 derivatives. Hydrogen bonding of the Schiff bases likewise makes non-zwitterionic the 3-hydroxypyridine derivatives; accordingly the writer proposes that the form absorbing at 278 to 285 mμ is the hydrogen-bonded Schiff base which has lost the double bond in conjugation with the ring, presumably by the same process by which the non-hydrogen-bonded form appears to have lost its double bond. The dissociations of this form lie at *p*H values supporting a hydrogen-bonded structure. Hydration to the carbinolamine is presently regarded as the most probable reason for the loss of the bathochromic effect of the double bond in conjugation. Presumably the aldimine structure is stabilized by H-bonding, and tends to change to the carbinolamine form when the H-bonding is weakened.

Under this interpretation the addition of a hydrogen ion to the colorless form III (Fig. 1) would tend to cause a carbinolamine-to-aldimine conversion. Spectral changes during protonation of cupric pyridoxylidinevaline were attributed to a similar conversion. Jenkins and Sizer[12] have reported in a preliminary note that a change occurs in the spectrum of glutamic-oxaloacetic transaminase in going from *p*H 8.5 to 4.5, with the appearance of a yellow color, like that observed here on the titration of plp-Leu·Gly·Gly. A difference is that the λ_{max} for the transaminase at *p*H 8.5 lies at 363 rather than at 330–335 mμ. Presumably, as they propose, this Schiff base remains largely in the aldimine form (IV), not hydrogen bonded until the $[H^+]$ is increased. The present results suggest that the behavior they report for this enzyme might arise from the presence of an N-terminal amino group, spatially situated in this enzyme so as to form the non-hydrogen-bonded Schiff base with plp, which is already bound at an adjacent point. An interesting question is whether this arrangement facilitates or hampers Schiff base formation with glutamate. Furthermore, the observed reactivity of various proteins with pl and plp must complicate the biological vitamin B_6 economy. It may also explain the presence of plp in phosphorylase.[13]

Why do the peptide and protein derivatives pass over to the "330" form? The same tendency to assume this colorless form was observed with amino acid amides and esters. For example the spectrum of plp-L-leucine amide was very similar to that of plp-L-Leu·L-Leu. Glycine ethyl ester and glycine amide reacted strongly with the aldehydes (formation constants from plp losses, about 1800 and 3700, respectively) forming peaks at 330 as well as at 284 and 414 mμ. Also in analogy to several of the glycine peptides, glycine amide and glycine ethyl ester gave far higher densities at 284 mμ maxima than at 415 mμ.

Structures other than –COOR and –CONHR which intensified the formation of the non-hydrogen-bonded product included $-CHRNH_3^+$ (ethylenediamine, diaminopropionic acid, α,γ-diaminobutyric acid) and $-C_6H_5$ (in aniline but scarcely in benzylamine). All these effective groups are sufficiently strong electron-attracting groups to lower the pK' of the amino group to the neighborhood of 8 or lower; the lowered electronegativity of the imine-nitrogen presumably prevents strong hydrogen-bonding. An intermediate proportion of the colorless product was obtained with pyridyl-2-alanine. Not effective were $-CH_2OH$ (in ethanolamine, α-methylserine and tris-(hydroxymethyl)-aminomethane), $-CH_2Br$ (in bromoethylamine) of $CH_2 = CH-$ (in allylamine). These are in general weaker electron-attracting groups, presumably not able to prevent the imine-N from hydrogen bonding.

One apparent inconsistency was the ineffectiveness of tris-(hydroxymethyl)-aminomethane in permitting conversion to the colorless form. The electron-withdrawing effect here is enough to bring the pK' to a value very close to that for glycylglycine, yet the latter produces considerably more absorption at 330 mμ. Steric factors may discriminate against formation of the non-hydrogen-bonded product in this case. For example, the methyl ester of α-aminoisobutyric acid formed the lowest proportion of this colorless product of any α-amino acid ester studied. It is concluded that the electron-attracting influence of neighboring groups is the principal factor in encouraging passage to the form absorbing at about 330 mμ, but that other structural features are significant.

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Experimental

Chemicals.—Pyridoxal·HCl (a gift of Merck and Co.) was converted to the free base with KOH as usual. Pyridoxal-5-phosphate was obtained from the California Foundation for Biochemical Research, and the purity confirmed by the spectrum in 0.1 *N* HCl. For significant observations solutions prepared within 2 hours were used, although freshly frozen solutions could be used a day or two later. To spare material, small weighings were made and the re-

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sultant concentration checked spectrophotometrically, preferably at 295 mμ in 0.1 *N* acid.

The peptides used were of excellent appearance; several were checked and gave the theoretical titration results. The DL-leucylglycylglycine was from two sources, Hoffman-La Roche and Nutritional Biochemicals. The other peptides were from either of these sources or the Mann Research Laboratories, except that α-glutamylglutamic acid was a gift of Dr. J. W. Hinman, The Upjohn Co. Histidylhistidine was not labeled as to configuration; other optically active peptides were DL-forms except for Glu·Glu, Leu·Leu and Ala·Leu which were indicated to have the L-configuration.

Crystalline bovine serum albumin was obtained from the Armour Co.; crystalline zinc insulin was the gift of Dr. Otto Behrens of the Eli Lilly Co. The following were prepared in this Laboratory: α-aminoisobutyric acid methyl ester, *m*-nitrophenylglycine, α-methylserine and crystalline ovalbumin and β-lactoglobulin. L-Leucine amide acetate was a gift of Dr. Paul Zamecnik; 2-pyridylalanine, of Dr. Carl Niemann.

Instruments.—A Beckman DU ultraviolet spectrophotometer was used with silica cells, l = 1 cm. The temperature throughout was regulated at 24 ± 1°. A Beckman model G *p*H meter and glass electrode were used.

Procedures.—Peptide solutions were adjusted to *p*H 7.50 (or other values) using KOH or perchloric acid. Buffering was by the peptide itself (*p*H 3 to 5, 8 to 10) or acetate, phosphate or bicarbonate–carbonate buffers. The comparisons at *p*H 7.50 were made at $\Gamma/2 = 0.20$, established by the potassium phosphate; comparisons of spectra at various *p*H values were made at $\Gamma/2 = 0.10$, established by the buffer plus $KClO_4$ as required. The ionic strength contributed by zwitterions was neglected in these values. Electrometric titrations were made with *N* KOH or HCl delivered by an Agla syringe buret.

Spectral study was made between 1 and 2 hr. after mixing except as stated, the optical densities being checked for stability. For the determination of stability constants, proline was introduced into the solutions to maintain a constant zwitterion concentration, usually 0.05 or 0.10 *M*. The loss of plp at 380 mμ, or of pl at 317 mμ was used to calculate the extent of reaction of the aldehyde. A preliminary value for the absorbancy of the products at complete reaction was obtained at the highest peptide level chosen; the resultant preliminary constant permitted an improved value for the absorbancy of complete reaction and in turn successively more accurate constants. The molar absorbancies of the various types of product did not appear to be constant enough from peptide to peptide to justify calculation of equilibrium constants for each reaction course.

Preparation of Potassium Salt of Pyridoxylidene-DL-Leu·Gly·Gly.—The tripeptide and pl (0.1 millimole of each) were dissolved in 0.1 ml. of *M* KOH in methanol. After 2 hours, dropwise addition of ether caused crystallization in rosettes of needles. The product showed a 1:1:1 theoretical content of pyridoxal,[14] Leu·Gly·Gly (method of Moore and Stein,[15] Leu·Gly·Gly standard) and of potassium (by flame photometry). Infrared spectra were obtained in Nujol mulls. The parallel derivatives of DL-Ala·Gly·Gly and Gly·Gly·Gly were obtained in a similar way, as was also a pyridoxal derivative of histidylhistidine. The infrared spectra of pl-Leu·Gly·Gly and pl-Ala·Gly·Gly were very similar, whereas those of the other 2 products were distinctive.

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOLOGY, BROOKHAVEN NATIONAL LABORATORY, THE ATOMIC ENERGY PROJECT UNIVERSITY OF CALIFORNIA AT LOS ANGELES AND THE BIOLOGICAL LABORATORIES, HARVARD UNIVERSITY]

Absence of Phosphotriester Linkages in Tobacco Mosaic Virus

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The protein coat of tobacco mosaic virus was separated from its ribonucleic acid by the action of detergent in the presence of H_2O^{18}. Assay of the RNA phosphate showed no detectable incorporation of O^{18} as a result of the separation. It is concluded that few, if any, triply esterified phosphates are present in the tobacco mosaic virus and that the drop in *p*H observed during detergent action probably is due to unmasking of acid groups.

It has been observed that solutions of tobacco mosaic virus (TMV) undergo a considerable fall in *p*H upon heating[1] or treatment with long chain alkyl sulfates.[2] The magnitude of this reaction suggests the unmasking or formation of large numbers of acidic groups of an unknown nature. This may involve the nucleic acid or protein moieties or both. One possibility is that upon denaturation and dissociation of the virus nucleoprotein the free nucleic acid undergoes phosphate ester bond hydrolysis. Since the molecular weight of the nucleic acids isolated by these various techniques is high, from 2.5×10^5 [3] to 1.7×10^6,[4] major hydrolysis of backbone phosphate ester bonds is precluded. However, the possibility of phosphotriester bonds, either with active groups on the protein or with active groups in the nucleotides, has not been excluded. While the mildness of the chemical treatment might ordinarily suggest unmasking rather than covalent bond rupture, the marked chemical lability of phosphotriesters[5] and the fact that thio[6] and amido[7] phosphates are even more labile than the oxygen esters means that the occurrence of phosphotriesters in the native TMV cannot be excluded as an explanation of the *p*H shift.

Because of its importance to virus structure and protein synthesis, the presence or absence of these bonds was tested by dissociating the ribonucleic acid (RNA) of TMV from its protein coat in the presence of H_2O^{18}. The presence and number of triester bonds would then be indicated by the O^{18} content of the isolated RNA phosphate.

Experimental

Separation of RNA from TMV in Presence of H_2O^{18}.—TMV was isolated from the juice of infected tobacco leaves by repeated differential centrifugation. The clear pellets, 0.5-g. and 1.0-g. portions, were suspended in 35 ml. of water

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