The synthetic racemic monoester had the same absorption spectrum. Optically active sodium monoethyl α-acetamidomalonate (experiment L24) showed, in D_2O, absorption bands at 2.95–3.05 μ (w), 5.82 (m), 6.18 (s), 6.78–6.90 (w), 7.25 (m), 7.34 (m), 7.55 (w). The synthetic racemic salt had the same absorption spectrum.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF CHICAGO, CHICAGO, ILLINOIS]

Reaction of Pyridoxal-5-phosphate with Aminothiols

BY MARY V. BUELL AND RAYMOND E. HANSEN

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A study of the reaction between pyridoxal-5-phosphate and aminothiols was prompted by the observation that pyridoxal-5-phosphate is released in the form of a complex with cysteine when muscle phsphorylase *a* is incubated in a cysteine buffer at *p*H 6.8. In aqueous media over a wide range of *p*H values the 4-formyl group of pyridoxal-5-phosphate reacts with the sulfhydryl group of an addend that contains no amino group to form a highly dissociable thiohemiacetal; it also reacts with the amino group of an addend that contains no sulfhydryl group to form a highly dissociable Schiff base. When both functional groups are present in suitable proximity on the same addend, the product is a relatively stable complex containing a thiazolidine ring. The second order constants describing the rates of complex formation between pyridoxal-5-phosphate and both cysteine and its ethyl ester have been determined as a function of *p*H and compared with those found for the same reactions with 5-deoxypyridoxal. The results are believed to bear upon the bonding of pyridoxal-5-phosphate in muscle phosphorylase.

Introduction

When aldehydes react with amines highly dissociable Schiff bases are formed; when they react with mercaptans thiohemiacetals are formed that are also highly dissociable. Schubert[1] described a relatively stable product containing a thiazolidine ring that was formed when aldehydes react in aqueous solution with those aminothiols in which the two functional groups are in suitable proximity. Heyl, *et al.*,[2] studied the products formed when pyridoxal reacts in organic solvents with twenty-three amino acids; only with three, namely cysteine, histidine and penacillamine, was condensation with ring closure observed. These reactions were not carried out in aqueous solutions or with other 4-formyl pyridine analogs; nor were the products characterized spectrophotometrically.

Extensive studies of the absorption spectra of 3-hydroxypyridine analogs have been made by Harris, *et al.*,[3] Peterson and Sober,[4] and Metzler and Snell.[5] The latter authors discussed changes in spectra associated with ionization of the phenolic hydroxyl group, loss of the proton from the pyridinium nitrogen and introduction of a formyl group at position 4. They pointed out the marked similarities between the spectra of pyridoxal-5-phosphate and 5-deoxypyridoxal. Similarities in spectra are to be expected since the structures differ only in the substituents at position 5, namely a methylene phosphate group in the former, a methyl group in the latter. Importantly, neither of these groups can form a hemiacetal with the formyl group at position 4, in contrast with the 5-hydroxymethyl group of pyridoxal. Unlike pyridoxal, which at *p*H 7.0 shows little absorbancy near 380 mμ, the other 4-formyl analogs mentioned have bands with high absorbancies and maxima at or near this wave length. This fundamental difference is attributed to the predominance of the hemiacetal structure of pyridoxal,[6] in which the carbonyl group is not free.

Studies of the mechanism whereby pyridoxal-5-phosphate functions as coenzyme in the decarboxylation of amino acids have not revealed the direct participation of the essential phosphate group in this type of reaction.[7] More recently pyridoxal-5-phosphate was shown by Baranowski, *et al.*,[8] to be a prosthetic group in muscle phosphorylase, an enzyme that catalyzes a totally different type of reaction. Cori and Illingworth[9] demonstrated that it restores activity to the apoenzymes of both phosphorylases *a* and *b*. Its phosphate group does not exchange either with that of glucose-1-phosphate or with inorganic phosphate in the phosphorolytic reaction.[10] The bonding of pyridoxal-5-phosphate to the apoenzyme has been partially elucidated by Fischer, *et al.*,[11] who showed the formyl carbon to be bonded to the protein both through the ε amino group of a lysyl residue and also through a second group designated as X. The present paper is primarily concerned with model complexes that are formed in aqueous solutions when pyridoxal-5-phosphate and 5-deoxypyridoxal react with aminothiols. It is suggested that in certain respects these complexes simulate those native to muscle phosphorylase.

Advantage is taken of changes in the absorption spectra of pyridoxal-5-phosphate and 5-deoxypyridoxal as they react with aminothiols. In aqueous solutions the carbonyl carbon of both of these pyridine analogs is sp^2-bonded, in contradistinction to that of pyridoxal, which is sp^3-bonded as a consequence of hemiacetal formation.[6] Absorption spectra at *p*H 7.0 of the sp^2-bonded carbonyl car-

(1) N. P. Schubert, *J. Biol. Chem.*, **111**, 671 (1935); *ibid.*, **114**, 341 (1936).

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

(3) S. A. Harris, T. J. Webb and K. Folkers, *ibid.*, **62**, 3198 (1940).

(4) E. A. Peterson and H. A. Sober, *ibid.*, **76**, 169 (1954).

(5) D. E. Metzler and E. E. Snell, *ibid.*, **77**, 2431 (1955).

(6) D. Heyl, E. Luz, S. A. Harris and K. Folkers, *ibid.*, **73**, 3430 (1951).

(7) S. Mandeles, R. Koppelman and M. E. Hanke, *J. Biol. Chem.*, **209**, 327 (1954).

(8) T. Baranowski, B. Illingworth, D. H. Brown and C. F. Cori, *Biochim. Biophys. Acta*, **25**, 16 (1957).

(9) C. F. Cori and B. Illingworth, *Proc. Natl. Acad. Sci.*, *U. S.*, **43**, 547 (1957).

(10) B. Illingworth, H. S. Jansz, D. H. Brown and C. F. Cori, *ibid.*, **44**, 1180 (1958).

(11) E. H. Fischer, A. B. Kent, E. R. Snyder and E. G. Krebs THIS JOURNAL, **80**, 2906 (1958).

bon in this type of compound are characterized by a band with maximum between 375 and 415 mμ; with sp^3-bonding the maximum appears at shorter wave lengths, between 315 and 335 mμ. Combined as imine, the carbonyl carbon is also sp^2-bonded, but after ring closure it becomes sp^3-bonded and the maximum of the absorption band shifts to shorter wave lengths.

Experimental

Spectrophotometric Measurements.—A Cary recording spectrophotometer Model 11 was used only for rapid scanning of spectra during the course of certain reactions, as noted. Otherwise, absorption spectra and kinetic data were taken with a Beckman spectrophotometer Model D.U., in which the calibration of wave length was checked with the lines of hydrogen appearing in the near ultraviolet. Unless otherwise specified the wave lengths mentioned are those observed with the Beckman instrument.

Complex between Pyridoxal-5-phosphate and Cysteine.—The pyridoxal-5-phosphate used was Lot 5440 from the California Corp. for Biochemical Research and Lot 8838 from the Nutritional Biochemicals Corp. When chromatographed on paper each product gave a single spot absorbing ultraviolet light; the absorption spectrum of each product conformed closely to the values found in the literature.[4]

When pyridoxal-5-phosphate reacts with cysteine in aqueous solutions over a wide *p*H range its absorption band with maximum at 380 mμ disappears and the absorbancy of the band with maximum at 325 mμ increases without change in wave length. At *p*H 7.0, when the molar concentration of cysteine was about four times that of pyridoxal-5-phosphate, the rate of reaction was slow enough to be followed conveniently in the Cary instrument. Figure 1 illustrates this experiment in which the procedure was typical of many others. Both cells contained 3.0 ml. of phosphate buffer, $4 \times 10^{-2}M$, *p*H 7.0; to the experimental cell was added a small volume (about 10 μl.) of a freshly prepared stock solution of pridoxal-5-phosphate to make its concentration about $1.5 \times 10^{-4}M$. Curve 1, Fig. 1, shows the spectrum of pyridoxal-5-phosphate taken with the Cary instrument in absence of addend, *i.e.*, at zero time. A small volume, usually ten to twenty μl., of a concentrated solution of cysteine hydrochloride was added to each cell to give the desired relative concentrations of reactants in the experimental cell. Figure 1 shows the changing spectra as the reaction progressed. All curves were started at the long wave length end of the spectrum, the scanning time being about seven minutes. Intervals between the times at which the curves were started varied between ten and twenty minutes. With curve 6 the reaction is approaching equilibrium. In this type of experiment any point on the curves represents the absorbancy only at the instant when it was recorded. Nevertheless, certain significant facts are apparent: (1) The wave lengths at which maxima occur do not change. (2) The decrease in absorbancy at 385 mμ (Cary) is proportional to its increase at 330 mμ (Cary). (3) The isosbestic point suggests that a single equilibrium is being observed between the two chromophores directly concerned in the reaction. (Actually four chromophoric species are present). (4) The band with maximum at 385 mμ (Cary) almost disappears. When homocysteine was used as addend a family of curves was obtained that was qualitatively identical with that found with cysteine but the rate of reaction was much slower.

Reaction of Pyridoxal-5-phosphate with Addends of Different Types.—Figure 1 suggests a reaction between the carbonyl group of pyridoxal-5-phosphate and the sulfhydryl group of cysteine. To test this point experiments were done, with both the Cary and Beckman instruments, with a series of addends that included several types; (1) those having an amino but no sulfhydryl group, (2) those in which sulfhydryl was the only functional group, (3) those having a sulfhydryl as well as other functional groups, but no amino group, (4) those having both an amino and a sulfhydryl group. With alanine and serine at a concentration of 3 $\times 10^{-2}M$ or less the spectra showed the presence of Schiff bases but no increase in absorbancy at 325 mμ. This observation rules out the unlikely possibility that the hydroxyl group of serine might react analogously with the sulfhydryl group of cysteine. With 1-thiopropane and 2-thiopropane the concentrations attainable were limited by solubility

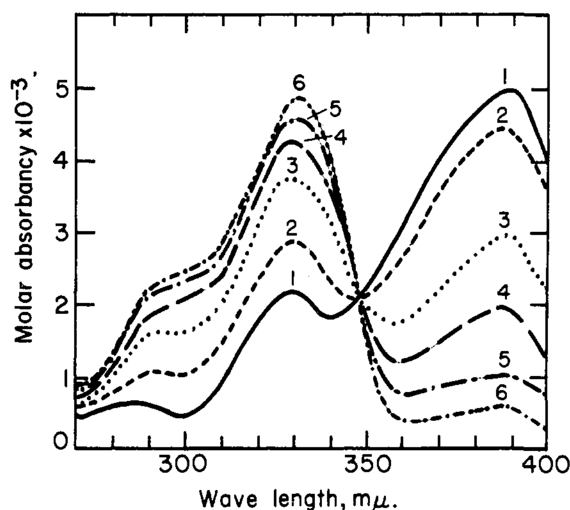


Fig. 1.—Absorption spectra of pyridoxal-5-phosphate scanned with a Cary recording spectrophotometer at intervals during the course of its reaction with four equivalents of cysteine at *p*H 7.0. The numbers beside the curves indicate the order in which the spectra were recorded.

but rapid reaction was clearly indicated by decreased absorbancy at 380 mμ and by increased absorbancy at 325 mμ. With 2-thioacetic acid, 3-thiopropionic acid and 2-thioethanol an increase in absorbancy at 325 mμ was observed only when the concentration of addend was exceedingly high relative to that of pyridoxal-5-phosphate, and at no attainable concentration was the absorbancy of the "380 mμ band" reduced by more than 50 per cent. Equilibrium was attained rapidly but the adduct was highly dissociable. This series of experiments shows that in aqueous solutions at *p*H 7.0 the carbonyl group of pyridoxal-5-phosphate reacts rapidly with an amino group to form a highly dissociable Schiff base and with a sulfhydryl group to form a thiohemiacetal that is also highly dissociable.

When the addend contained both functional groups, as in 2-thioethylamine, cysteine, cysteine ethyl ester and cysteinylglycine the product fell into neither category mentioned. For example, at *p*H 7.0 the relatively stable complex formed with cysteine has an absorption maximum at 325 mμ that almost coincides with that of the thiohemiacetals; this conforms with the premise concerning the spectra of compounds of this type in which the carbonyl carbon is sp^3-bonded. There are such vast differences, however, in the rates at which the products are formed, and more particularly in their stability, that a different type of product must result from reaction with aminothiols, namely one containing a thiazolidine ring. As seen in Fig. 1, the reaction goes almost to completion in the presence of four equivalents of cysteine; with the same amount of an addend having only one of the functional groups no detectable change in spectrum is observed at this concentration.

Stability of Complexes of Aminothiols with Pyridoxal-5-phosphate.—The four aminothiols mentioned above form complexes in which both the nitrogen and sulfur of the addend enter into ring formation with the carbonyl carbon of pyridoxal-5-phosphate. Tables I and II list some of the properties of the complexes formed with pyridoxal-5-phosphate at *p*H 7.0 under standardized conditions. To a solution of pyridoxal-5-phosphate, $1.41 \times 10^{-4}M$ in phosphate buffer, $4 \times 10^{-2}M$, were added 4.5 equivalents of an aminothiol. When complex formation was complete, as judged by the disappearance of absorbancy at 380 mμ, an absorption spectrum was taken to determine the wave length of maximal absorbancy of each complex. To an aliquot of each solution was added a "sulfhydryl reagent" in an amount equimolar with that of the addend; when possible, both the half-time was noted that was required for breaking the thiazolidine ring and also the maximum wave length of the absorption band of the resulting imine. In separate experiments, to be described in a later section, second order constants were determined, describing the rate of complex formation of each addend with pyridoxal-5-

TABLE I

PROPERTIES OF AMINOTHIOLS AND THEIR COMPLEXES WITH PYRIDOXAL-5-PHOSPHATE[a]

	Compound	R group	Stability of addend on standing[b]	Max. rate constant × 10^{-4} mole^{-1} sec.$^{-1}$ of complex formation, 20°[c]	pH of max. rate	Stability of complex on standing (hr.)[d]	Wave length max. of complex (mμ)
I	2-Thioethylamine	–H	+[1]	3.2	9.0	2	326
II	Cysteine	$-COO^-$	+[2]	2.6	9.0	24	325
III	Cysteinylglycine	$-CONHCH_2COO^-$	+[2]	6.4	6.8	>24	330
IV	Cysteine ethyl ester	$-COOC_2H_5$	+[3]	19.3	6.4	>24	329

[a] With the exception of the rate constants, all observations were made at 20° and *p*H 7.0, measured with the glass electrode. [b] The values are qualitative indications of stability judged by the times at which a precipitate appeared. [c] Second order rate constant measured as described in a later section of the text. [d] The values are qualitative indications of stability judged by the time at which a yellow color appeared.

phosphate. For convenience in making comparisons the *p*H at which the reaction rate was maximal and the value of the constant at this *p*H are included in Table I.

The addends themselves are somewhat unstable in buffered aqueous solutions at *p*H 7.0, as shown by the appearance of a precipitate on standing at room temperature (presumably the disulfide form of the compound). By this criterion, the stability of the addends, as numbered in Table I, decreases in the order IV>III>II>I. The relative stability of the complexes was tested in several ways. Visual inspection showed the complexes with IV and III to be colorless after standing at room temperature for twenty-four hours, in contrast to the complex with I which became yellow after two hours. Differences in the wave lengths of the maxima of the absorption bands are small but suggest greater stability of the complexes formed with IV and III as compared with II and I. The complexes formed with all four addends were stable in the presence of iodoacetamide for twenty-four hours; in the presence of parachloromercuribenzoate all four were broken at a rate too fast to measure. Toward N-ethylmaleimide the stability of the complexes varied; results are reported as the time when the reaction was half-completed. An accurate value could not be found for the complex with IV because of an unexpected difficulty in following the reaction (which is being investigated). The relative stability of the complexes with the other addends was III>II>I.

Imine as Requisite Precursor of Complex Formation.—Theoretically, either imine or thiohemiacetal formation might preceed ring closure. Experiments to be described suggest that the former rather than the latter is the intermediate. When 2-thioethylamine, cysteinylglycine or cysteine ethyl ester reacts with pyridoxal-5-phosphate the spectrum of the reaction mixture clearly shows the presence of imine, the amounts observed increasing in the order in which the compounds are mentioned. The absence of Schiff base in the spectrum when the addend is cysteine or homocysteine does not preclude the possibility that imine formation is essential to ring closure; rather, it suggests that the rate of Schiff base formation with these addends limits the rate at which the thiazolidine derivative may be formed. Many factors, to be discussed later, affect the rate of complex formation; among these is the rate of imine formation.

Additional evidence pointing to precedence of imine formation follows. When the addend was histidine no reaction, other than Schiff base formation, was immediately apparent in the spectrum at *p*H 7.0. After ten hours at room temperature, however, imine was no longer observed and ring closure was indicated by increased absorbancy in the band at shorter wave length, the maximum occurring at 321 mμ in this product. No similar product was observed with imidazole either at *p*H 7.0 or at *p*H 5.0, the *p*K value describing the loss of the imidazole proton being 6.95. In the presence of other amino acids in the same reaction mixture the rate of complex formation with cysteine was decreased. For example, when the molar ratios of cysteine, histidine and lysine, relative to that of pyridoxal-5-phosphate, were 10, 25 and 60, respectively, Schiff bases were visible in the spectrum but the rate of complex formation with cysteine was reduced to about one-fourth of that found in the absence of histidine and lysine. These observations, considered together, point to imine formation as an essential step in the reaction of pyridoxal-5-phosphate with aminothiols.

No complex formation was observed at *p*H 7.0 with thiourea or with glutathione; failure of the latter to react may be due to the fact that the cysteinyl residue is not terminal or that its potential sulfhydryl group is in the form of a thiazoline ring.[12]

TABLE II

REACTION OF COMPLEXES WITH "SULFHYDRYL REAGENTS"[a]

	Compound	Half-time required for breaking complex by "sulfhydryl reagents" (min.) CMB[b]	NEM[c]	Wave length max. of imine formed by "sulfhydryl reagent" (mμ) CMB	NEM
I	2-Thioethylamine	<1.0	2.3	388	388
II	Cysteine	<1.0	19.0	385	380
III	Cysteinylglycine	<1.0	22.0	382	381
IV	Cysteine ethyl ester	<1.0	..	375	..

[a] The "sulfhydryl reagents" were present in amounts equimolar with aminothiol. [b] CMB indicates parachlormercuribenzoate. These reactions were carried out at *p*H 8.0 to keep the reagent in solution. [c] NEM indicates N-ethylmaleimide; *p*H 7.0.

Complex Formation with Other Pyridine Analogs.—When cysteine was added to pyridoxal at *p*H 7.0 no change was observed in the absorption spectrum, either in the near ultraviolet where Schiff bases absorb or at shorter wave lengths were the complexes absorb. Perhaps no reaction should be expected inasmuch as the absorbancy of pyridoxal at *p*H 7.0 is minimal at and near 380 mμ, the wave length of maximal absorbancy of the active ionic species of pyridoxal-5-phosphate. Also, at *p*H 10.0 there was no spectrophotometric evidence of complex formation with cysteine in spite of the fact that at this *p*H there is appreciable absorbancy of pyridoxal in the region near 380 mμ and pyridoxal is known to form Schiff bases with many amines and amino acids.[13] The fact should not be overlooked, however, that it might be difficult to detect the products of reaction spectrophotometrically if Schiff base formation were the rate-limiting step, because pyridoxal has an absorption band with maximum close to that expected of the product. Slow reaction was observed at *p*H 11.0 between cysteine ethyl ester and pyridoxal when 100 equivalents of ester were used. These conditions favor imine formation because the free aldehyde, with a band peaking at 385 mμ, is present in maximal amounts at this *p*H and the large quantities of addend promote reaction by mass action. After an hour the absorbancy of the "385 mμ band" decreased to 40% of its initial value without change in wave length of the maximum; hence no Schiff base accumulated in the reaction mixture. There was evidence of ring closure since the maximum of the hemiacetal form of pyridoxal at 300 mμ had shifted to 315, the wave length expected of the complex at this *p*H. It seems probable, therefore, that the failure of cysteine or its ethyl ester to react readily with pyridoxal at physiological *p*H values is due to the fact that imine formation does not occur at a significant rate.

Complex formation between 5-deoxypyridoxal and both cysteine and its ethyl ester was observed over a wide *p*H range.

Rates of Complex Formation.—In many experiments the rates of complex formation between pyridoxal-5-phosphate

(12) M. Calvin in S. Colowick, *et al.*, "Glutathione, A Symposium," Academic Press, New York, N. Y., 1954, pp. 21–26.

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

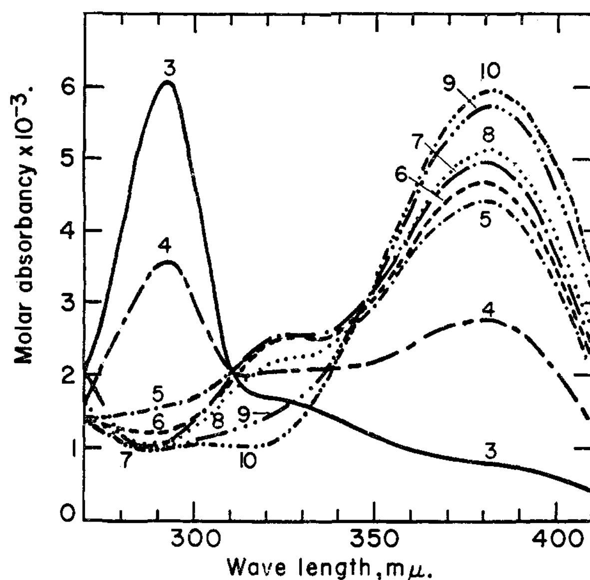


Fig. 2.—Absorption spectra of pyridoxal-5-phosphate, taken with a calibrated Beckman D.U. spectrophotometer. The numbers beside the curves indicate the *p*H values of the buffer-solvent. The buffers used were: all 4×10^{-2} *M;* acetate at *p*H 5 and below; carbonate at *p*H 9 and above; phosphate at intermediate values. The molar absorbancy at 380 mμ and *p*H 7.0, used in the calculations, was 4900.[4] Readings were made at intervals of 5 mμ except in the regions near maxima, where the interval was 1 mμ.

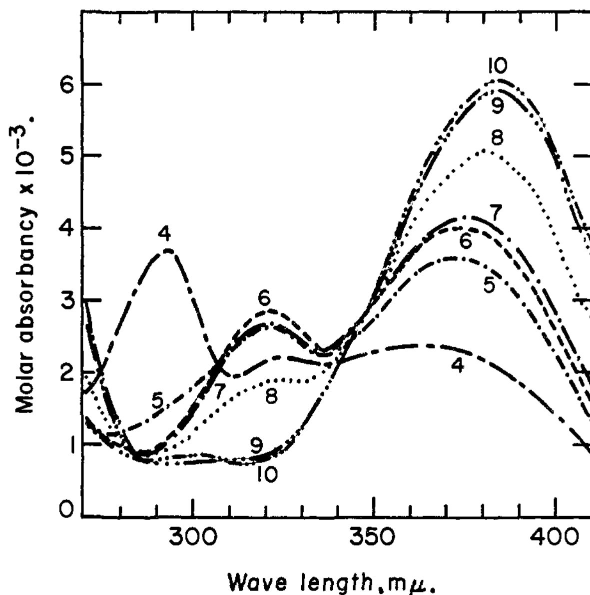


Fig. 3.—Absorption spectra of 5-deoxypyridoxal, taken under the conditions described for Fig. 2. The molar absorbancy at 380 mμ and *p*H 7.0, used in the calculations, was 4000.

and cysteine were followed by observing both the decrease in absorbancy at 380 mμ and its increase at 325 mμ as a function of time and also as a function of *p*H. In all cases the changes in absorbancy observed at each wave length were proportional to each other, the negative values at 380 mμ being 1.5 times the positive values at 325 mμ at *p*H 7.0. In later esperiments only the absorbancies at 380 mμ were recorded. First, an absorption spectrum was taken of the pyridine analog at a concentration of 1.5×10^{-4}*M* in a

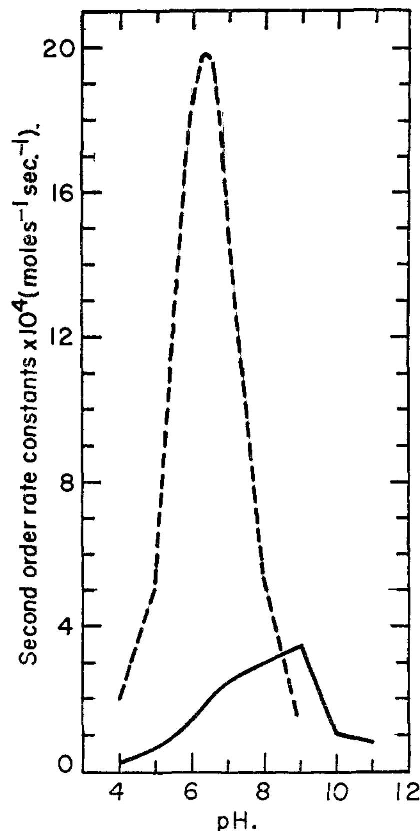


Fig. 4.—Second order rate constants of complex formation with pyridoxal-5-phosphate at 20° as a function of *p*H. Solid line cysteine, dashed line cysteine ethyl ester; values determined at intervals of 1 *p*H unit except near *p*H 6.0 where the interval was 0.2 unit.

buffer, 4×10^{-2}*M*, suitable for maintaining the desired *p*H. Next, a small volume of a concentrated solution of cysteine, or its ethyl ester, was added to each cell and the decrease in absorbancy at 380 mμ in the experimental cell was recorded as a function of time. The slopes of the curves used in calculating the rate constants were estimated from data taken at the end of each minute of reaction between the first and the tenth minute.

The rate of each reaction was studied first at *p*H 7.0 in a series of experiments in which the concentration of aminothiol relative to that of pyridine analog was varied. With more than one equivalent of addend the reactions followed second order kinetics accurately until equilibrium was approached. With less than one equivalent this was not the case; here the initial rates approximated first order kinetics with reference to the disappearance of the free aldehyde form of the pyridine analog. In six experiments in which the equivalents of cysteine were varied between 1.95 and 12.20 with respect to the concentration of pyridoxal-5-phosphate the second order rate constant at *p*H 7.0 was found to be 2.64 (± 0.10) $\times 10^{-4}$ mole^{-1} sec.$^{-1}$ at 20°. Variations of similar magnitude were observed in the values determined for the second order rate constants of the other reactions. The results of this type of experiment were used in selecting the concentration of addend appropriate for use in subsequent experiments in which only the *p*H was varied.

Figures 2 and 3 show that the amounts of total free aldehyde (measured by the absorbancy of the "380 mμ band") of both pyridine analogs are highly *p*H-dependent. Since this band affords an independent measure of the progress of the reaction, as shown in Fig. 1, the initial absorbancy at 380 mμ was used as a measure of the concentration of the pyridine analog at each *p*H in calculating the second order rate constants. Thus, these constants, shown as a function of *p*H in Figs. 4 and 5, are essentially independent of variations in the *total* amount of free aldehyde as the *p*H

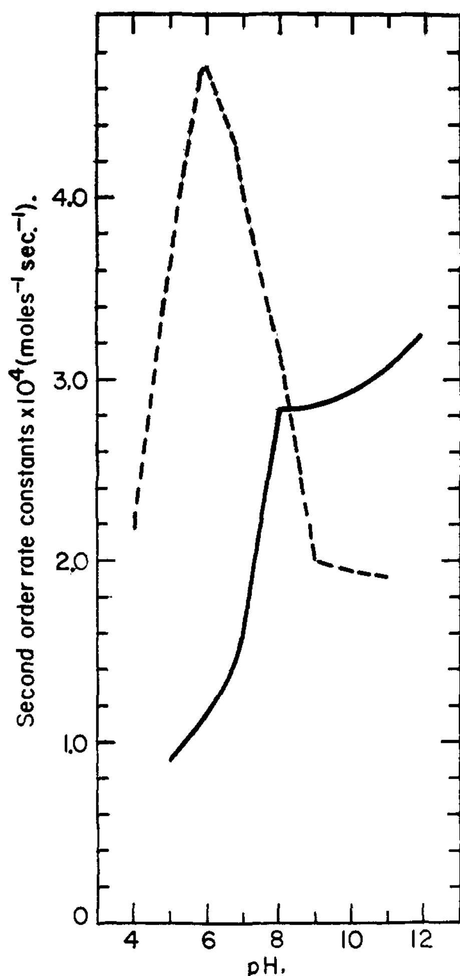
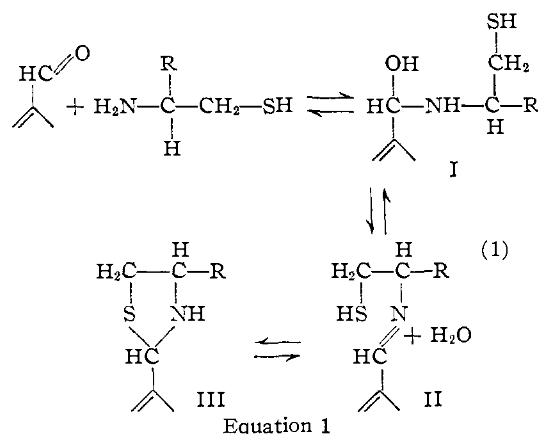


Fig. 5.—Second order rate constants of complex formation with 5-deoxy-pyridoxal at 20° as a function of *p*H. Solid line cysteine, dashed line cysteine ethyl ester; values determined at intervals of 1 *p*H unit except near *p*H 6.0 where the interval was 0.4 unit.

was varied. The distribution of the reactive species of the addend as a function of *p*H could not be determined directly; therefore its concentration was taken as the total amount present in the reaction mixture. Since the concentration of the reactive ionic species of the aminothiols is *p*H-dependent the rate constants, thus calculated, should reflect two factors; (1) changes associated with the distribution of the reactive ionic species of each addend and (2) changes in the quantities of the more reactive aldehyde form resulting from the presence of the phosphate group in pyridoxal-5-phosphate as opposed to its absence in 5-deoxypyridoxal.

Discussion

The reaction resulting in complex formation between either pyridoxal-5-phosphate or 5-deoxypyridoxal and aminothiols is believed to occur in three steps involving addition, dehydration and ring closure. In equation 1 only the reactive formyl group at position 4 of the pyridine analog is shown and only the essential structure of the aminothiol is indicated. Many factors may affect the speed of this sequence of reactions at a given temperature. Figures 4 and 5 show the *p*H profiles of the rate of complex formation when each pyridine analog reacts with two aminothiols of different type. With cysteine R is an ionized carboxyl



Equation 1

group carrying a formal negative charge distributed between the two oxygen atoms. With cysteine ethyl ester R is $-COOC_2H_5$; not only has the formal negative charge been eliminated but resonance between the oxygens is no longer possible, with the result that the carbonyl group is highly polar. Figures 4 and 5 show that the rates of formation of the complex III in the four reactions described are highly *p*H-dependent and differ widely among themselves. Consideration of these results suggests the factors (to be discussed) that appear to influence the over-all rates: (1) The rate of formation of the imine II limits the rate of formation of the complex. (2) The ionic species of cysteine, or its ethyl ester that reacts to form the addition compound I has an uncharged amino group. (3) The ionic species of the imine that reacts to form the thiazolidine ring has an uncharged sulfhydryl group. (4) The charge on each pyridine analog affects the rate at which each charged aminothiol may approach the reactive carbonyl group to form the addition product. (5) The phosphate group of pyridoxal-5-phosphate has a stimulatory or catalytic effect on imine formation that is lacking in 5-deoxypyridoxal. These five factors are interrelated and in some cases opposing; no one set of reaction rates illustrates the unique effect of a single factor.

Charges on Pyridine Analogs.—In strong acid both pyridoxal-5-phosphate and 5-deoxypyridoxal have a net formal charge of +1, associated with the pyridinium nitrogen.[5,14] Each dissociation of the phosphate group contributes a negative charge to pyridoxal-5-phosphate and the dissociation of the phenolic hydroxyl group contributes one negative charge to each analog. When the latter dissociation is complete the net charge on 5-deoxypyridoxal is zero until the positive charge on the pyridinium nitrogen is lost, when the formal charge becomes −1. Throughout the *p*H range of formation of the complex the formal charge on pyridoxal-5-phosphate is approximately one to two units more negative than that on 5-deoxypyridoxal; the electron density of the greater negative charge on the former is centered about the phosphate group.

Charges on the Addends.—At all *p*H values at which the rates of complex formation are significant the only structural difference between cysteine

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

and its ethyl ester is the presence of an ionized carboxyl group in cysteine in contrast to the un-ionized, but highly polar ethyl carboxylic acid group in the ester. Since the groups participating directly in the reaction are sulfhydryl and amino, any difference in distribution of the ionic species must reflect the effect of this unique difference in structure on the *pK* values of the reacting groups. Benesch and Benesch,[15] using a spectrophotometric technique for measuring the charged sulfide group, estimated the microscopic *pK* values of each dissociation of each group of cysteine and of its ethyl ester. At *p*H 7 cysteine exists predominately as $HSRNH_3^+$ (form a) and above *p*H 12 as $\bar{S}RNH_2$ (form d). At *p*H 9.5 $\bar{S}RNH_3^+$ (form b) and $SRNH_2$ (form c) both occur maximally; the former represents about 60% of the total and the latter about 30%. Both *pK* values describing the dissociations of the ammonium group of cysteine are reduced by about two units in cysteine ethyl ester; both *pK* values of the sulfhydryl group of cysteine are reduced by about one unit in the ethyl ester. Consequently, the *p*H of maximal concentration of forms b and c is about 1.5 units lower for the ethyl ester than for cysteine.

Effects of the Phosphate Group on Absorption Spectra.—Figures 2 and 3 reveal not only the striking similarities between the spectra of 5-deoxypyridoxal and pyridoxal-5-phosphate, pointed out by Metzler and Snell,[5] but also two apparent differences that are doubtless interrelated. In each compound the band peaking at or near 380 mμ represents free aldehyde in contradistinction to the band with maximum at or near 325 mμ that represents hydrated aldehyde. The first difference concerns the location of the maxima in the analogous bands in the near ultraviolet. In each compound the molar absorbancy of the "380 mμ band" increases during progressive dissociation of the phenolic hydroxyl group and loss of the pyridinium proton. Figure 3 shows that with 5-deoxypyridoxal the maximum is shifted 14 mμ toward longer wave lengths as the *p*H is raised from 5 to 10. Metzler and Snell[5] attribute this shift to contributions from two mesomeric forms of the free aldehyde. Surprisingly, as shown in Fig. 2, the maximum of the analogous band of pyridoxal-5-phosphate remains at 380 mμ throughout the *p*H range in question; even at *p*H 14 the band peaks at 380 mμ.

The second difference between the absorption spectra of the two compounds is concerned with the amount of free aldehyde relative to that of hydrated aldehyde in each compound. With pyridoxal-5-phosphate, particularly in and near the physiological *p*H range, the ratio of free to hydrated aldehyde is larger than with 5-deoxypyridoxal. Figure 6 illustrates the effect of the phosphate group over the *p*H range of the reaction on the amount of *total* free aldehyde as measured by the absorbancy of each compound at 380 mμ, but does not identify the more reactive form of the free aldehyde. Below *p*H 8.0 the amount of *total* free aldehyde is greater with pyridoxal-5-phosphate than with 5-deoxypyridoxal; relatively little change is apparent in the former, however, during the secondary phosphate dissociation. These differences in spectra must represent an inductive effect of the phosphate group on the conjugated system. Molecular models of pyridoxal-5-phosphate show that on rotation the phosphate group can come within atomic distances of the carbonyl group. It seems plausible, therefore, that the inductive effect is exerted through the carbonyl function rather than through the methylene group by means of which the phosphate group is attached to the pyridine ring.

(15) R. E. Benesch and R. Benesch, THIS JOURNAL, 77, 5877 (1955).

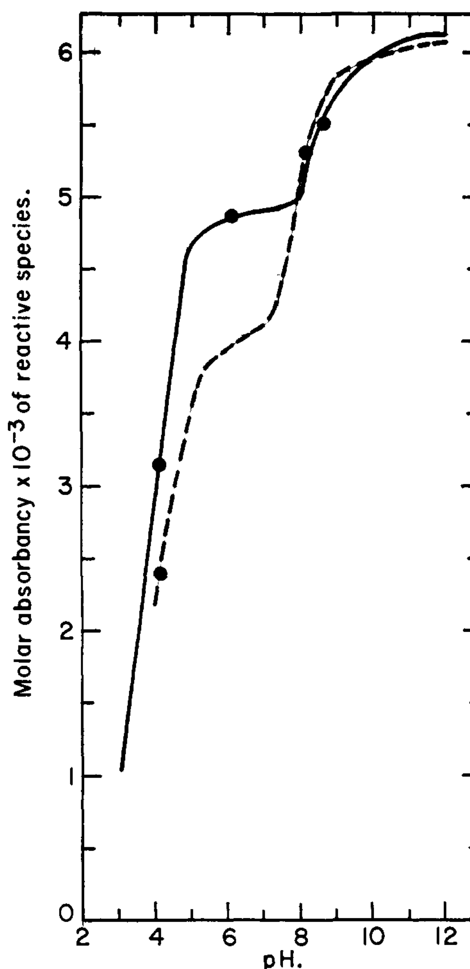


Fig. 6.—Effect of *p*H on the relative amounts of *total* free aldehyde as measured by absorbancy at 380 mμ. Dotted line, 5-deoxypyridoxal; solid line, pyridoxal-5-phosphate. The points on the curves indicate reported *pK* values.[5,14]

Effect of Charge of Addend on *p*H of Maximal Rate.—So many variables affect the rates of formation of the complexes (Figs. 4 and 5) that no one of them may be evaluated quantitatively; certain qualitative deductions, however, may be made. Equation 1 indicates that the over-all rate at which III is formed is dependent upon the rates of formation of I and II. The sharp peaks in the *p*H profiles—particularly those involving cysteine ethyl ester where the rate of imine formation is known not to limit the rate of ring closure—implicate the rates of both the addition and dehydration reactions on the rate of imine formation. The former requires an uncharged amino group for reaction with the

carbonyl group. With crysteine ethyl ester the sharp drop in rate on the "acid side" of the peak is primarily an expression of the increasing paucity of the uncharged species of addend as the *p*H is lowered. Thus, the lower *p*H of maximal rate of complex formation with cysteine ethyl ester than with cysteine may to some degree reflect the lower *p*H at which the uncharged form of the ester occurs maximally. The sharp rise in rate as the peak is approached from the "alkaline side" would be expected if the dehydration step were *p*H-dependent and "acid catalyzed." This point is not demonstrated experimentally in this system because the reaction does not stop with II, but in similar reactions in which the end product is analogous to imine the dehydration step is known to be *p*H-dependent. For example, in the reaction of several aldehydes and ketones with hydroxylamine and other "carbonyl reagents" dehydration of the primary addition product was shown by Jencks[16] to be "acid-catalyzed." Finally, evidence has already been presented pointing to the dependency of ring closure on imine formation. Presumably, an unionized sulfhydryl group is essential for this reaction but it may be pointed out that the *pK* describing the dissociation of the sulfhydryl group of the imine is not known and is not necessarily the same as that of the aminothiol from which it was formed.

Effect of Charge of Pyridine Analog on Rates.—In any analysis of the rate of complex formation between a specific pyridine analog and a specific aminothiol another factor must be considered, namely, the mutual interaction of charges on each reactant. For example, the rate of complex formation between 5-deoxypyridoxal and cysteine (Fig. 5) rises steeply with increasing *p*H as more uncharged amino groups become available until the *p*H is reached at which negative charges are developed on both reactants concomitant with loss of the pyridinium proton and ionization of the sulfhydryl group. These charges, by mutual repulsion, would be expected to slow the approach of the reactive groups and thus to decrease the rate of formation of I and subsequent steps. This effect is suggested by the break in the curve at *p*H 8.0. Fig. 4 shows that the effect is even greater when cysteine reacts with pyridoxal-5-phosphate; this would also be expected because in alkaline solutions the formal charge on pyridoxal-5-phosphate is about two units more negative than on 5-deoxypyridoxal. One effect of the phosphate group directly correlated with charge is to limit the rate at which pyridoxal-5-phosphate reacts with negatively charged aminothiols. This effect is clearly manifest in alkaline solutions because the negative charge on both reactants is increased simultaneously but it is not necessarily limited to this *p*H region. The repulsion between negative charges may also be a factor contributing to the slower reaction of cysteine than of its ethyl ester with pyridoxal-5-phosphate.

Catalytic Effect of the Phosphate Group on Rates.—A different type of effect of the phosphate group is seen in the four-fold increase in rate of complex formation when cysteine ethyl ester reacts with pyridoxal-5-phosphate as compared with 5-deoxypyridoxal. Not only is the rate of reaction increased but the *p*H range of maximal rate occurs within somewhat narrower limits. These differences are specifically referable to the phosphate group of pyridoxal-5-phosphate and occur during its secondary dissociation; in this case they cannot be attributed to the phosphate of the buffer[16] since both reactions were carried out in the same buffer and there was no discontinuity in the curves when buffers were changed. The peaks of both curves occur at approximately the same *p*H. Figure 4 shows that the rate curve with pyridoxal-5-phosphate and cysteine ethyl ester is symmetrical about the peak and that both limbs of the curves are linear functions of *p*H. Since the presence of imine is apparent in the spectrum as pyridoxal-5-phosphate reacts with cysteine ethyl ester the rate-limiting step in complex formation in this case must be ring closure. The effect of the phosphate group is therefore concerned primarily with the rates of formation of I or II or both; the observed rate curve might result from any one of these possibilities. A suggestion that the formation of I may be catalyzed to some degree by the phosphate group of pyridoxal-5-phosphate comes from the absorption spectra. The larger amounts of *total* free aldehyde near *p*H 6.0 (Fig. 6) with pyridoxal-5-phosphate than with 5-deoxypyridoxal could account for only a small part of the observed increase in rate. Although the inductive effect of the phosphate group on the spectrum cannot be interpreted in terms of reactivity of the carbonyl function it does indicate a difference in the properties of the free aldehyde forms of the two pyridine analogs.

In no case was evidence found of accumulation of I. This intermediate, with sp^3-bonding of the carbonyl carbon, would be expected to have an absorption band with maximum at or near 325 mμ; if it accumulated to a significant degree at the same time that III was being formed the observed proportionality between the decrease in absorbancy at 380 and increase at 325 mμ would not obtain.

Rates of Reaction of Other Aminothiols with Pyridoxal-5-phosphate.—The factors bearing on the rates of complex formation of cysteine and its ethyl ester with the biologically important pyridoxal-5-phosphate find further experimental support in the data presented in Fig. 7. 2-Thioethylamine has the simplest structure essential for complex formation; its maximal rate of complex formation ($K = 7.5 \times 10^{-4}$ mole^{-1} sec.$^{-1}$) occurs at *p*H 9. The maximal rate with cysteine is also found at *p*H 9, but the rate constant is reduced by half as a consequence of the negative charge of the carboxyl group. With cysteine ethyl ester, in which the negative charge has been removed and the polar carbonyl group is in close proximity to the reactive amino group, radical changes are found both in the *p*H (6.4) and rate constant (19.3×10^{-4} mole^{-1} sec.$^{-1}$) of maximal reaction. Cysteinyl-glycine resembles cysteine ethyl ester in having a carbonyl group in the same position relative to the reactive amino group. It was of particular interest to find maximal rate of reaction at *p*H 6.8 as might have been anticipated from the dissociation constants of the reactive groups.[15] Also the maximal rate constant has been reduced to

(16) W. P. Jencks, THIS JOURNAL., 81, 475 (1959).

one-third of that found for cysteine ethyl ester; a reduction in rate would be expected as a consequence of the inhibitory effect of the ionized carboxyl group of the glycyl residue.

Complexes as Models for Phosphorylase.—The absorption band of phosphorylase *b* with maximum at 333 mμ (measured in a Cary spectrophotometer) was shown by Kent, *et al.*[17] to represent pyridoxal-5-phosphate in its natural environment in the enzyme. The spectra, stability, *p*H and rates of formation of the complexes described suggest that this type of compound may serve as a model for the bonding of pyridoxal-5-phosphate in muscle phosphorylase. More specifically, the complex formed with cysteinylglycine closely mimics that of the native enzyme with respect to its absorption maximum; furthermore, the *p*H limits within which it may be formed correspond closely to the *p*H-activity curves of phosphorylase.[17] The points of attachment of the carbonyl group of pyridoxal-5-phosphate to the enzyme could be the ε amino group of a peptide-bound lysyl residue[18] and the sulfhydryl group of a peptide-bound cysteinyl residue. In this case no thiazolidine ring would be formed but the spectrum resulting from this type of bonding should closely resemble both that of the model complex with cysteinylglycine and that found in phosphorylase. Although the evidence at present points in this direction the possibility still must be considered that a thiazolidine ring structure may be present in the native enzyme. The properties of the complexes formed between pyridoxal-5-phosphate and aminothiols are highly dependent upon the structural environment surrounding the essential aminothiol structure.

5-Deoxypyridoxal, but neither pyridoxamine nor pyridoxamine-5-phosphate, would be expected to combine with the apoenzyme of phosphorylase *a*, as was observed experimentally by Illingworth, *et al.*[10]

The suggested bonding implicates four cysteinyl residues in phosphorylase *a* so located that each sulfhydryl group may function together with the ε amino group of a lysyl residue to form a complex with the carbonyl carbon of pyridoxal-5-phosphate. It does not follow, however, that the amino acid residues providing the functional groups are necessarily located sequentially, or even on the same peptide chain.

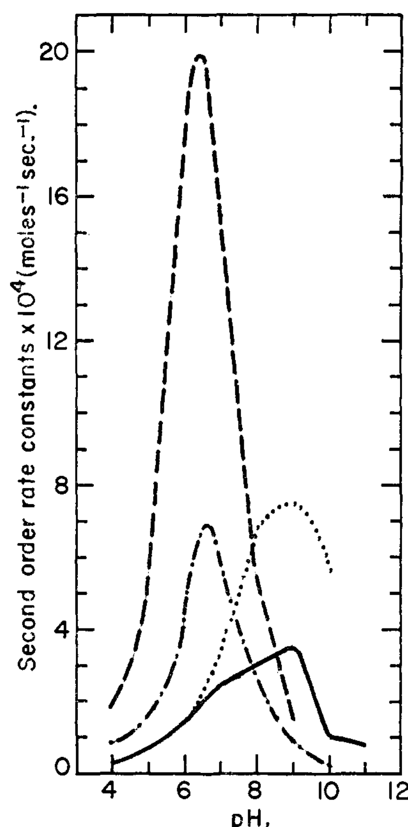


Fig. 7.—Second order rate constants of complex formation with pyridoxal-5-phosphate as a function of *p*H, 20°. Solid line cysteine, dotted line 2-thioethylamine, dashed line cysteine ethyl ester, dot-dashed line cysteinylglycine. Values were determined at intervals of 1 *p*H unit, with several additional values at intervals of 0.2 unit on each side of the peaks that appear between *p*H 6 and 7.

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(17) A. B. Kent, E. G. Krebs and E. H. Fischer, *J. Biol. Chem.*, **232**, 549 (1958).

(18) E. H. Fischer, A. B. Kent, E. R. Snyder and E. G. Krebs, THIS JOURNAL, **80**, 2906 (1958).