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THE NUCLEOPHILIC ATTACK OF THIOLS ON PYRIDOXAL-5'-PHOSPHATE

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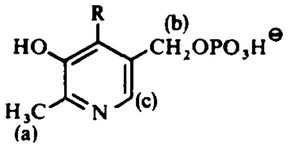
Abstract—The results of temperature jump relaxation studies on the reaction of 2-mercaptoethanol with pyridoxal-5'-phosphate in aqueous solution are presented together with NMR and UV spectra. The observed concentration and pH dependence of the relaxation time are explained by a three step mechanism for hemimercaptal formation.

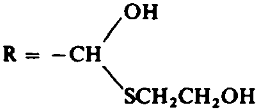
INTRODUCTION

THE addition of functional groups, important in enzymic reactions, e.g. amino, hydroxyl and mercapto groups, to the CO group of pyridoxal-5'-phosphate have been described,[1-3] but as the rates of the primary addition step are too fast to be measured by conventional kinetic methods only thermodynamic data have been reported. In this paper the results of kinetic measurements for the reaction of the thiol mercaptoethanol (ME) with pyridoxal-5'-phosphate (PLP) in aqueous solutions, obtained by the temperature jump relaxation technique, are presented. A mechanism is proposed for these reactions.

FORMULA



(d)
1: R = $-CHO$

2:

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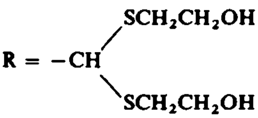
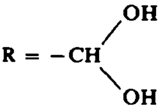

$R = -CH(SCH_2CH_2OH)(SCH_2CH_2OH)$

4:

$R = -CH(OH)(OH)$

Materials and methods. Standard analytical grade PLP monohydrate was obtained from Merck (Darmstadt), ME from Schuchardt (Munich), and 2-Mercaptoacetic acid (MAA) from Fluka (Buchs, Switzerland). The substances were used without further purification. The buffers used were: In the pH range $2{\cdot}0 \leqslant pH \leqslant 4{\cdot}0$: citrate/HCl; in the range from $5{\cdot}0 \leqslant pH \leqslant 6{\cdot}0$: citrate/NaOH and at $pH = 7{\cdot}0$:

phosphate. The pH of all other solutions was adjusted by addition of N/100 HCl or N/100 NaOH.

Doubly distilled water was used throughout. pH measurements were made with a standard pH-Meter ("Radiometer", Type 22) and glass electrodes (Radiometer, GK 2021B).

UV spectra were recorded with a Cary 14 spectrophotometer. Measurements were made 30 min after mixing the solns in thermostated cuvettes at the temps indicated (25° for equilibrium measurements, 15° and 25° for temperature difference spectra). Quartz cells (1 cm lightpath) were used.

NMR spectra were recorded in D_2O soln with a Varian HA 100 spectrometer at the Institute for Molecular Biology, Biochemistry and Biophysics, Stöckheim, West Germany. t-Butanol, was used as the internal standard. For comparison with TMS the chemical shifts were corrected by adding the frequency difference $\delta_{TMS} - \delta_{t-BuOH} = 129$ ppm. The pD of the solns was adjusted by addition of NaOD and D_3PO_4 soln (Merck, Darmstadt). The pD was measured on a standard pH-peter with glass electrodes, the values were corrected using the relation pD = pH + 0·4.[10]

The principles of the temp jump relaxation technique have been previously described.[11] The results presented in this paper were obtained from measurement made with an improved temp jump apparatus.[12] Cells especially designed for small volumes were used (1·8 ml cells with 1 cm lightpath and 0·9 ml cells with 0·7 cm lightpath). The discharge of 31 kV from a $5{\cdot}5 \cdot 10^{-8}$ F condensor produced a temp jump of about 6°. The starting temp was 19°, thus allowing the relaxation process to be observed at 25°. A typical oscillogramm is shown in Fig. 1.

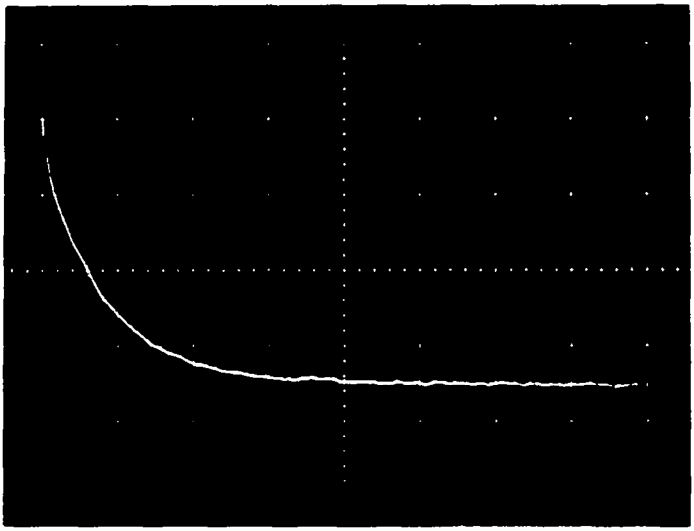


FIG 1. "Typical" oscillogram of a temperature jump relaxation experiment for hemimercaptal formation from PLP and ME ($C^\circ_{PLP} = 2{\cdot}10^{-4}$ M, $C^\circ_{ME} = 1{\cdot}10^{-2}$ M, pH = 9·9, $\tau = 37$ μsec). The ordinate represents the transmission of the solution at 406 mμ (increasing with time). Scale: 50 μsec/large div.

RESULTS

1. *NMR spectra.* NMR spectra of PLP were recorded in D_2O with and without addition of ME. In acidic soln (pD ~ 1·0) and at neutral pD values the signal of the

aldehydic proton disappeared completely on addition of about 1 M ME. A corresponding increase in the signal of the methin proton on C4' in the range of δ(TMS) ~ 6·5 was observed. The chemical shifts of this methin proton in **2**, **3**, and in hydrated **4** were similar. Since chemical equilibrium was rapidly established only one signal was observed. The chemical shifts of all the other protons in the molecule remained essentially unchanged upon addition to the CO group.

TABLE 1. NMR SPECTRA OF PYRIDOXAL-5'-PHOSPHATE IN D_2O WITH AND WITHOUT MERCAPTOETHANOL

pD	C_{ME}	chemical shifts δ (ppm)*				
		a	b	c	d	
7·3	0	2·57	5·1†	7·71	6·27 (17%)	10·40 (83%)
6·7	1 M	2·41	4·88	7·69	6·40	

* The numbers of the protons are shown in 1.

† The signal is partially hidden in the HDO signal.

Although almost all the PLP was present as free aldehyde at high pD values (pD ~ 10) no addition reaction was detected by NMR spectroscopy at these pD values.

2. *UV spectra.* Hemimercaptal formation **2** was followed spectroscopically in the UV region, and when it occurred, the first absorption band of PLP at pH = 7 (λ_{max} = 388 mμ, ε_{max} = 5400) shifted towards shorter wave lengths (λ_{max} = 322 mμ). The equilibrium constant for this reaction was obtained from the usual "Hildebrand-Benesi"-plot.[4] The relationship remained linear up to relatively high concentrations (Fig 2) of ME (1 M). This indicates that only hemimercaptal formation occurs. The

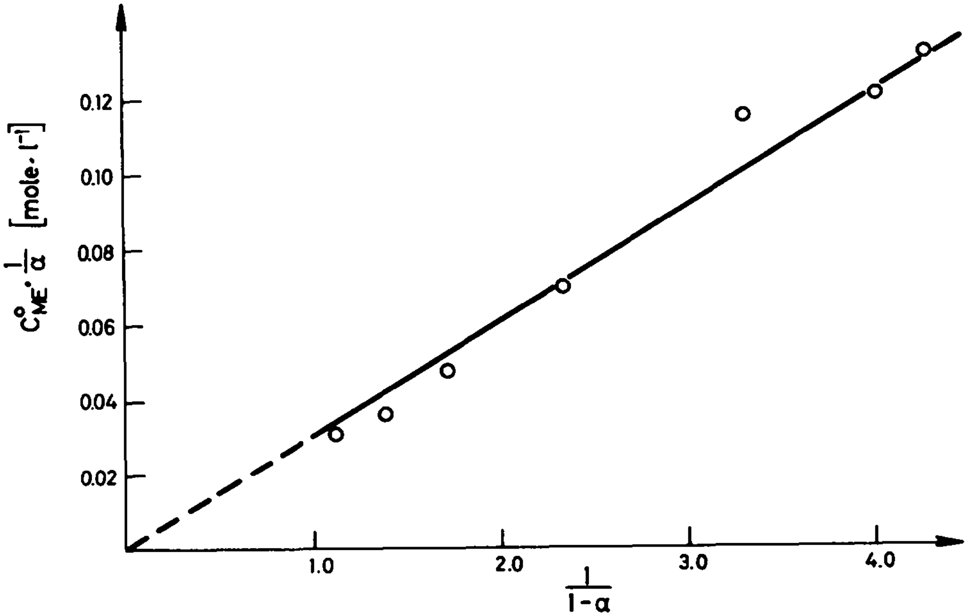
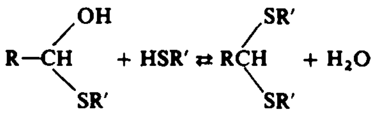


FIG 2. "Hildebrand-Benesi"-plot[4] for hemimercaptal formation from PLP and ME (λ = 388 mμ, $\alpha = [E(C_{ME}=O) - E(C_{ME})]/[E(C_{ME}=O) - E(C_{ME}=\infty)]$)

equilibrium constant was calculated to be $K = 42\ 1 \times \text{mole}^{-1}$ (corrected for hydration: $K = K_{obs}(1 + K_{hyd})$).

If mercapto acetic acid was used instead of ME, in addition to the fast primary step a slow reaction was observed.[2] This slow process has been attributed to mercaptal formation (1). With ME this slow process was not found. This is in agreement with the equilibrium measurements.



$$\mathrm{R{-}\overset{OH}{\underset{SR'}{CH}}} + \mathrm{HSR'} \rightleftarrows \mathrm{R\overset{SR'}{\underset{SR'}{CH}}} + \mathrm{H_2O} \qquad (1)$$

Addition of ME to alkaline solutions of PLP (pH ~ 11·5) did not affect the UV or the NMR spectra.

3. *Relaxation measurements.* Aqueous solutions of PLP in the absence of other reagents exhibited two distinct relaxation processes which were observed by the temperature jump technique.[5] An additional relaxation time was observed in the presence of ME. In the pH-range 5·0–8·0 and at the concentrations used ($C^{\circ}_{PLP} = 2{\cdot}10^{-4}$ M, $C^{\circ}_{ME} = 1{\cdot}10^{-2}$ M) the latter relaxation process had the largest amplitude of the three relaxation processes observed. At higher pH-values the amplitude decreased (Fig 3) until the relaxation process disappeared completely at pH ~ 11·0. This is in agreement with UV and NMR spectra, which indicated that at these pH-values no addition of ME to the CO group occurs. Between pH = 4·0 and pH = 11 the reaction of ME with PLP is faster than the hydration process. Around pH = 4·0 the relaxation times for hydration and ME addition are similar, but at lower pH-values hydration is faster than hemimercaptal formation. In more acidic solutions the amplitude of the relaxation process due to the addition of ME decreases rapidly (Fig. 3).

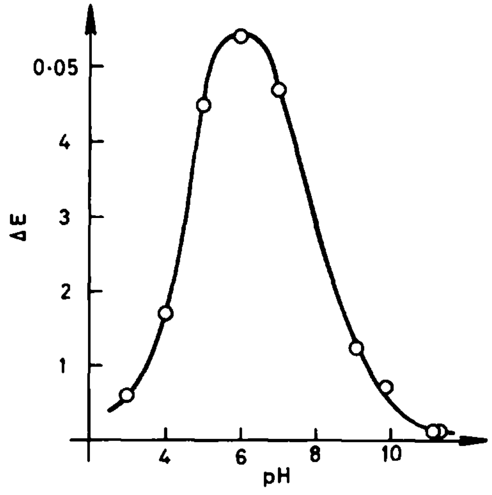


FIG 3. pH-dependence of the relaxation amplitude for hemimercaptal formation from PLP and ME (λ = 406 mμ)

The relaxation times measured do not depend on the buffer concentration within the limits of experimental error. This was shown by dilution of the buffer at pH = 7·0.

The dependence of the relaxation amplitude on wavelength at pH = 7·0 is shown in Fig 4 and is in accord with the UV temperature difference spectrum obtained. This result together with the NMR data show that the observed relaxation process corresponds to a nucleophilic attack by ME on the CO group of PLP.

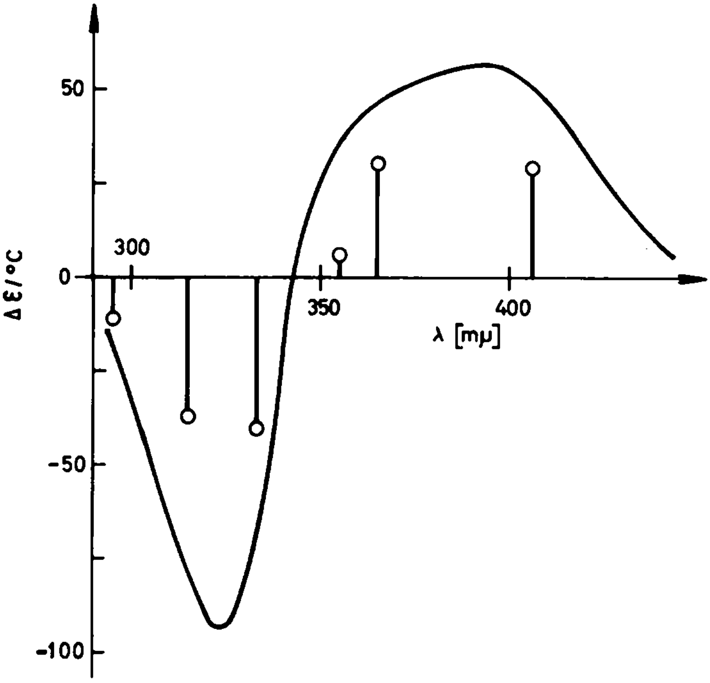


Fig 4. Wave length dependence of the change in extinction coefficient with temperature in a solution of PLP and ME ("over all" reaction observed in the UV-spectrum: ——— solid line; amplitudes from temperature jump relaxation for the individual step at different wave lengths: ○○○ open circles) ($C^\circ_{PLP} = 2{\cdot}10^{-4}$ M, $C^\circ_{ME} = 1{\cdot}10^{-2}$ M, pH = 7·0)

At constant pH (pH = 5·0, 6·0 and 7·0) the relaxation time shows a linear concentration dependence which is typical for a second order reaction:

$$\mathrm{PLP} + \mathrm{ME} \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} \mathrm{PLP-ME}$$

$$\tau^{-1} = k_{12}(C_{PLP} + C_{ME}) + k_{21} \qquad (2)$$

Since the association constant of PLP and ME is small and $C_{PLP} \ll C_{ME}$, the sum of the concentrations of the reactants (Equ 2) may be replaced by the total concentration of ME.

$$C_{PLP} + C_{ME} \sim C_{ME} \sim C^\circ_{ME}$$

The rate constants k_{12} and k_{21} were obtained from the slopes and intercepts of plots of τ^{-1} against total concentration of ME (e.g. Fig 5 for pH = 7·0). These values together with the equilibrium constant, $K = k_{12}/k_{21}$, are given in Table 2. The values obtained for K are in good agreement with those derived from spectroscopic measurements in the UV region ($K = 42$ 1 mole^{-1}).

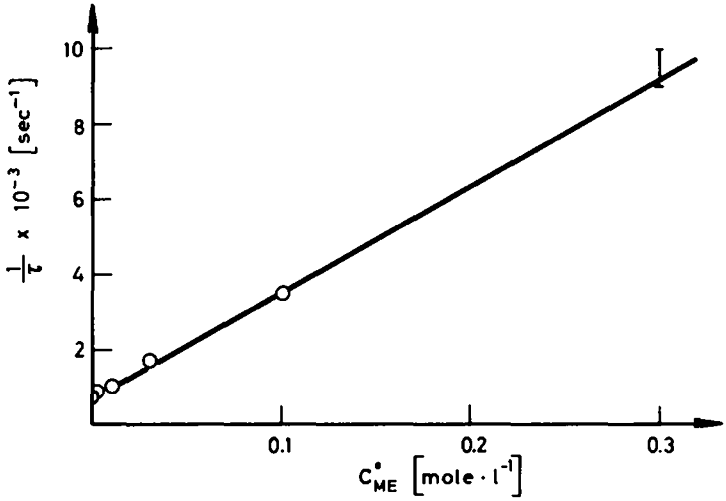


FIG 5. Concentration dependence of the relaxation time for hemimercaptal formation from PLP and ME (λ = 406 mμ, $C^{\circ}_{PLP} = 2 \cdot 10^{-4}$ M, pH = 7·0)

The pH-dependence of the relaxation time at constant ME concentration is shown in Fig 6. A logarithmic plot of $-\log \tau$ against pH results in regions where the curve follows a linear relationship with a slope, $\alpha = 1$:

$$-\log \tau = \alpha \cdot \text{pH} - \beta$$

The linear portions are connected by curves with points of inflection near the pK-values of PLP. Above the pK-value of ME, $-\log \tau$ remains essentially constant (Fig 6).

TABLE 2. RATE CONSTANTS AND EQUILIBRIUM CONSTANT FOR HEMIMERCAPTAL FORMATION FROM PLP AND ME AT DIFFERENT pH-VALUES

pH	$k_{12} \cdot 10^{-3}$ (l · mole^{-1} · sec^{-1}	$k_{21} \cdot 10^{-2}$ (sec^{-1})	K (l · mole^{-1})
5·0	1·2	0·27	46
6·0	5·7	1·5	38
7·0	29	7·2	40

DISCUSSION

Lienhard and Jencks[13] pointed out that the addition of thiols to CO groups is catalyzed by both hydronium and hydroxide ions. Their measurements were restricted Since we were able to extend our measurements to much higher pH values (pH ~ 11),

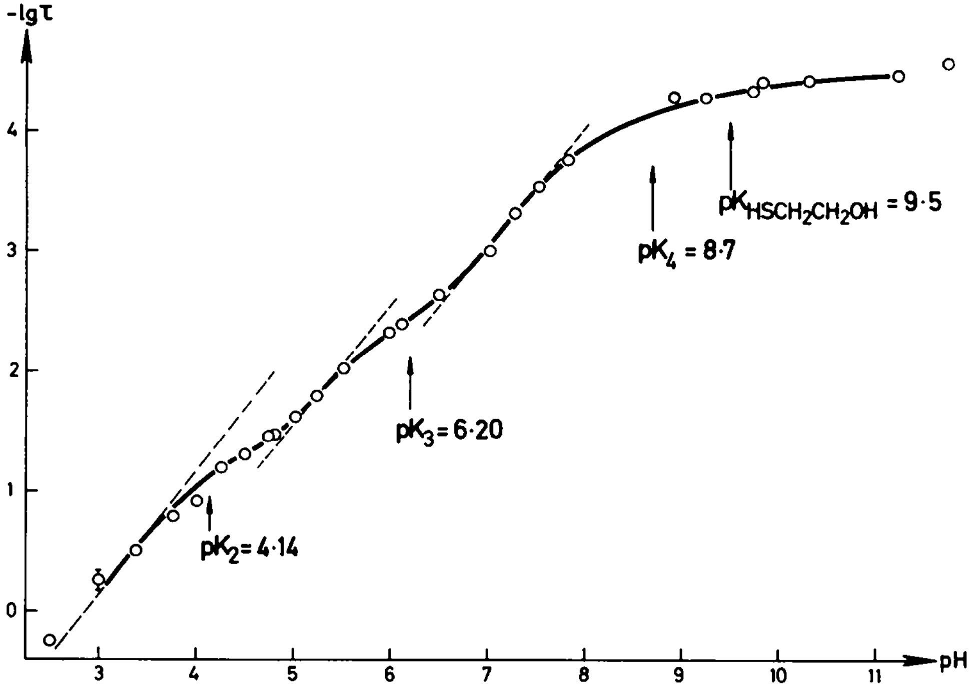
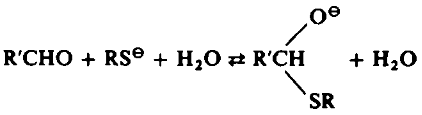


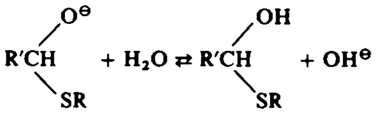
FIG 6. pH-dependence of the relaxation time for hemimercaptal formation from PLP and ME (λ = 406 mμ, $C^0_{PLP} = 2 \cdot 10^{-4}$ M, $C^0_{ME} = 1 \cdot 10^{-2}$ M)

we are now able to offer proof of the three step mechanism for $OH^{\ominus}$ catalysis previously proposed (4).

$$R'CHO + RSH + OH^{\ominus} \rightleftarrows R'CHO + RS^{\ominus} + H_2O \qquad (4a)$$



$$R'CHO + RS^{\ominus} + H_2O \rightleftarrows R'CH\begin{matrix} O^{\ominus} \\ SR \end{matrix} + H_2O \qquad (4b)$$



$$R'CH\begin{matrix} O^{\ominus} \\ SR \end{matrix} + H_2O \rightleftarrows R'CH\begin{matrix} OH \\ SR \end{matrix} + OH^{\ominus} \qquad (4c)$$

The wavelength-dependence of the relaxation amplitude (Fig 3) indicates that the process observed is an "over all" inter-conversion of aldehyde and hemimercaptal. In the pH-range below the pK-value of the thiol, (4b) rate determining, for the above mechanism to be valid the relaxation time has to obey the relationship

$$\tau^{-1} = \{k_{\leftarrow} + k_{\rightarrow}(C_{R'CHO} + C_{RSH})\} C^{\ominus}_{OH} \qquad (5)$$

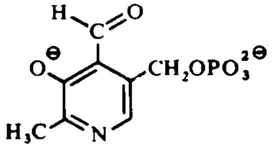
Complete agreement with our results is obtained, if the different straight lines in Fig 6 are assigned to the different protonation states of PLP.

Our measurements indicate that attack by the thiol anion on the CO group (4b) is rate determining. The dissociation of the thiol (4a) is expected to be subject to general base catalysis by buffer anion.[14] Since the relaxation time was found to be independent of buffer concentration, rate determining dissociation of the thiol (4a) was excluded. The reaction (4a) is essentially eliminated at pH values above the pK of the thiol since the thiol is predominantly present as R–S$^-$ anion under these conditions.

$$\tau^{-1} = k_{\rightarrow}(C_{RS^{\ominus}} + C_{R'CHO}) + k_{\leftarrow}(C_{R'CH(SR)OH} + C^{\ominus}_{OH}) \tag{6}$$

The relaxation time for the two step process (4b, 4c) with (4b) rate determining, becomes essentially independent of pH above pH = 9·5 which corresponds to the pK of ME.

The decrease in amplitude observed at high pH values indicates, that once the second proton has been removed from the pyridine nucleus of PLP the remaining molecule (7) does not react appreciably with HS-reagents. This is in agreement with the very low reactivity of CO groups in negatively charged molecules with conjugated π-electron systems e.g. mesoxalic acid.[8, 9]



(7)

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