Kinetic Study of the Schiff-base Formation between Glycine and Pyridoxal 5'-Phosphate (PLP), Pyridoxal (PL), and 5'-Deoxypyridoxal (DPL)

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The formation of Schiff bases between pyridoxal 5'-phosphate (PLP), pyridoxal (PL), 5'-deoxypyridoxal (DPL), and glycine has been investigated as a function of proton concentration at 25 °C and 0.1 mol dm^{-3} ionic strength. Ionization constants as well as equilibrium and microscopic kinetic constants of the ionic species present in solution have been determined.

The results show that all the protonatable groups of the aldehyde molecule are involved in the intramolecular acidic catalysis of the dehydration process undergone by the α-hydroxyamine intermediate. Results also reveal that the formation of Schiff base with pyridoxal is greatly affected by the formation of the cyclic hemiacetal of this aldehyde.

Pyridoxal 5'-phosphate (PLP) is one of the active forms of the vitamin B_6 group, and it is involved in many enzymic reactions. In PLP-dependent enzymes, coenzyme (PLP) is bound to protein through a Schiff-base linkage with the amine terminal group of a lysine residue.[1,2]

Schiff bases of PLP and its analogues with amino acids and primary amines have been extensively investigated, because of their important biological role in the metabolism of amino acids. Their stability constants[3–6] and ionization constants[7,8] have been determined in some cases and quantitative descriptions of their absorption spectra[9,10] have been carried out. Recently, kinetic studies on the formation of Schiff bases of PLP with some amino acids have been reported.[1,11]

The Schiff-base formation is accepted to proceed through the attack of amine on the aldehyde molecule to give an intermediate α-hydroxyamine. The α-hydroxyamine loses a molecule of water to yield the imine; dehydration involves expulsion of an OH^- ion.[12–14]

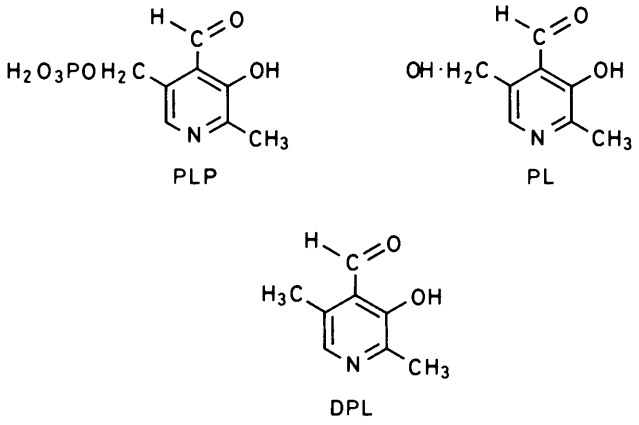
Although a great number of kinetic studies on PLP-Schiff base formation have been carried out, in most of them only the overall rate constants have been measured and no attempts have been made to assign the observed rate to the different ionic species existing in solution.

This paper describes the results obtained in a kinetic study of the formation and hydrolysis of the Schiff base formed by an amino acid, glycine, with PLP and two of its analogues, pyridoxal (PL) and 5'-deoxypyridoxal (DPL) over a wide range of pH values.

These two analogues have been chosen in order to compare the kinetic results because pyridoxal is able to activate the α, β, and γ groups of amino and keto acids in non-enzymic reactions, such reactions being a reasonably good model for the enzymic ones.[1] 5'-Deoxypyridoxal does not show catalytic activity but it has the three chemical groups which are considered to be fundamental for catalytic activity: an aldehyde group, a pyridine nitrogen, and a 3'-hydroxy group.

Methods and Materials.—5'-Deoxypyridoxal (DPL) was synthesized from pyridoxine hydroxychloride by the method of Iwata.[15] All other chemicals used were obtained from Merck.

Experiments were carried out in different buffered systems (chloroacetate, acetate, phosphate, and carbonate, at 0.02 mol dm^{-3}) keeping the ionic strength constant at 0.1 mol dm^{-3} by adding the appropriate amount of KCl.[16] PLP, PL, and DPL solutions were prepared daily in a suitable buffer and kept in the dark. Their exact concentration was determined by dilution with 0.1 mol dm^{-3} NaOH (DPL) or 0.1 mol dm^{-3} HCl (PL, PLP) and subsequent measurement of their absorbance.[17,18] The concentration thus found was *ca.* 5×10^{-4} for PLP and DPL and 5×10^{-3} mol dm^{-3} for PL. Glycine solutions were also prepared daily by diluting the appropriate amount of concentrated amino acid with the corresponding buffer and adjusting the pH with HCl or NaOH. Their concentrations ranged between 5×10^{-3} and 0.5 mol dm^{-3}.



pH measurements were made with a Crison pH-meter, using a Metrohm EA120 combined electrode previously calibrated with aqueous buffers at 25 °C. As verified for each experiment, the differences between the initial and final pH of the reaction cell were never greater than ±0.04 pH units.

Reactions were started by adding *ca.* 0.2–1.6 cm^3 of the glycine solution to the cell, which already contained the thermostatted aldehyde solution, prepared in the same buffer and at the same pH. The amino-acid concentration in the measuring cell was 50–1 000 times greater than the aldehyde concentration. The reaction was monitored by measuring the increase in absorbance at 420 nm (PL, PLP) or 280 nm (DPL). A Zeiss DMR11 spectrophotometer equipped with thermostat-

Table 1. Best kinetic constants pK and K_M obtained in the fitting of experimental values of k_1, k_2, and K_{pH} to equations (3)–(6) for PLP + glycine.

pK_{2P}	8.33	$\log k_1^3$	2.50
pK_{1P}	5.90	$\log k_1^2$	3.79
pK_{0P}	3.58	$\log k_1^1$	5.52
pK_{0L}	9.76	$\log k_1^0$	7.30
pK_{-1L}	2.37		
pK_{3B}	11.35	$\log k_2^4$	1.16
pK_{2B}	6.36	$\log k_2^3$	−0.55
pK_{1B}	5.46	$\log k_2^2$	0.676
pK_{0B}	2.84	$\log k_2^1$	0.584
pK_{-1B}	2.16	$\log k_2^0$	0.259
		$\log k_2^{-1}$	−0.365
$\log K_M$	1.33		

ted cells of 0.1 dm light path was used in every experiment. The temperature was kept constant at 25.00 ± 0.05 °C throughout.

The overall reaction between the aldehyde and glycine can be represented by:

$$R^1CHO + R^2NH_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} R^1CH{=}NR^2 + H_2O$$

From the Beer–Lambert law the integrated rate equation for this reaction is:

$$\ln\frac{A_\infty + A_0}{A_\infty - A} = -\ln\frac{ab - xx_e}{x_e^2} + k_{obs}t \qquad (1)$$

$$k_{obs} = \{[k_2 + k_1(a + b)]^2 - 4abk_1^2\}^{\frac{1}{2}} \qquad (2)$$

where a and b are the initial aldehyde and amino acid concentrations, respectively; x and x_e are the Schiff-base concentrations at time t and infinite time, respectively; A_0, A, and A_∞ are absorbances at 0, t, and infinite times, respectively; and k_1 and k_2 are the overall rate constants of formation and hydrolysis for the Schiff base.

k_1 and k_2 values were calculated using equation (2) from the k_{obs} values obtained at a given pH and different values of a and b. The equilibrium constant corresponding to each pH, K_{pH}, was calculated as the ratio k_1/k_2.

Results and Discussion

The overall rate constants for hydrolysis and formation of the Schiff base can be described in terms of the rate constants for individual ionic species present in each case.[4,6,19] For the PLP system the ionic species existing in solution over the pH range studied are given in Scheme 1 in which subscripts (−2, −1, 0, 1, 2, 3, 4) indicate the number of net negative charges on the molecule.

In aqueous solution, four different species have been considered for PLP. PLP_3 must be identified with the completely deprotonated aldehyde molecule, which bears three net negative charges: two on the phosphate group and one on the 3′-hydroxy group. Successive addition of protons gives the PLP_2, PLP_1 and PLP_0 forms.[20] Because of the carboxylic group on the amino acid and the imine nitrogen, two additional protonatable groups must be considered for the Schiff-base molecule and six different imine ionic species exist in aqueous solution: $BPLP_4$, $BPLP_3$, $BPLP_2$, $BPLP_1$, $BPLP_0$, and $BPLP_{-1}$. Their structural formulae have been assigned according to the literature which exists for this sort of compound.[3,5,7,8]

Equations (3)–(6) can be derived from Scheme 1, where a_H is the proton concentration and P_w the ionization equilibrium constant of water.

Pyridoxal and 5′-deoxypyridoxal have no phosphate group and their molecules have only two different ionization constants

$$k_1 = \frac{k_1^3 + k_1^2(a_H/K_{2P}) + k_1^1(a_H^2/K_{2P}K_{1P}) + k_1^0(a_H^3/K_{2P}K_{1P}K_{0P})}{(1 + a_H/K_{0L} + a_H^2/K_{0L}K_{-1L})(1 + a_H/K_{2P} + a_H^2/K_{2P}K_{1P} + a_H^3/K_{2P}K_{1P}K_{0P})} \qquad (3)$$

$$k_2 = \frac{k_{0H} + k_2^3(a_H/K_{3B}) + k_2^2(a_H^2/K_{3B}K_{2B}) + k_2^1(a_H^3/K_{3B}K_{2B}K_{1B}) + k_2^0(a_H^4/K_{3B}K_{2B}K_{1B}K_{0B}) + k_2^{-1}(a_H^5/K_{3B}K_{2B}K_{1B}K_{0B}K_{-1B})}{a_H/K_{3B} + a_H^2/K_{3B}K_{2B} + a_H^3/K_{3B}K_{2B}K_{1B} + a_H^4/K_{3B}K_{2B}K_{1B}K_{0B} + a_H^5/K_{3B}K_{2B}K_{1B}K_{0B}K_{-1B}} \qquad (4)$$

$$k_{0H} = k_2^4 + (k_{0H}^2P_w/k_{3B}) \qquad (5)$$

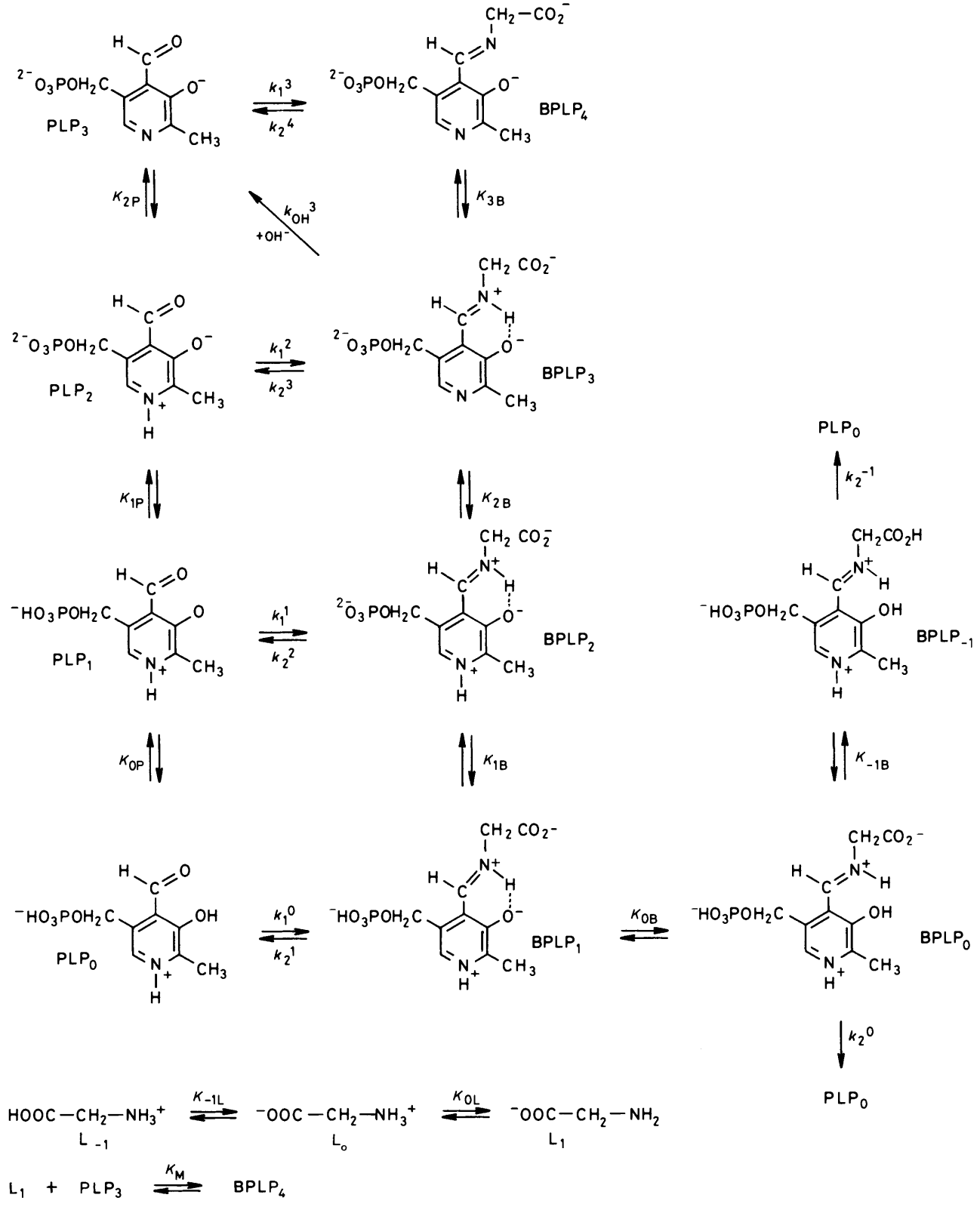
$$K_{PH} = \frac{K_M(1 + a_H/K_{3B} + a_H^2/K_{3B}K_{2B} + a_H^3/K_{3B}K_{2B}K_{1B} + a_H^4/K_{3B}K_{2B}K_{1B}K_{0B} + a_H^5/K_{3B}K_{2B}K_{1B}K_{0B}K_{-1B})}{(1 + a_H/K_{0L} + a_H^2/K_{0L}K_{-1L})(1 + a_H/K_{2P} + a_H^2/k_{2P}K_{1P} + a_H^3/K_{2P}K_{1P}K_{0P})} \qquad (6)$$

$$k_1 = \frac{k_1^1 + k_1^0(a_H/K_{0P}) + k_1^{-1}(a_H^2/K_{0P}K_{-1P})}{(1 + a_H/K_{0L} + a_H^2/K_{0L}K_{-1L})(1 + a_H/K_{0P} + a_H^2/K_{0P}K_{-1P})} \qquad (7)$$

$$k_2 = \frac{k_{0H} + k_2^1(a_H/K_{1B}) + k_2^0(a_H^2/K_{1B}K_{0B}) + k_2^{-1}(a_H^3/K_{1B}K_{0B}K_{-1B}) + k_2^{-2}(a_H^4/K_{1B}K_{0B}K_{-1B}K_{-2B})}{a_H/K_{1B} + a_H^2/K_{1B}K_{0B} + a_H^3/K_{1B}K_{0B}K_{-1B} + a_H^4/K_{1B}K_{0B}K_{-1B}K_{-2B}} \qquad (8)$$

$$k_{0H} = k_2^2 + k_{0H}^2P_w/K_{1B} \qquad (9)$$

$$K_{PH} = \frac{K_M(1 + a_H/K_{1B} + a_H^2/K_{1B}K_{0B} + a_H^3/K_{1B}K_{0B}K_{-1B} + a_H^4/K_{1B}K_{0B}K_{-1B}K_{-2B})}{(1 + a_H/K_{0L} + a_H^2/K_{0L}K_{-1L})(1 + a_H/K_{0P} + a_H^2/K_{0P}K_{-1P})} \qquad (10)$$



Scheme 1.

corresponding to the 3′-hydroxy group and pyridine nitrogen. The kinetic scheme for these two aldehydes is shown in Scheme 2, in which the structural formulae of aldehyde and Schiff base have been omitted but PL_1 and DPL_1 are analogous forms to PLP_3; PL_0 and DPL_0 are similar to PLP_2, and PL_{-1} and DPL_{-1} are similar to the PLP_0 form.

Equations (7)–(10) can be derived from Scheme 2.

The experimental data of k_1, k_2, and K_{pH} were fitted to equations (3)–(6) or (7)–(10) in each case by means of a non-linear regression method, minimizing the U_i function:

Table 2. Best kinetic constants pK and K_M obtained in the fitting of experimental values of k_1, k_2, and K_{pH} to equations (7)–(10) for DLP + glycine and PL + glycine.

DPL				PL			
pK_{0P}	8.02	$\log k_1^{1}$	3.03	pK_{0P}	8.65	$\log k_1^{1}$	2.25
pK_{-1P}	4.09	$\log k_1^{0}$	3.94	pK_{-1P}	4.23	$\log k_1^{0}$	2.79
pK_{0L}	9.78	$\log k_1^{-1}$	7.00	pK_{0L}	9.77	$\log k_1^{-1}$	5.29
pK_{-1L}	2.38			pK_{-1L}	2.36		
pK_{1B}	11.22	$\log k_2^{2}$	1.97	pK_{1B}	10.75	$\log k_2^{2}$	1.28
pK_{0B}	6.43	$\log k_2^{1}$	−0.326	pK_{0B}	5.96	$\log k_2^{1}$	−0.343
pK_{-1B}	3.04	$\log k_2^{0}$	0.449	pK_{-1B}	3.02	$\log k_2^{0}$	0.435
pK_{-2B}	2.12	$\log k_2^{-1}$	0.300	pK_{-2B}	2.21	$\log k_2^{-1}$	−0.048
		$\log k_2^{-2}$	−1.05			$\log k_2^{-2}$	−1.14
$\log K_M$	1.09			$\log K_M$	0.962		

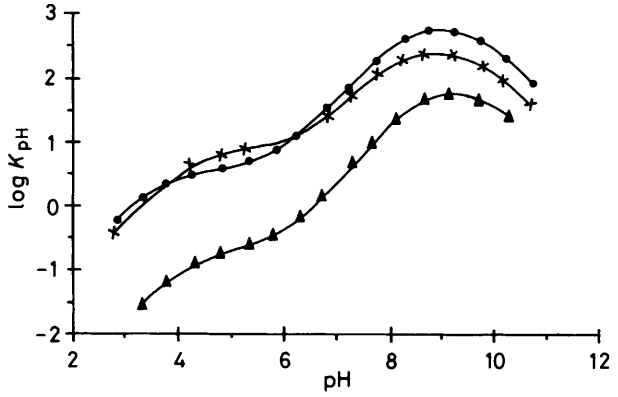


Figure 1. Variation of log K_{pH} as a function of pH for Schiff bases of glycine and: ●, PLP; ▲, PL; and ×, DPL. Points are experimental values and continuous lines are the theoretical fitting to equations (6) or (10), respectively.

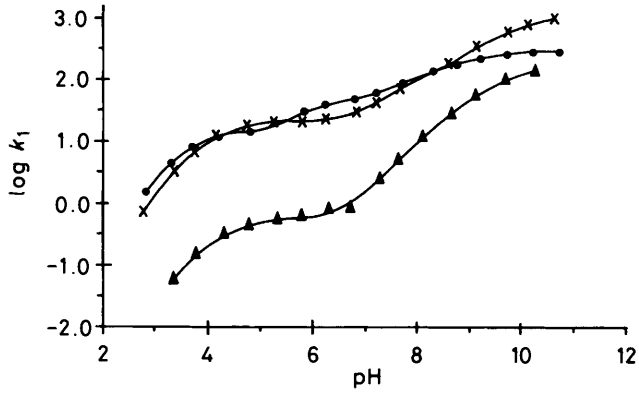


Figure 2. Variation of log k_1 as a function of pH for Schiff bases of glycine and: ●, PLP; ▲, PL; and ×, DPL. Points are experimental values and continuous lines are the theoretical fitting to equations (3) or (7), respectively.

$$U_i = \Sigma(\log k_{i,e} - \log k_{i,t})^2 \qquad (11)$$

where i = 1, 2, or pH and subscripts 'e' and 't' refer to experimental and theoretical data respectively.

Figures 1, 2, and 3 show the variation with pH of the logarithm of the overall kinetic and equilibrium constants of the Schiff bases formed between glycine and PLP, PL, and DPL. Points are experimental values and lines are the theoretical functions obtained according to equations (3), (4), and (6) for PLP system and (7), (8), and (10) for PL and DPL systems and using the pK and microscopic constant values given in Tables 1 and 2.

The shapes of the k_1, k_2, and K_{pH} functions are very similar for the three different aldehyde–amino acid systems, which means similar behaviour for these aldehydes. As with other examples of PLP Schiff-base formation, k_1 increases with pH, k_2 has a minimum value between pH 7.5 and 8.5, and K_{pH} shows a maximum in the basic pH region. Nevertheless, some differences can be observed if we compare the corresponding curves; for example, the equilibrium constant of PLP and DPL Schiff bases are larger than the corresponding quantities for the equilibrium of PL Schiff bases and this is mainly due to the lower k_1 values. The same behaviour has been described for the imines of PLP, PL, and DPL with primary amines[20] and amino acids.[5]

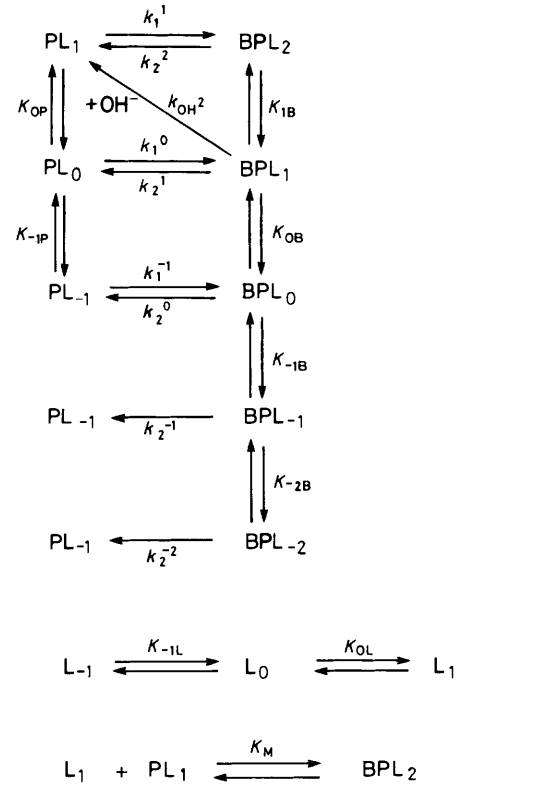
The low k_1 values of PL Schiff bases might be due to the presence of hemiacetal forms which are unable to produce the Schiff base.[1]

The k_2 values of PL and DPL Schiff bases show a minimum at pH 8. On the other hand, this minimum is more marked and slowly shifted for the PLP system; this shows the largest stability of these imines at basic pH.

As we said above, the variation of equilibrium constant with pH shows a maximum at pH 9. For the Schiff bases of these aldehydes with hexylamine, such maxima are present at pH 9.8.[20] According to Metzler *et al.*,[3] a maximum in the plot of log K_{pH} *versus* pH must lie in the pH range between the most basic aldehyde pK and the amine pK. In this case, glycine has a pK value of 9.78 and hexylamine has pK 10.75, hence the more basic hexylamine pK could explain the shift of K_{pH} maximum at lower pH.

The different microscopic and ionization constants obtained from the best-fitting of experimental data to the theoretical equations are given in Tables 1 and 2. For PLP as well as for PL and DPL systems, an increase in k_1^{i} can be observed on the successive addition of protons to the aldehyde molecule, which means an involvement of all the protonatable groups of the aldehyde in the intramolecular acid catalysis of the dehydration of the corresponding intermediate α-hydroxyamine, in a similar way to the three aldehydes studied here. It has been suggested that such catalysis is promoted by the 3′-hydroxy group.[19]

k_1^{i} values for PL Schiff base formation are not as high as k_1^{i} for PLP and DPL Schiff bases, these last being quite similar to each other. This fact can be rationalized on the basis of n.m.r. measurements,[21] which have shown that increased internal cyclic hemiacetal formation of PL between the 5′-OH group and 4-carbonyl reduces the concentration of free aldehyde, which is required for Schiff base formation.[22]



Scheme 2.

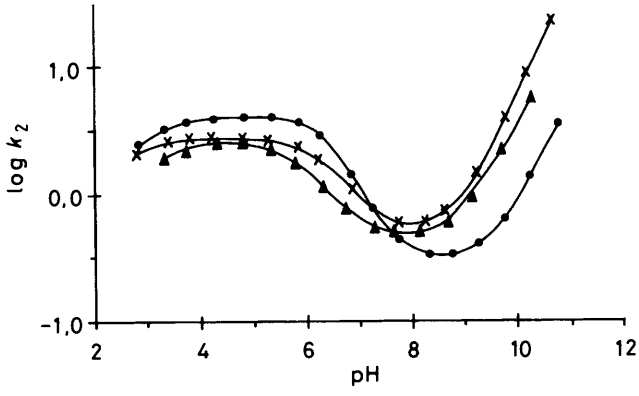


Figure 3. Variation of log k_2 as a function of pH for Schiff bases of glycine and: ●, PLP; ▲, PL; and ×, DPL. Points are experimental values and continuous lines are the theoretical fitting to equations (4) or (8), respectively.

A Brönsted plot of log k_1^i as a function of aldehyde pK, (Figure 4) gives similar slopes for PLP and DPL ($\alpha = 0.77$) which seems to indicate the minimal influence of a phosphate group upon the efficiency of intramolecular general catalysis for Schiff-base formation. The α value of the Brönsted plot for the PL system is lower than the preceding values and this is due to the significant amount of the hemiacetal form of PL unable to yield Schiff base.

The hydrolysis constant k_2^3 of the PLP Schiff base (or k_2^2 of the PL and DPL Schiff bases) can be calculated from the ratio k_1^3/K_M (or k_1^1/K_M). The values thus obtained are almost equal to the corresponding k_{OH} value, *i.e.* under these conditions $k_{OH}^2 P_w/k_{3B}$ ($k_{OH}^2 P_w/k_{1B}$) should be much less than k_2^3 (k_2^2) [see equations (5) and (9)]. For the three Schiff bases, k_{OH} values are larger than k_2^i and this indicates the ease with which this kind of Schiff base can be hydrolysed at high pH.

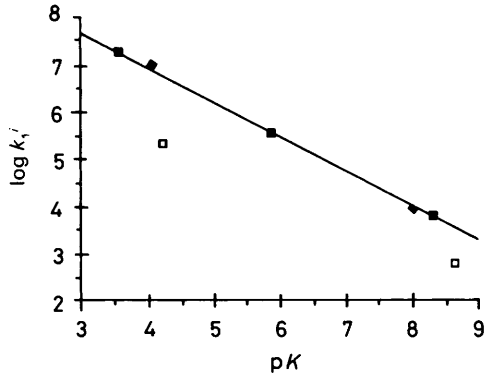


Figure 4. Plot of log k_1^i the second-order rate constants for the Schiff-base formation of: PLP, ■; PL, □; and DPL, ◆ *versus* pK. The values are given in Tables 1 and 2.

On the other hand, successive addition of protons to the Schiff base of glycine does not have the same effect upon the hydrolysis process: incorporation of the first proton on the imine nitrogen [4,8] stabilizes the molecule, whereas incorporation of a second proton again increases the hydrolysis constant. log k_2^i is not a linear function of pH, which means that a much more complicated mechanism operates for the hydrolysis process.

We should expect the ionization constant of the phosphate group of the Schiff base to be quite close to that of the aldehyde molecule, pK_{2P} 5.90. pK_{2B} and pK_{1B} obtained are 6.36 and 5.46, higher and lower than pK_{2P} respectively, and it is not clear which pK value can be identified with phosphate group protonation. Neither PL nor DPL has a phosphate group and for the Schiff bases of these two aldehydes values of 5.96 and 6.43 have been estimated for pK_{0B}, which are indeed due to ring pyridine ionization. Furthermore, on the basis of absorption spectra, pK for the ring nitrogen of 3-hydroxypyridine-4-carboxaldehyde has been estimated to be *ca.* 6.4 [3,7] and it is little affected by structure or by solvent. We think, therefore, that in spite of the low value of pK_{1B}, it must be assigned to the ionization constant of the phosphate group and the value of pK_{2B} = 6.36 is due to ionization of the pyridine nitrogen. According to this assignment, protonation of the ring nitrogen increases the resistance of the Schiff-base molecule to hydrolysis.

The first pK value of the PL Schiff base (10.75) is lower than pK_{2B} of the PLP Schiff base (11.35), and lower than pK_{2B} of the DPL Schiff base (11.22). This is probably due to some stabilizing interaction in the completely deprotonated Schiff-base molecule, perhaps involving hydrogen bonding between the imine group and the 5′-hydroxy group as suggested by Metzler *et al.*[3] For the three Schiff bases the pK value of the carboxy group is slightly lower than the pK of the group in the free amino acid and it is practically independent of the aldehyde.

Gout *et al.*[5] compared rate constants of formation and hydrolysis of imines from several carbonylic compounds, primary amines, and amino acids. They found that PLP has the largest rate constant for imine formation and one of the lowest constants for imine hydrolysis. We have also found the same behaviour at any pH, even at very high pH; K_M(PLP) > K_M(DPL) > K_M(PL), and that makes it possible to obtain high concentrations of imines of PLP in neutral or moderately basic solutions. Our results show that DPL is the best PLP analogue and it can be taken as a PLP model for some kinetic studies.

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