Kinetic Study of the Reaction of Pyridoxal 5′-Phosphate with Hydrazino Compounds of Pharmacological Activity

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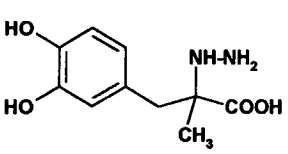
The kinetics of the reaction between pyridoxal 5′-phosphate (PLP) with carbidopa, hydralazine, and isoniazid, in aqueous solution at variable pH and constant ionic strength of 0.1M was studied spectrophotometrically. The rate constants of formation and hydrolysis of the resulting *Schiff* base, and its stability were determined in a wide range of pH. A comparison is made of the formation rate constants with those of PLP with hydrazine. The reactivity shows the sequence isoniazid > hydrazine > carbidopa > hydralazine in the whole range of pH studied. The *Schiff* bases studied are more stable than those formed by PLP and hexylamine and as stable as those described for the reactions of PLP with poly(L-lysine) or copolypeptides containing L-lysine.

Introduction. – Pyridoxal 5′-phosphate (PLP) is one of the different forms of vitamin B_6 and plays an important role as coenzyme of a wide range of different reactions as transaminations, deaminations, decarboxylations, and others [1–3]. Its action is by forming a carbinolamine (1-amino alcohol) intermediate by bonding its carbonyl group to the ε-amino group of L-lysine residue of the polypeptide chain [1][4]. The carbinolamine loses a molecule of H_2O to yield the *Schiff* base in an acid-catalyzed process [4][5]. The first step of most the PLP-dependent enzymes is a transimination reaction, namely, the conversion of the PLP-lysine imine to the PLP-substrate imine [6].

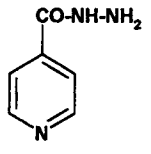
In attempts to obtain information about the PLP-dependent enzymatic process, several reaction models have been studied, using differents primary amines [7–12], amino acids [13–14], and polypeptides [15–18]. Pyridoxal (PL) and 5′-deoxypyridoxal (DPL) have been used as model of PLP [11][13][19][20] in order to verify the role of the phosphate group. To emulate the more or less hydrophobic environment of enzymatic process, the reaction models have been studied in aqueous (H_2O/EtOH, H_2O/dioxane) [21–24] and non-aqueous media (pure and mixed solvents) [25–27]. These studies have provided information about the formation and hydrolysis of *Schiff* bases and also about their stability.

Several hydrazine (H_2N-NH_2) derivatives are widely used as therapeutic chemicals, for example, carbidopa (used in combination with levodopa for the treatment of *Parkinson*'s disease), hydralazine (antihypertensive drug), and isoniazid (the most important drug worldwide for the treatment of all types of tuberculosis). It is known that biochemical interactions occur between PLP and hydrazino compounds. In most cases,

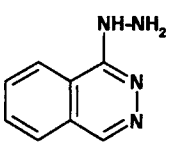
hydrazino compounds behave as inhibitory compounds towards PLP-dependent enzymes, because they react with PLP forming *Schiff* bases competing with enzymatic process [28][29].



Carbidopa



Isoniazid



Hydralazine

In this paper, we report the kinetic results of the reaction of pyridoxal 5′-phosphate with carbidopa (= 2-hydrazino-2-methyl-3-(3,4-dihydroxyphenyl)propionic acid; PLP-CD system), hydralazine (= 1-hydrazinophthalazine; PLP-HL system), and isoniazid (= pyridine-4-carbohydrazide; PLP-ISO system). Also a comparison is made with the reactions of pyridoxal 5′-phosphate with hydrazine (PLP-HY system) [30], with poly(L-lysine) [15–17], and co-polypeptides containing L-lysine [16–18].

Materials and Methods. – Pyridoxal 5′-phosphate (PLP) and the chemicals used in the buffer solutions (acetate, phosphate, and carbonate) were of reagent grade and purchased from *Merck*. Carbidopa, hydralazine, and isoniazid were from *Laboratorio Chile* and were used as purchased.

Acetate, phosphate, and carbonate buffers were used in appropriate pH ranges. The ionic strength was kept constant and equal to 0.1M.

Solns. of PLP and hydrazinic compounds were prepared daily in appropriate buffers and were stored refrigerated in the dark. The exact concentrations of PLP was determined by dilution [31] with 0.1M HCl.

The kinetics of formation of the *Schiff* bases were monitored by measuring the variation of the absorption at 395 nm for hydralazine, in the range 295–370 nm for isoniazid, and 400–425 nm for carbidopa, as a function of pH using a *Perkin Elmer Lambda-3* or a *Uvikon 941-Plus* spectrophotometer furnished with thermostated cells of 1-cm light path. The reaction was started by adding a known volume of PLP-buffered soln. to prethermostated hydrazino-compound solns. at 25.00 ± 0.05°. The observed pseudo-first-order rate constants (k_{obs}) were determined by the infinite method. The pH on the reaction cell was monitored throughout and was found not to vary by more than 0.03 pH units. pH Measurements were made by using a *Crison* pH-meter furnished with a *Metrohm EA120* electrode or a *Radiometer PHM-62* with *GK-2401C* electrode calibrated previously with aq. buffers at 25°.

The overall reaction between an aldehyde and an amine can be depicted as follows:

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

where k_1 and k_2 are the overall rate constants of formation and hydrolysis of the *Schiff* base, respectively. The procedure used to calculate these two constants from the k_{obs} values is described in detail in [10]. The ratio between them represents the equilibrium constant ($K_{pH} = k_1/k_2$). Nucleophilic rate constants (k_N) were obtained from slopes of linear plots k_{obs} *vs.* free-amine concentration, or by dividing the k_1 values by their corresponding free-amine molar fraction.

The pK_a values of hydralazine and carbidopa were determined potentiometrically by titration with 0.1M NaOH of 0.01M solutions of the corresponding amine and 0.01M of HCl, by using a *Radiometer* automatic titrator provided with *PHM-62* pH-meter, *ABU-11* automatic burette, *TTT-60* titrator, *REA-160* recorder, *TTA-60* thermostatic support, *G-2040* glass electrode, and *K-4040* calomel electrode. The exper. conditions were those of

the kinetic measurements. For hydralazine, $pK_a = 7.25$ was found. For carbidopa, two pK_a values were determined, 9.85 corresponding to the catechol group (assigned by comparison with that described for levodopa [32]) and 7.20 corresponding to the hydrazinic group.

The pK_a value of isoniazid was determined spectrophotometrically by measuring the absorbance of a 0.01M of isoniazid at different pH values. The absorbance was measured at 230 nm in the 3.10–4.63 pH range and at 320 nm in the 10.12–11.76 pH range, under the same conditions as the kinetic measurements. The pH measurements were made in a *Radiometer PHM-62* pH-meter and the absorbances in a *Perkin-Elmer Lambda-3* spectrophotometer. The values found are 3.61 and 11.10, corresponding to the pyridine nitrogen and hydrazino group, respectively.

Results and Discussion. – *Figs. 1–3* show the logarithms of rate constant of formation, rate constant of hydrolysis, and equilibrium constant, log k_1, log k_2, and log K_{pH}, as a function of pH for the *Schiff* bases of PLP with carbidopa, hydralazine, and isoniazid (PLP-CD, PLP-HL, and PLP-ISO systems). They also include the values of the reaction of PLP with hydrazine (PLP-HY system) [30].

In *Fig. 1*, it can be observed that the *Schiff*-base formation of PLP-CD and PLP-HL system show the same behavior as PLP-HY system as a function of pH. The PLP-CD system seems to form *Schiff* bases faster than the PLP-HY system at pH < 8.5, whereas the PLP-HL system shows a slower rate of formation than the reference system in the whole pH range studied. For the PLP-ISO system, log k_1 values decrease to a minimum at pH 8 and then increase again as the pH increases. Nevertheless, the PLP-ISO system shows log k_1 values smaller than those of the PLP-HY system in pH range of 5.5–10.0. This different behavior is due to the different basicities of hydrazino-group bearers.

To be independent of the formation rate constant from the free-amine molar fraction and to compare the reactivities of different hydrazino compounds to PLP, the k_N values for the three systems at different pH values were calculated. *Fig. 4* shows the behavior of log k_N for the different systems as function of pH, and also summarizes the

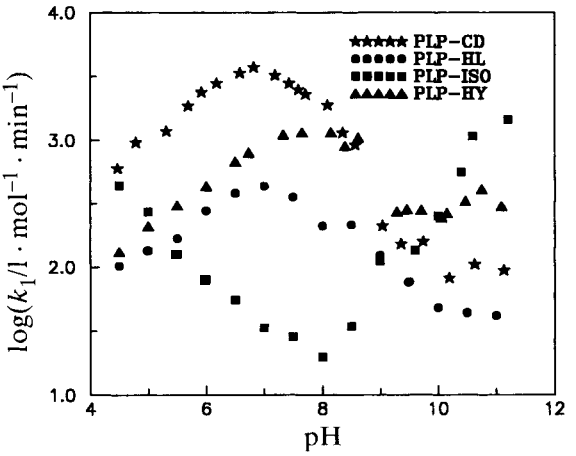


Fig. 1. *Plot of log* k_1 vs. *pH for different adducts of PLP with carbidopa* (★), *hydralazine* (●), *isoniacid* (■), *and hydrazine* (▲) [30]

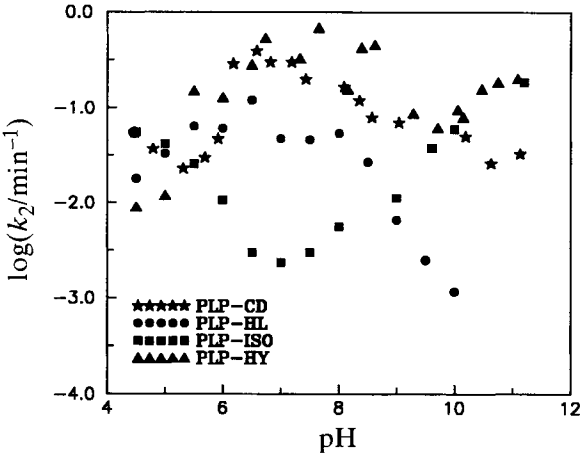


Fig. 2. *Plot of log* k_2 *vs. pH for different adducts of PLP with carbidopa* (★), *hydralazine* (●), *isoniazid* (■), *and hydrazine* (▲) [30]

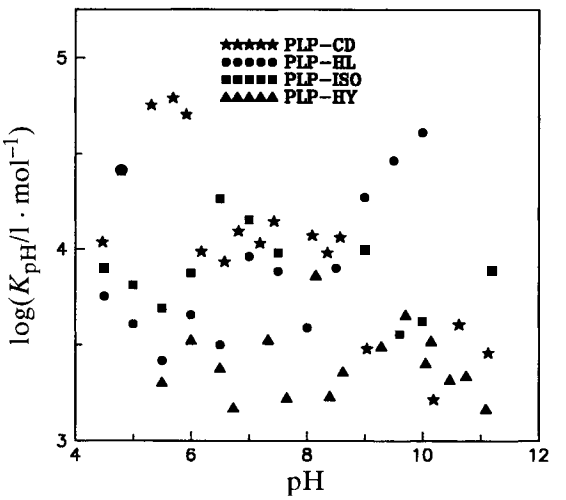


Fig. 3. *Plot of log* k_{pH} *vs. pH for different adducts of PLP with carbidopa* (★), *hydralazine* (●), *isoniazid* (■), *and hydrazine* (▲) [30]

values for the PLP-HY system [30]. The reactivity shows the sequence

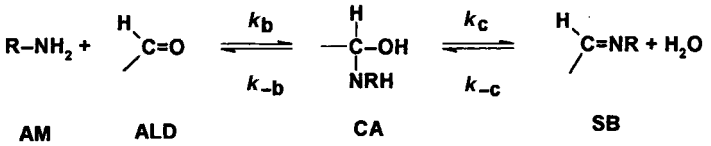
isoniazid > hydrazine > carbidopa > hydralazine

in the whole pH range studied, revealing a greater reactivity for isoniazid.

This result explains the adverse reactions of isoniazid with PLP in malnourished patients and those predisposed to neurophaty [33]. If vitamine B_6 is not given concurrently, peripheral neuritis is the most common reaction to isoniazid. Toxic effects can be minimized by prophylactic therapy with vitamine B_6 (15 to 50 mg per day) [34].

For the systems studied, an increase of log k_N as pH decreases is observed as in the PLP-HY system. It is known that *Schiff* bases (SB) are formed *via* a two-step mechanism involving the initial formation of a 'carbinolamine' (CA; 1-amino alcohol) by attack of an amine (AM) to the carbonyl group of an aldehyde (ALD), followed by dehydratation [4] (*Scheme 1*).

Scheme 1



If $K_b = k_b/k_{-b}$ is defined as the equilibrium formation constant of carbinolamine, the following expression will hold for the observed rate constant:

$$k_{obs} = \frac{k_c \cdot K_b \cdot [AM]}{(1 + K_b \cdot [AM])} + k_{-c}$$

The linearity of plots of k_{obs} *vs.* [AM] permits to assume that $K_b \cdot [AM] << 1$; therefore, the overall rate constant of formation is $k_1 = k_c \cdot K_b \cdot [AM]$, and involves the carbinolamine-formation equilibrium constant.

Fig. 4 shows that k_N increases as pH decreases for the four systems shown, in accord with an intramolecular catalytic process [35]; therefore, it is possible to conclude that in the PLP-CD, PLP-HL, and PLP-ISO systems the carbinolamine dehydratation is the rate-determining step, as in most of the reactions of PLP and its analogues studied [7][10][11][13][17][19][20].

It is interesting to remark that PLP-CD, PLP-HL, and PLP-HY systems show a parallel behavior (*Fig. 4*) in the whole pH range studied, including the PLP-ISO system at pH > 7.0; nevertheless, the latter showing a more pronounced increase than the others as pH decreases in acidic media. This result is a consequence of the structural differences of hydrazino-group bearers and due to the presence of other protonable groups.

The reactivity sequence found

isoniazid > hydrazine > carbidopa > hydralazine,

is the same sequence shown by the pK_a values of the hydrazino group. However, a quantitative comparison of the reactivities by using a structure-reactivity relationship is not possible due to the fact that the amines studied are not structurally homogeneous. The PLP-HL system is about one order of magnitude less reactive than the PLP-CD system in the whole pH range, although both amines have similar pK_a. This behavior can be explained by assuming a steric hindrance in the attack of the amine to the carbonyl group. It is due to the fact that the hydrazino group is bonded to a polycyclic aromatic ring in hydralazine. At this point, it is necessary to remember that, although the rate-determining step is the dehydratation of the carbinolamine intermediate, as it was men-

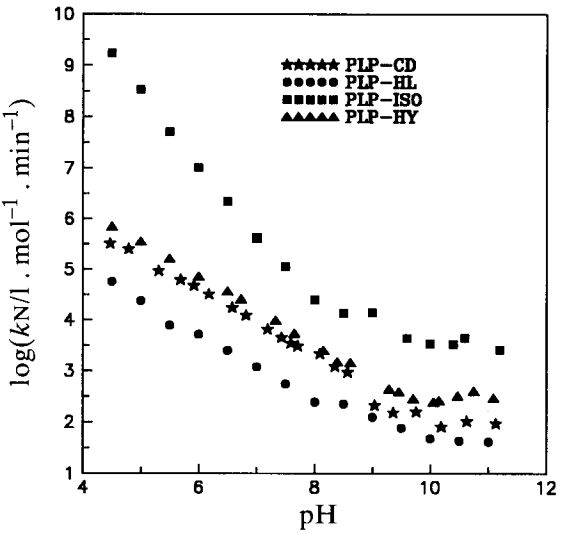
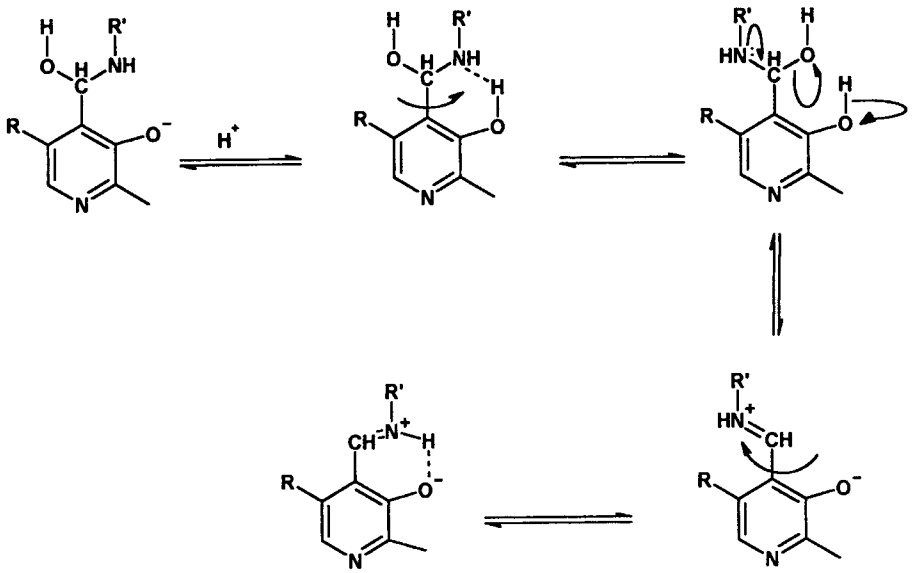


Fig. 4. *Plot of log* k_N *vs. pH for different adducts of PLP with carbidopa* (★), *hydralazine* (●), *isoniazid* (■), *and hydrazine* (▲) [30]

tioned before, there are several previous equilibrium steps whose equilibrium constants are involved in k_1 and also k_N (see *Scheme 2*).

In *Fig. 2*, it can be observed that the log k_2 *vs.* pH plot is similar for the PLP-HY, PLP-CD, and PLP-HL systems, showing a maximum k_2 value near to pH 7.0–8.0. It must be emphasized that this behavior is in contrast to that observed for the reactions

Scheme 2



of the PLP with hexylamine [11], amino acids [13], homo- and copolypeptides of L-lysine, [15][17][18], and also for other systems of PLP analogues as PL and DPL with the same amino-group bearers [11][13][20]. All these systems show a minimum in the log k_2 *vs.* pH plots. Nevertheless, the PLP-ISO system, displays a minimum in the log k_2 *vs.* pH plot, as the described model reactions. The differences observed in behavior can be related to the different pK_a values of *Schiff* bases derived from the different amino-group bearers, since carbidopa, hydrazine, and hydralazine are less basic than the isoniazid and the other amino-group bearers used as models (amines, amino acids, and polypeptides).

In *Fig. 3*, it can be observed that the log K_{pH} values for different systems studied are comprised between the values of log $K_{pH} = 3-5$ in the pH range used in the study and concretely between log $K_{pH} = 3-4$ at the nearness of the physiological pH range (pH 7–8). These values are greater than those of the other systems as PLP-amines [11], and PLP-amino acids [13] (these systems show values of log $K_{pH} \approx 2-3$ in the same pH range), and closely similar to the described values for the reactions of PLP with homo- and copolypeptides of L-lysine [15][17][18], (log $K_{pH} = 3-4$).

The major portion of vitamin B_6 in human plasma occurs in the phosphorylated form as biologically active PLP and bound to albumin forming a stable *Schiff* base. Therefore, the therapeutical chemicals studied in this work form *Schiff* bases with PLP as stable as those formed in the reaction of PLP with polypeptides, considered by us as the more realistic enzymatic model described.

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