184. Kinetic and Thermodynamic Parameters for *Schiff*-Base Formation between 5′-Deoxypyridoxal and Hexylamine[1])

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The thermodynamic parameters involved in the formation of *Schiff* bases between 5′-deoxypyridoxal and hexylamine were determined at different pH values and a constant ionic strength (0.1M). The overall and individual rate constants of formation and hydrolysis at 10, 15, 20, 25, and 30° were also determined. The enthalpy of the overall formation process was found to be negative at all the pH values assayed except the neutral, while its entropy was always positive. The results obtained show the great relevance of the phosphate group at C(5′) to the stabilization of the *Schiff* bases of pyridoxal 5′-phosphate.

Introduction. – Pyridoxal-5′-phosphate (PLP) and pyridoxal (PL) are two forms of vitamin B_6. These compounds can activate groups at the α, β, and γ positions of amino acids in enzymatic (PLP) and nonenzymatic (PL) processes [1]. 5′-Deoxypyridoxal (DPL) is considered to be a good analogue of PLP and PL [2], as it bears three chemical groups (–CH=O, –OH, =N–) regarded as essential to catalytic activity in amino-acid metabolism. Both PLP and PL, and DPL bear groups liable to be protonated, so they can occur in different tautomeric equilibria [3].

PLP binds to PLP-dependent proteins *via* its aldehyde group, which reacts with the terminal amino group of an L-lysine residue in the polypeptide chain to form an aldimine or *Schiff* base.

The properties of the *Schiff* bases of PLP bound to the active sites of proteins have been studied by investigating reactions between PLP (or its analogues) and some primary amines. This is essential in order to determine the thermodynamic parameters involved in the formation of *Schiff* bases.

In [4] [5], we reported thermodynamic parameters involved in the formation of *Schiff* bases between PLP and hexylamine, and studied the influence of protonable groups in PLP on the kinetic parameters of the formation and hydrolysis processes (PLP and hexylamine, PL and hexylamine, and DPL and hexylamine). We also studied the influence of the solvent polarity on the stability of *Schiff* bases of PLP [6] and DPL [7] with hexylamine.

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In this work, we studied the formation and hydrolysis of the *Schiff* bases formed between 5′-deoxypyridoxal and hexylamine at different pH values and temperatures, and a constant ionic strength. The results obtained were compared with those found for *Schiff* bases of PLP and hexylamine under the same conditions [4] [8]. This study allowed us to calculate overall thermodynamic and kinetic parameters, as well as those corresponding to the different ionic species involved in the process. It also allowed us to evaluate the influence of the phosphate group on the thermodynamic parameters involved in the formation of the *Schiff* bases of PLP.

Materials and Methods. – 5′-Deoxypyridoxal was synthesized from pyridoxine hydrochloride (*Merck*), according to *Iwata* [9], while hexylamine hydrochloride was prepared from hexylamine (*Merck*). All other reagents used were reagent-grade *Merck* chemicals. Acetate, phosphate, or carbonate buffers adjusted to 0.1M ionic strength were used as required to obtain the different pH values [10]. DPL solutions were made daily in appropriate buffers and were kept in the dark. Their exact concentration was determined by dilution with 0.1M NaOH and subsequent measurement of their absorbance at 395 nm ($\varepsilon = 6400$ l/mol·cm) [11]. The measured concentrations ranged between $5 \cdot 10^{-5}$ and $1 \cdot 10^{-4}$ M.

Hexylamine solutions were also made daily by diluting the appropriate amount of hexylamine hydrochloride in the corresponding buffer. Their concentration ranged between $5 \cdot 10^{-2}$ and $1 \cdot 10^{-3}$ M.

The kinetics of formation of the *Schiff* bases was monitored by measuring the variation of their absorption at 280 nm on a *Zeiss DMR11* spectrophotometer furnished with thermostated cells of 1-cm light path.

The imines were formed by adding volumes containing known amounts of hexylamine hydrochloride to buffered solutions of DPL previously thermostated at 25 ± 0.05°. The amine concentration the measuring cell was 50–100 times higher than that of DPL, although this ratio was lower at basic pH values.

The pH on the reaction cell was monitored throughout and was found not to vary by more than ±0.03 units. It was measured with the aid of a *Crison* pH-meter furnished with a *Metrohm EA120* combined electrode.

The overall reaction between aldehyde and amine is

$$R^1{-}CHO + NH_2{-}R^2 \underset{k_2}{\overset{k_1}{\leftrightarrows}} R^1{-}CH{=}N{-}R^2 + H_2O \tag{1}$$

Constants k_1 and k_2 were determined as reported in [6] [7].

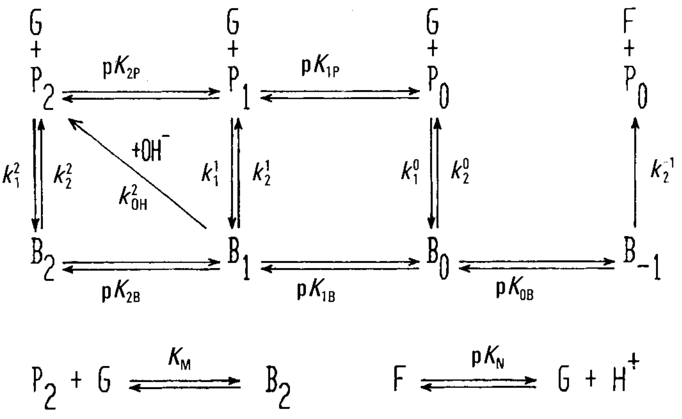
The equilibrium constants (K_{pH}) were determined either as the ratio between k_1 and k_2 or from *Eqn. 2* [12]:

$$A_0/(A_\infty - A_0) = [\varepsilon_P/(\varepsilon_B - \varepsilon_P)] + [\varepsilon_P/((\varepsilon_B - \varepsilon_P) \cdot K_{pH} \cdot b_e)] \tag{2}$$

were ε_P and ε_B are the molar absorptivities of the aldehyde and *Schiff* base, respectively, at this pH and wavelength, b_e the amine concentration at equilibrium, and A_0 and A_∞ are the absorbances at times 0 and ∞, respectively.

The ionic species occurring in solution over the pH range studied are included in the *Scheme*, where P and B denote the aldehyde and its aldimine, respectively, and the subscripts -1 indicate the number of net negative charges on the aldehyde molecules, F and G being the monoprotonated form and unprotonated form of the amine, respectively.

Scheme



The overall rate constants of hydrolysis and formation of the aldimine can be described in terms of those of the individual ionic species concerned. k_1^i (i = 0,1, or 2) and k_2^i (i = −1,0,1, or 2) are the individual rate constants of formation of the aldimine, and of hydrolysis of the aldimine by H_2O, and k_{OH}^2 is the rate constant of hydrolysis of B_1 (aldimine with no net charge) by OH^- ions.

K_N is the ionization constant of the hexylamine, pK_{2P} and pK_{1P} are the pK values of the aldehyde, pK_{2B}, pK_{1B}, and pK_{0B} are the pK values of the aldimine.

The equilibrium formation constant for the aldimine at very high pH values is given by $K_M = k_1^2/k_2^2$.

The rate of formation of the aldimine will be given by:

$$v_1 = [G]\sum_{i=0}^{2} k_1^i[P_i] = k_1[RNH_2]_T[DPL]_T \tag{3}$$

were T denotes the concentration of all species.

The hydrolysis of the aldimine conforms to *Eqn. 4*:

$$v_2 = k_{OH}^2[B_1][OH^-] + \sum_{i=-1}^{2} k_2^i[B_i] = k_2[\text{aldimine}]_T \tag{4}$$

The equilibrium constant K_{pH} is given by:

$$K_{pH} = [\text{aldimine}]_T/([RNH_2]_T[DPL]_T) \tag{5}$$

Taking into account the equilibria in the *Scheme*, *Eqns. 3–5* can be readily transformed into *Eqns. 6–8*:

$$k_1 = \frac{k_1^2 + k_1^1 \cdot a/K_{2P} + k_1^0 \cdot a^2/(K_{2P} \cdot K_{1P})}{(1 + a/K_N)\,(1 + a/K_{2P} + a^2/(K_{2P} \cdot K_{1P}))} \tag{6}$$

$$k_2 = \frac{k_{OH} + k_2^1 \cdot a/K_{2B} + k_2^0 \cdot a^2/(K_{2B} \cdot K_{1B}) + k_2^{-1} \cdot a^3/(K_{2B} \cdot K_{1B} \cdot K_{OB})}{1 + a/K_{2B} + a^2/(K_{2B} \cdot K_{1K}) + a^3/(K_{2B} \cdot K_{1B} \cdot K_{OB})} \tag{7}$$

$$K_{pH} = \frac{(1 + a/K_{2B} + a^2/(K_{2B} \cdot K_{1B}) + a^3/(K_{2B} \cdot K_{1B} \cdot K_{OB}))\,K_M}{(1 + a/K_N)(1 + a/K_{2P} + a^2/(K_{2P} \cdot K_{1P}))} \tag{8}$$

$$k_{OH} = k_2^2 + k_{OH}^2(K_W/K_{2B}) \tag{9}$$

where $a = 10^{-pH}$ and K_W is the ionic product of H_2O.

The experimental values of k_1, k_2, and K_{pH} were fitted simultaneously to *Eqns. 6–8* by a nonlinear regression method involving minimization of the functions U_i and U_{pH}:

$$U_i = \Sigma(\log k_{i,e} - \log k_{i,t})^2 \quad i = 1 \text{ or } 2 \tag{10}$$

$$U_{pH} = \Sigma(\log K_{pH,e} - \log K_{pH,t})^2 \tag{11}$$

where the subscripts e and t denote experimental and theoretical data, respectively. The initial values of the protonation constants of DPl and hexylamine listed in *Table 1* were used for this purpose. The protonation constants of the *Schiff* bases were estimated from literature data for similar systems [4] [5].

Table 1. p*K Values and Temperature Coefficients for 5'-Deoxypyridoxal and Hexylamine Determined by Different Techniques at 0.1* M *Ionic Strength*

	pK_{1P}[a])	pK_{2P}[a])	pK_N[b])
$T = 25°$	4.15	8.22	10.50
$\frac{d(pK)}{dT}$	0.10	−0.13	−0.16

[a]) Determined by spectrophotometric techniques. [b]) Determined by potentiometric techniques.

Results and Discussion. – The formation *Schiff* base is known to take place *via* the addition of the NH_2 group to a C=O compound to yield a 'aminocarbinol' (intermediate product), which releases one H_2O molecule to become an imine. The rate-determining step in the process is the dehydration of the 'aminocarbinol' [13] [14].

Table 2 lists the rate constants of formation (k_1) and hydrolysis (k_2) of the *Schiff* bases formed between DPL and hexylamine at different pH values and temperatures (10, 15, 20,

Table 2. *Kinetic Parameters for* Schiff-*Base Formation*[a])

$T = 10°$					$T = 15°$				
pH	k_1	k_2	K_{pH}[b])	K_{pH}[c])	pH	k_1	k_2	K_{pH}[b])	K_{pH}[c])
4.62	1.037	0.0845	12.27	11.02	4.04	0.785	0.129	6.07	6.34
4.91	1.300	0.0796	16.33	15.27	4.34	1.178	0.131	8.98	9.30
5.26	1.535	0.0700	20.05	25.06	4.77	1.732	0.132	13.14	14.62
5.78	1.754	0.0605	25.06	26.89	5.30	2.268	0.126	16.83	17.84
6.26	2.009	0.0493	33.19	37.01	5.68	2.382	0.121	19.64	19.60
6.65	2.427	0.0326	49.21	47.89	6.28	2.897	0.100	28.97	35.41
7.05	3.451	0.0390	88.51	95.13	6.66	3.715	0.080	46.55	49.27
7.44	5.768	0.0326	177	158	7.08	5.875	0.063	92.90	98.80
7.87	11.17	0.0295	379	389	7.50	10.94	0.053	206	228
8.38	24.04	0.0306	785	813	7.83	20.09	0.051	394	463
8.74	38.02	0.0360	1069	1134	8.24	33.96	0.053	644	677
9.09	58.75	0.0472	1244	1285	8.67	58.48	0.065	906	940
9.44	99.31	0.0735	1352	1342	9.09	99.31	0.097	1028	940
9.86	199	0.151	1315	1311	9.46	173	0.169	1028	1086
10.20	359	0.281	1276	1286	9.98	441	0.454	971	995
10.60	764	0.679	1125	1101	10.20	653	0.700	933	903
10.8	1084	1.059	1024	1047	10.60	1327	1.371	968	954

Table 2 (cont.)

$T = 20°$

pH	k_1	k_2	K_{pH}[b])	K_{pH}
3.90	1.064	0.175	6.07	6.25
4.12	1.479	0.185	7.98	8.00
4.38	2.014	0.194	10.38	9.79
4.94	2.985	0.200	14.93	14.16
5.24	3.288	0.198	16.63	16.51
5.79	3.802	0.180	21.13	19.82
6.19	4.467	0.199	22.43	28.10
6.67	6.266	0.117	53.60	48.10
7.11	10.72	0.093	116	110
7.50	19.41	0.085	230	228
7.91	36.48	0.082	447	414
8.42	69.82	0.096	730	749
8.80	108	0.129	838	824
9.32	230	0.264	869	886
9.70	505	0.603	841	1008
10.20	1000	1.306	766	790
10.50	1884	2.987	631	653

$T = 25°$

pH	k_1	k_2	K_{pH}[b])	K_{pH}[c])
4.23	2.692	0.288	9.35	10.40
4.53	3.428	0.306	11.21	12.65
4.85	4.131	0.313	13.18	14.27
5.18	4.581	0.312	14.69	15.51
5.47	4.977	0.303	16.44	18.03
5.78	5.432	0.275	19.72	19.10
6.23	6.474	0.232	27.93	23.59
6.54	8.318	0.194	42.85	28.23
6.84	11.03	0.169	65.38	42.45
7.08	16.79	0.152	111	111
7.32	23.71	0.143	166	155
7.69	43.85	0.142	310	302
7.91	59.84	0.143	419	415
8.43	112	0.174	644	661
8.69	153	0.210	729	713
8.94	221	0.275	804	855
9.27	352	0.447	787	823
9.63	682	0.871	783	792
9.96	1230	1.690	728	753
10.20	2158	3.396	635	697

$T = 30°$

pH	k_1	k_2	K_{pH}[b])	K_{pH}[c])
3.63	2.037	0.372	5.47	5.38
4.18	4.198	0.462	9.08	8.88
4.57	5.598	0.492	11.38	10.61
5.02	6.668	0.495	13.46	13.88
5.37	7.261	0.468	15.52	15.09
5.77	8.147	0.427	19.10	25.71
6.23	10.35	0.345	30.00	25.54
6.67	15.85	0.269	58.88	63.72
7.05	26.73	0.236	114	116
7.50	56.83	0.219	260	242
7.91	97.05	0.229	424	403
8.42	181	0.299	424	403
8.81	299	0.432	693	646
9.17	524	0.753	695	653
9.57	1072	1.585	676	667
10.10	1000	1.306	766	736
10.50	1584	2.2987	631	643

[a]) k_1: $l \cdot mol^{-1} \cdot min^{-1}$. k_2: min^{-1}. K_{pH}: $l \cdot mol^{-1}$. [b]) Calculated from k_1/k_2. [c]) Obtained from *Eqn. 2*.

25, and 30°). As can be seen, k_1 increases with increasing pH, while the variation of k_2 shows a minimum between pH 7 and pH 8. These values are consistent with those found for the *Schiff* bases of PLP and hexylamine [4] [8]. However, the k_1 values of the *Schiff* bases of DPL are smaller than those of the bases of PLP at any pH and temperature, while the k_2 values of the former are always larger than those of the latter bases. The *Figure* shows the variation of K_{pH} as a function of pH. The K_{pH} values found were always smaller

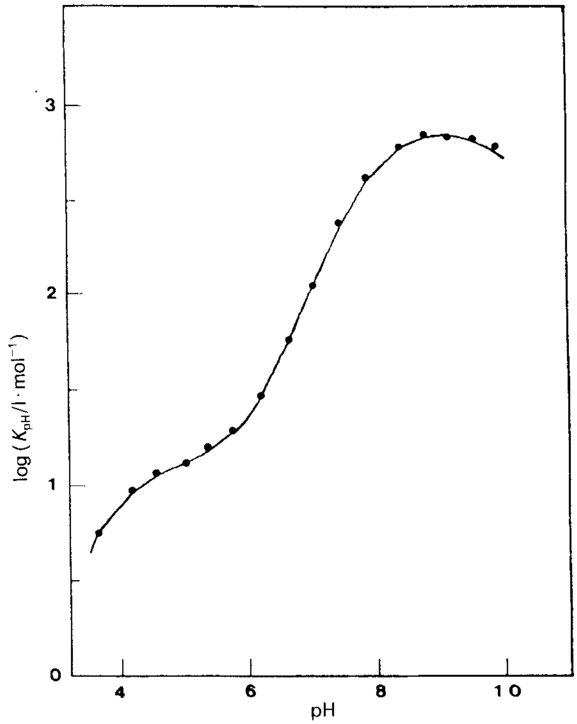


Figure. *Variation of log* K_{pH} *as a function of the pH at 30°.* ●: Experimental values; fine line: theoretical fitting calculated from *Eqn. 8* and the parameters given in *Table 4.*

than those of the *Schiff* bases of PLP. The values of the equilibrium constant of the process, K_{pH}, calculated by the two above-described procedures, were quite consistent.

The data given in *Table 2* were used to calculate the energies of activation of the overall formation ($E_a(k_1)$) and hydrolysis ($E_a(k_2)$) processes, as well as the enthalpy of formation of the *Schiff* base of DPL and hexylamine (*Table 3*). The energies of activation process were of the same order as those reported for the system formed between PLP and ε-aminocaproic acid or L-serine (between 28 and 54 kJ/mol) [15] and for that of PLP and hexylamine (between 46 and 66 kJ/mol) [4]. The formation of the *Schiff* base between DPL and hexylamine was exothermic at acidic and basic pH values. The process was

Table 3. *Energy of Activation for* Schiff*-Base Formation, and Hydrolysis and Enthalpy of Formation Process* (standard errors for 95% confidence level)

pH	$E_a(k_1)$/kJ·mol^{-1}	$E_a(k_2)$/kJ·mol^{-1}	ΔH/kJ·mol^{-}1
10	72.3 ± 3.6	101.0 ± 4.2	−28.7 ± 1.7
9	78.0 ± 3.7	86.4 ± 3.8	−8.4 ± 1.6
8	69.6 ± 3.6	77.0 ± 2.7	−7.4 ± 1.4
7	72.9 ± 3.4	64.6 ± 3.6	8.4 ± 1.3
6	55.0 ± 2.7	61.2 ± 3.6	−6.0 ± 1.2
5	54.1 ± 2.6	66.3 ± 2.2	−12.2 ± 1.1
4	52.4 ± 3.2	59.0 ± 2.1	−6.4 ± 1.2

endothermic at a neutral pH over a narrower interval than that of the *Schiff* base of PLP and the same amine. This can be accounted for on the basis of the absence of a phosphate group, the protonation of which is a endothermic process [16], in the DPL molecule.

By fitting the data given in *Table 2* to *Eqns. 6–8*, we calculated the individual rate constants of formation (k_1^i) and hydrolysis (k_2^i and k_{OH}), as well as the different pK values of DPL, hexylamine, and *Schiff* base. As can be seen, the experimental results were quite consistent with those obtained by using the parameters listed in *Table 4* in *Eqns. 6–8* (*Fig.*).

Table 4. *Best Kinetics Constants, p*K*'s and* K_M *Values Obtained in the Fitting of Experimental Values of* k_1, k_2, *and* K_{pH} *to* Eqns. 6–8

T/°C	10	15	20	25	30
$\log k_1^0$	6.99	7.08	7.22	7.30	7.46
$\log k_1^1$	4.43	4.56	4.70	4.80	4.95
$\log k_1^2$	3.58	3.70	3.81	3.92	4.08
$\log k_2^{-1}$	−1.36	−1.28	−1.20	−1.12	−1.02
$\log k_2^0$	−1.08	−0.875	−0.675	−0.485	−0.283
$\log k_2^1$	−1.61	−1.35	−1.15	−0.905	−0.712
$\log k_{OH}$	1.78	1.84	1.88	1.93	2.03
pK_{1P}	4.46	4.34	4.23	4.14	4.04
pK_{2P}	8.35	8.22	8.09	7.98	7.86
pK_{OB}	3.74	3.63	3.53	3.39	3.30
pK_{1B}	6.52	6.37	6.31	6.23	6.11
pK_{2B}	12.54	12.25	11.93	11.69	11.44
pK_N	11.21	11.05	10.90	10.75	10.62
$\log K_M$	1.80	1.87	1.93	1.99	2.06
$\delta(k_1)$[a])	0.002	0.002	0.002	0.003	0.003
$\delta(k_2)$[a])	0.001	0.004	0.003	0.003	0.003
$\delta(K_{pH})$[a])	0.010	0.020	0.020	0.020	0.008

[a]) δ(i) is the standard deviation of fitting of the respective equation.

The decrease in k_1^i with increase in the pH observed at all the temperatures assayed (*Table 4*) was a result of intramolecular acid catalysis on the dehydration of the 'aminocarbinol' [17]. The *Schiff* bases of DPL and hexylamine have k_{OH} values tenfold those of the bases of PLP [4], *i.e.* the former are more readily hydrolysed at high pH values. The k_{OH} values obtained were greater than those of k_2^i (i = 1, 0, −1). The incorporation of the first proton into the *Schiff* base (species B_1 in the *Scheme*) stabilized the molecule.

The *Arrhenius* plots of the rate constants k_1^i and k_2^1, and the *Vant'Hoff* plot of the corresponding K_{pH}^i constants allowed us to calculate the activation energy, the enthalpy, and the entropy of the individual formation and hydrolysis the *Schiff* bases (*Table 5*). The formation of *Schiff* base of DPL was more exothermic than that of the base of PLP ($\Delta H = 21.3$ and -12.2 kJ/mol for the unprotonated and monoprotonated form, respectively) [4].

Irrespective of the number of protons bonded to the *Schiff* base resulting from DPL and hexylamine, the entropy of the individual process was always positive, in contrast to those of the *Schiff* bases of PLP [4] [15] (ΔS was always negative for the protonated *Schiff*

Table 5. *Energy of Activation and Thermodynamic Parameters Corresponding to the Elementary Processes*a)

		r	δ_{xy}			r	δ_{xy}
$E_a(k_1^0)$	38.4 ± 3.5b)	0.999	0.66	ΔH^0	−27.3 ± 0.3b)	0.998	0.17
$E_a(k_1^1)$	42.4 ± 3.0b)	0.998	0.66	ΔH^1	−31.9 ± 0.2b)	1.000	0.05
$E_a(k_1^2)$	40.3 ± 2.7b)	0.998	0.59	ΔH^2	20.9 ± 1.4b)	0.998	0.30
$E_a(k_2^{-1})$	27.8 ± 1.1b)	0.998	0.20				
$E_a(k_2^0)$	65.7 ± 1.2b)	0.937	0.59	ΔS^0	49.4 ± 15.4c)	0.998	0.17
$E_a(k_2^1)$	74.2 ± 3.0b)	0.972	0.65	ΔS^1	8.2 ± 4.4c)	1.000	0.05
$E_a(k_2^2)$	19.5 ± 1.0b)	0.998	0.29	ΔS^2	112.0 ± 28.1c)	0.998	0.30

a) r is the correlation coefficient and δ_{xy} the deviation standard of the corresponding plot (*Arrhenius* or *Vant'Hoff*). The intervals given beside the E_a, ΔH, and ΔS are the standard errors for 95% confidence level.

b) In $kJ \cdot mol^{-1}$.

c) In $J \cdot mol^{-1} \cdot k^{-1}$.

bases). This divergent behaviour should be ascribed to the absence of a phosphate group in the DPL molecule, as its presence appears to constrain the motion of *Schiff*-base molecule and, hence, result in a more orderly structure.

The results obtained in this work allow us to conclude that the presence of the phosphate group in the *Schiff* base not only favours the occurrence of electrostatic interactions between the base and the environment of the active site in PLP-dependent enzymes, as reported in [18] [19], but also increases the stability of the base at any pH and temperature (K_{pH}(PLP) > K_{pH}(DPL)). The PLP molecule features the greater rate constants of formation and the smaller of hydrolysis of *Schiff* bases.

REFERENCES

[1] D. L. Leusing, in 'Vitamin B-6 Pyridoxal Phosphate. Chemical, Biochemical and Medical Aspects', Eds. D. Dolphin, R. Poulson, and O. Avramovic, J. Wiley & Sons, New York, 1986, Part A, Chapt. 4, p. 49.

[2] Y. C. Chang, R. D. Scott, D. J. Graves, *Biochemistry* **1987,** *26,* 360.

[3] M. Cortijo, J. Llor, J. M. Sanchez Ruiz, *J. Biol. Chem.* **1988,** *263,* 17960.

[4] M. A. Garcia del Vado, J. Donoso, F. Muñoz, G. Echevarria, F. Garcia Blanco, *J. Chem. Soc., Perkin Trans. 2* **1987,** 445.

[5] M. A. Vazquez, J. Donoso, F. Muñoz, F. Garcia Blanco, M. A. Garcia del Vado, G. Echevarria, *Bull. Soc. Chim. Fr.* **1988,** 361.

[6] M. A. Garcia del Vado, G. Echevarria, A. Garcia-Espantaleon, J. Donoso, F. Muñoz, F. Garcia Blanco, *J. Mol. Catal.* **1988,** *44,* 313.

[7] M. A. Vazquez, J. Donoso, F. Muñoz, F. Garcia Blanco, M. A. Garcia del Vado, G. Echevarria, *J. Mol. Catal.* **1990,** *59,* 137.

[8] J. M. Sanchez Ruiz, J. M. Rodriguez Pulido, J. Llor, M. Cortijo, *J. Chem. Soc., Perkin Trans. 2* **1982,** 1425.

[9] C. Iwata, *Biochem. Prep.* **1968,** *12,* 117.

[10] V. Cerda, C. Mongay Fernandez, *An. Quim.* **1980,** *76 B,* 154.

[11] E. A. Peterson, H. A. Sober, *J. Am. Chem. Soc.* **1954,** *89,* 169.

[12] D. S. Auld, T. C. Bruice, *J. Am. Chem. Soc.* **1967,** *89,* 2083.

[13] E. N. Cordes, W. P. Jencs, *J. Am. Chem. Soc.* **1963,** *76,* 2843.

[14] S. A. Hershey, D. L. Leussing, *J. Am. Chem. Soc.* **1977,** *99,* 1992.

[15] H. Wiesinger, H. J. Hinz, *Arch. Biochem. Biophys.* **1984,** *235,* 34.

[16] G. R. Echevarria, M. A. Garcia del Vado, F. Garcia Blanco, M. Menendez, J. Lainez, *J. Solution Chem.* **1986,** *15,* 151.

[17] D. S. Auld, T. C. Bruice, *J. Am. Chem. Soc.* **1973,** *95,* 4270.

[18] H. Wiesinger, H. J. Hinz, *Biochemistry* **1984,** *23,* 4921.

[19] D. Palm, H. W. Klein, R. Schinzel, M. Bühner, E. J. M. Helmreich, *Biochemistry* **1990,** *29,* 1099.