

One-Electron Redox Reactions of Water-Soluble Vitamins.

III. Pyridoxine and Pyridoxal Phosphate (Vitamin B₆)

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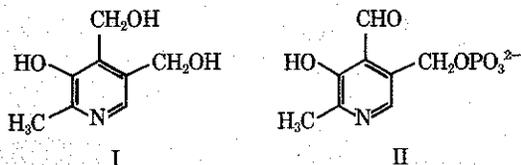
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Abstract: The one-electron reduction of pyridoxine (PH) and pyridoxal phosphate (PPH) in water by hydrated electrons, e_{aq}^- , and acetone ketyl radicals, $(CH_3)_2\dot{C}OH$, was studied using the fast-reaction technique of pulse radiolysis and kinetic absorption spectrophotometry. The reaction rate constants of e_{aq}^- , $(CH_3)_2\dot{C}OH$ and OH radicals with PH and PPH were determined at different pH values consistent with the ionization constants of these substrates. The optical absorption spectra and extinction coefficients of the free-radical intermediates produced from the one-electron reduction of pyridoxine and pyridoxal phosphate were obtained at different pH values. From the change in absorbance with pH at fixed wavelengths, the ionization constants of the radicals were derived. For the pyridoxine intermediate, pK_a values of 4.8 and 11.4 were obtained and the species assigned to $\cdot PH_3^+$, $\cdot PH_2$, and $\cdot PH^-$. The rate of protonation of $\cdot PH_2$ to $\cdot PH_3^+$ by protons was $k = 7.8 \pm 1.0 \times 10^9 M^{-1} sec^{-1}$. In the case of pyridoxal phosphate, pK_a values of 3.7 and 6.9 were obtained for the corresponding radicals $\cdot PPH_3^+$, $\cdot PPH_2$, and $\cdot PPH^-$. For both PH and PPH, the first proton loss in the radical is suggested to come from the ring nitrogen, and the second proton loss from the phenolic hydroxyl group. Support for the latter assignment was obtained from the one-electron reduction of 3-methoxypyridoxal phosphate. The properties of the substrates and of the radicals are discussed on the basis of their redox potentials.

Pyridoxal phosphate (vitamin B₆) and related compounds are essential coenzymes for a series of reactions involving α -amino acids, e.g., transamination, racemization, and decarboxylation reactions.^{2,3} They are present as Schiff bases bound to the ϵ -amino group of lysine in the protein moiety. Many of these reactions can be brought about nonenzymatically, and the assumption has been made that the mechanisms of these reactions are probably similar.^{2,3}

The absorption spectra,⁴ ionization constants,⁴ proton transfer kinetics,⁵ and polarography⁶ of pyridoxine (I) and pyridoxal 5-phosphate (II) have been studied in some de-



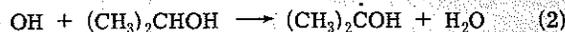
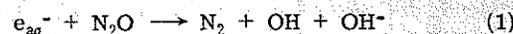
tail. Except for the polarographic reduction of these compounds, the nature of the intermediates produced from the one-electron redox reactions does not appear to have been studied.

The one-electron reduction of compounds I and II by hydrated electrons, e_{aq}^- , and by the acetone ketyl radical, $(CH_3)_2\dot{C}OH$, were investigated in aqueous solution using the fast-reaction technique of pulse radiolysis and kinetic absorption spectrophotometry. The assignment, spectral characteristics, ionization constants, and reactivity of the radical intermediates produced are presented below. The reaction of OH radicals has also been briefly examined.

Experimental Section

The pulse radiolysis technique and experimental set-up used have been described elsewhere.^{7,8} Single pulses of 2.3 MeV electrons and ~ 30 nsec duration were used (Febetron 705 machine).

The radiolysis of water produces $H_2O \xrightarrow{\gamma} e_{aq}^-$ (2.8), OH (2.8), and H (0.6), where the numbers in parentheses are the G values (yields per 100 eV of energy absorbed). One-electron reduction of compounds I and II was carried out at $\sim 22^\circ$ by using two methods: (a) by reaction with e_{aq}^- ; (b) by reaction with $(CH_3)_2\dot{C}OH$. For method (a) solutions were irradiated in the presence of argon (1 atm) and $\sim 1.0 M$ *tert*-butyl alcohol to scavenge the OH radicals produced from the radiolysis of water. The β -carbon radical produced⁷ from this alcohol was found not to interfere with the observations reported below; For method (b) solutions were irradiated in the presence of N_2O (1 atm) and $\sim 1.0 M$ isopropyl alcohol. The reactions occurring in this system are



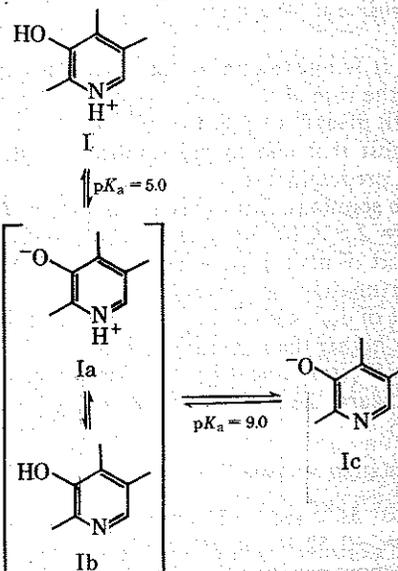
where $k_1 = 8.7 \times 10^9 M^{-1} sec^{-1}$ (ref 9) and $k_2 = 2.0 \times 10^9 M^{-1} sec^{-1}$ (ref 9). Under these conditions all the e_{aq}^- (>95%) reacted with N_2O and none with the substrates.

The chemicals used were the best research grade commercially available and were obtained from Calbiochem and Sigma Chemicals. Solutions were buffered with perchloric acid, potassium hydroxide, borate, and phosphates. Due to the sensitivity to light of the substrates,¹⁰ the solutions were prepared just prior to use and kept in the dark. Exposure to the monitoring light from a pulsed Xenon lamp was kept to a minimum by using a synchronized shutter (open for ~ 5 – 10 msec). Fresh solutions were used for each pulse.

The transient optical spectra obtained were corrected for depletion of the substrate at the appropriate wavelength and pH. The extinction coefficients given were derived⁷ on the basis of the G values given above.

Results and Discussion

Pyridoxine. Pyridoxine (PH) is present in aqueous solution at intermediate pH in two forms^{4,5} with a proton bound to the phenolic hydroxyl group or to the ring nitrogen. The



forms Ia and Ib (referred to as PH for simplicity) are very reactive to e_{aq}^- , with $k = 2.2 \times 10^{10} M^{-1} sec^{-1}$, at pH 6.8;

Table I. Reaction Rate Constants of e_{aq}^- , OH \cdot , and $(CH_3)_2COH$ Radicals with Pyridoxine and Pyridoxal Phosphate in Aqueous Solutions

Substrate ^d	e_{aq}^- ^a		OH \cdot ^b		$(CH_3)_2COH$ ^{b,c}	
	pH	Ionic form $k, M^{-1} sec^{-1}$	pH	Ionic form $k, M^{-1} sec^{-1}$	pH	Ionic form $k, M^{-1} sec^{-1}$
Pyridoxine, PH (5.0, 8.97)	6.8	PH 2.2×10^{10}	3.6	PH $_2^+$ 4.3×10^9	<i>e</i>	
	11.0	P $^-$ 2.5×10^9	7.2	PH 6.3×10^9		
			10.5	P $^-$ 7.4×10^9		
Pyridoxal phosphate, PPH (<2.5, ^f 4.14, 6.2, ^f 8.7)	6.3, 7.3	PPH 1.6×10^{10}	1.0	PPH $_2^+$ 5.8×10^8	5.8 $\times 10^8$	
	11.2	PP $^-$ 6.1×10^9	5.6	PPH 1.3×10^8	1.3 $\times 10^8$	
			10.0, 13.3	PP $^-$ 2.9×10^8	2.9 $\times 10^8$	

^a Determined in the presence of 0.1 M *t*-BuOH by monitoring decay kinetics of e_{aq}^- at 700 nm. ^b Determined by monitoring formation kinetics of transient species in solutions saturated with N $_2$ O. ^c In the presence of 1.0 M isopropyl alcohol. ^d Numbers in parentheses are the pK $_a$ values of the substrate. ^e $k < 10^7 M^{-1} sec^{-1}$ at all pH values 0–13.6. ^f Dissociation of phosphate group.

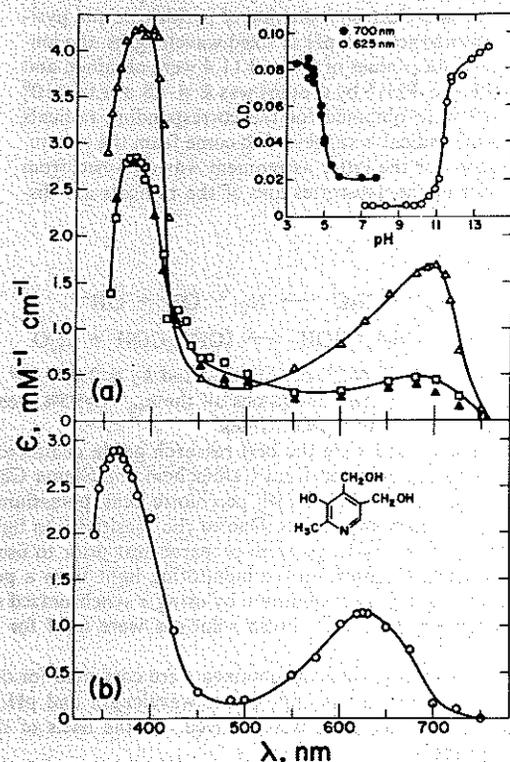
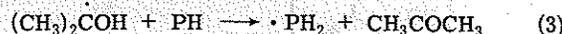


Figure 1. Absorption spectra of intermediates produced from the one-electron reduction by e_{aq}^- of pyridoxine (1–5 mM, in the presence of 1.0 M *tert*-butyl alcohol): (a) at pH 3.6 (transients T $_1$ (\blacktriangle) and T $_2$ (\triangle) read at $\sim 0.2 \mu sec$ and $\sim 3.0 \mu sec$ after the pulse, respectively) and at pH 7.0 (\square). Total dose ~ 6 krads/pulse; (b) at pH 13.3 (\circ). Insert: change in absorbance at 700 and 625 nm with pH.

see Table I. It is interesting to note that this rate is much higher than the rate of e_{aq}^- with pyridine⁹ ($3.0 \times 10^9 M^{-1} sec^{-1}$) or with phenol⁹ ($1.8 \times 10^7 M^{-1} sec^{-1}$). It would appear to suggest that due to the equilibrium the reactivity with form Ia predominates.

In alkaline solutions, form Ic is much less reactive toward e_{aq}^- , and $k = 2.5 \times 10^9 M^{-1} sec^{-1}$ at pH 11.0. This value is close to that of neutral pyridine ($3.0 \times 10^9 M^{-1} sec^{-1}$). It was, unfortunately, not possible experimentally to determine the rate of e_{aq}^- with form I. It is expected, however, to be somewhat higher than the rate with Ia.

The acetone ketyl radical and its anion were found to be unreactive toward the different forms of pyridoxine at pH 1, 7, and 13.5

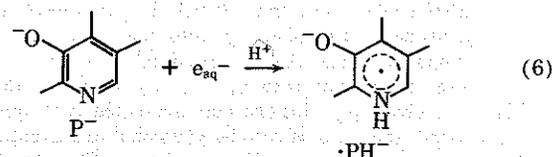
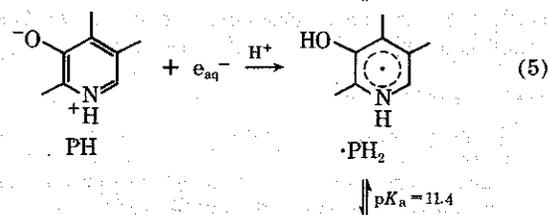
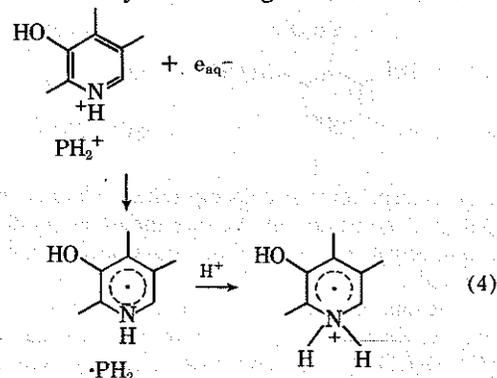


with $k_3 \ll 10^7 M^{-1} sec^{-1}$. The redox potential of pyridoxine⁶ is $E^{01} = -1.52$ V (at pH 7.0 and 25°C) and this is considerably more negative than the "kinetic" potential¹¹ of the $(CH_3)_2COH$ radical, $E_k^{01} = -0.82$ V. On the basis of these potentials, it is clear that reaction 3 cannot take place.

The reaction of e_{aq}^- with pyridoxine at pH 3.6 gave rise to a transient spectrum T $_1$ with maxima at 380 and 680 nm (Figure 1). At $\sim 3.0 \mu sec$ after the electron pulse, an increase in absorbance is observed to give a transient spectrum T $_2$ with maxima at 390 and 695 nm (Figure 1a). The formation kinetics of T $_2$ was found to be dependent on [H $^+$] but independent of phosphate buffer and of pyridoxine concentrations. The rate constant $k(T_1 + H^+ \rightarrow T_2) = 7.8 \pm 1.0 \times 10^9 M^{-1} sec^{-1}$.

On pulse radiolysis of aqueous solutions of pyridoxine at pH 7.0, a spectrum identical to the T $_1$ spectrum found at pH 3.6 is observed (Figure 1a). At pH 13.3, when pyridoxine is present as form Ic, the transient spectrum observed has maxima at 360 and 625 nm and is different from that observed at pH 7.0 (Figure 1b). On monitoring the change in absorbance with pH at 625 and 700 nm, two titration curves are obtained from which one derives pK $_a$ values of 4.8 and 11.4 (see Figure 1a).

Based on the above results, the following mechanism is suggested (reactions 4–6). Other equilibria between various forms of the radicals may be occurring.



The reaction of e_{aq}^- with PH $_2^+$ at pH 3.6 is shown in reaction 4; $\cdot\text{PH}_2$ is the initial transient (T $_1$) produced immediately after the pulse. Its protonation by H $^+$ to form $\cdot\text{PH}_3^+$ (T $_2$ transient) is kinetically observable with $k = 7.8 \times 10^9$

Table II. Absorption Maxima, Extinction Coefficients, Decay Kinetics, and Ionization Constants of Radicals Produced by One-Electron Reduction of Pyridoxine and Pyridoxal Phosphate in Water

Substrate ^a	pH	λ_{\max} , nm	ϵ , $\text{mM}^{-1} \text{cm}^{-1}$	$2k$, $M^{-1} \text{sec}^{-1}$	$\text{p}K_a$ (radical)	Suggested radical
Pyridoxine, PH	3.6	380, ^d 680 ^d	2.85, 0.45			$\cdot\text{PH}_2$
		390, ^e 695 ^e	4.30, 1.70	4.1×10^8 ^c	4.8	$\cdot\text{PH}_3^+$
	7.0	380, 680	2.85, 0.45	3.4×10^8 ^c	11.4	$\cdot\text{PH}_2^-$
Pyridoxal phosphate, PPH	13.0	360, 625	2.90, 1.15	2.5×10^8 ^c		$\cdot\text{PH}^-$
	1.0 ^b	395, ~480	$\geq 3.4, 1.5$	2.8×10^8 ^b		$\cdot\text{PPH}_3^+$
	5.5	~395, ^d ~620 ^d	7.0, 0.7		3.7	$\cdot\text{PPH}_2^-$ (species A)
	13.3	385, ^f 450, 620 ^f	6.2, 3.0, 0.7	2.5×10^8 ^b		$\cdot\text{PPH}_2^-$ (species B)
		410, 535	8.2, 2.2	8.7×10^6 ^{b,g}	6.9	

^a Intermediates produced by pulse radiolysis of the substrates in the presence of $\sim 1.0 M$ *t*-BuOH. ^b Intermediates produced by electron transfer from $(\text{CH}_3)_2\text{COH}$ radicals (see text). ^c Same decay rate for both bands. ^d Initial transient T_1 measured at "zero" time. ^e Second transient T_2 measured at ~ 30 μsec after the pulse. ^f Transient T_2 measured at ~ 10 μsec after the pulse. ^g Decay kinetics was not a perfect second-order process.

Table III. Absorption Maxima, Extinction Coefficients, and Decay Kinetics of Radicals Produced by Reaction of OH Radicals with Pyridoxine in Water^a

pH	λ_{\max} , nm	ϵ , $\text{mM}^{-1} \text{cm}^{-1}$	$2k$, $M^{-1} \text{sec}^{-1}$
3.6	325, 395, ~465	2.7, 2.4, 1.1	1.8×10^8
7.2	395, ~475	2.9, 1.3	3.4×10^8 ^b
13.3	355	3.1	1.3×10^8 ^b

^a $5 \times 10^{-4} M$ pyridoxine solutions saturated with N_2O . ^b Determined at pH 10.5. At pH 13.3 transient decays via mixed kinetics.

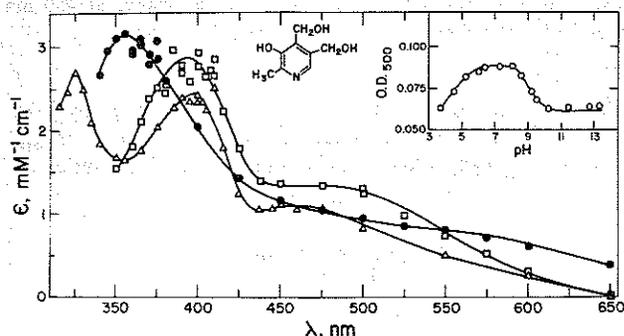


Figure 2. Absorption spectra of intermediates produced from the reaction of OH radicals with pyridoxine ($5 \times 10^{-4} M$, 1 atm N_2O) at pH 3.6 (Δ), pH 7.2 (\square), and pH 13.3 (\bullet). Insert: change in absorbance at 500 nm with pH. Total dose ~ 3 krad/pulse.

$M^{-1} \text{sec}^{-1}$. At pH 7.0 (reaction 5), the protonation of the initial species produced from the reaction of e_{aq}^- with PH to give the $\cdot\text{PH}_2$ radical is not observed. Protonation by water or the buffer is probably occurring with $k \geq 10^7 \text{sec}^{-1}$, but is not observable under our time resolution ($\tau \sim 0.2 \mu\text{sec}$). The spectral characteristics of the $\cdot\text{PH}_2$ radical at pH 7.0 are identical to those of the T_1 species formed at pH 3.6; see Figure 1a. In alkaline solutions the protonation of the species produced from the reaction of e_{aq}^- with P^- to give $\cdot\text{PH}^-$ was also not observed. The $\cdot\text{PH}^-$ radical can be in equilibrium with the proton on either the ring nitrogen or the phenolic OH group (only one structure is shown).

Recent studies¹²⁻¹⁴ have shown that the intermediates produced from the one-electron reduction of various aromatic nitrogen heterocyclic compounds by e_{aq}^- or $(\text{CH}_3)_2\text{COH}$ radicals undergo rapid protonation to give radical cations in neutral solution; e.g., pyrazine (Pz) produces the $\cdot\text{PzH}_2^+$ radical with a $\text{p}K_a = 10.5$. The relatively slow protonation of $\cdot\text{PH}_2$ presumably indicates that the formation of the $\cdot\text{PH}_3^+$ radical has some kinetic barriers. The change from $\cdot\text{PH}_2$ to $\cdot\text{PH}_3^+$ leads to a breakdown of the ring conjugation (as compared to $\cdot\text{PzH} \rightarrow \cdot\text{PzH}_2^+$), and the radical is no longer planar.

With an odd electron in the ring, the phenolic group can exhibit ketonic properties. The enol-keto tautomerism of

the radicals suggested to be formed (reactions 4-6) presumably accounts for the observed absorption bands in the visible region. In the radical $\cdot\text{PH}^-$, the negative charge can be localized on the oxygen atom and the electron spin density is delocalized over the conjugated ring.

Support for the above assignments can be derived from the properties of radicals produced from unsaturated aliphatic alcohols.^{15,16} Using 1,4-pentadien-3-ol it was found¹⁶ that the $\text{CH}_2=\text{CH}\dot{\text{C}}(\text{OH})\text{CH}=\text{CH}_2$ radical can be ionized to $\text{CH}_2=\text{CH}\dot{\text{C}}(\text{O}^-)\text{CH}=\text{CH}_2$ with a $\text{p}K_a = 8.9$. These radical species also absorb in the visible region. In the absence of conjugation the $-\dot{\text{C}}(\text{OH})-$ radicals are much weaker acids¹⁵ and absorb⁷ in the far-uv region.

These radicals decay by second-order kinetics (Table II), with quite similar rate constants.

Reaction of OH with Pyridoxine. The reaction rate constants of OH radicals with pyridoxine are dependent on the state of protonation of the molecule; see Table I. Deprotonation of the nitrogen increases the electrophilic properties of the molecule and its reactivity toward OH radicals, as was found for various amines.¹⁷ The rate constants are lower than those found for the reaction of OH radicals with phenolic compounds¹⁸ ($k \geq 10^{10} M^{-1} \text{sec}^{-1}$).

Figure 2 shows the transient optical absorptions observed at pH 3.6, 7.2, and 13.3. The spectra at pH 3.6 and 7.2 appear to be similar and reflect acid-base changes in the radicals produced. The change in absorbance with pH at 500 nm shows two titration curves with $\text{p}K_a$ values very close to those of pyridoxine (see insert, Figure 2). The OH radical is thought to: (i) add to pyridoxine, and may eventually lead to dehydration with the formation of a phenoxy type of radical, as was observed for phenolic compounds;^{19,18} and (ii) abstract an H atom from the CH_2OH and CH_3 groups. The pyridoxine- $\dot{\text{C}}\text{HOH}$ radical formed is expected to have spectral and acid-base properties similar to radicals formed from unsaturated aliphatic alcohols.¹⁶

Since more than one radical is formed, it is not possible to assign the spectral bands observed in Figure 2. Table III gives the spectral characteristics and decay kinetics of these intermediates.

Pyridoxal 5-Phosphate. Pyridoxal phosphate (PPH) in water also has two dissociation constants associated with the phenolic hydroxyl group and the ring nitrogen, with $\text{p}K_a$ values⁴ of 4.14 and 8.7. The various forms present in solution are the same as those given above for pyridoxine (forms Ia, Ib, Ic). In addition, the phosphate group ionizes with $\text{p}K < 2.5$ and 6.2. Infrared results²⁰ suggest that hydration of the aldehyde group in PPH is pronounced in acid but not alkaline solutions.

At pH 6.3 and 7.3, the reaction rate constant of e_{aq}^- with PPH is $1.6 \times 10^{10} M^{-1} \text{sec}^{-1}$, close to that with PH (see Table I). The small difference could be due to the dinegative charge on the phosphate. At pH 11.2, the rate is $6.1 \times$

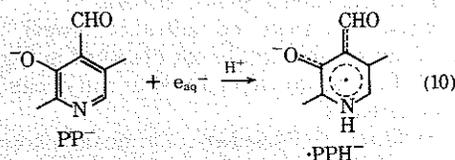
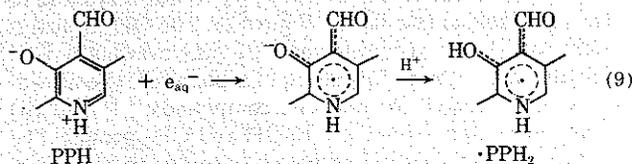
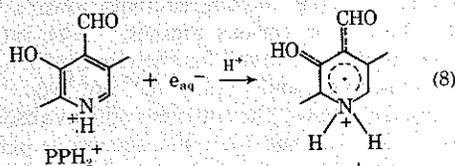
$10^9 M^{-1} sec^{-1}$ and significantly higher than that with pyridoxine ($2.5 \times 10^9 M^{-1} sec^{-1}$). Clearly, the presence of the 4-formyl group in PP^- accounts for this increase. Carbonyl groups are known²¹ to be reactive toward e_{aq}^- .

The $(CH_3)_2\dot{C}OH$ radical was found to react with pyridoxal phosphate at pH 1.0, 5.6, and 10.0, with rate constants of $1-6 \times 10^8 M^{-1} sec^{-1}$; see Table I. These rates were determined from the formation kinetics of the radicals produced from pyridoxal phosphate. The radicals were identical to those formed from the reaction with e_{aq}^- . The redox potential⁶ of PPH is $E^{01} = -0.516 V$ (at pH 7.0 and 25°), making it a stronger oxidant than PH and hence explains why it can be reduced with almost 100% efficiency by $(CH_3)_2\dot{C}OH$ radicals (see also above). Figure 3a shows the transient absorption spectrum of the radical produced from the reaction of $(CH_3)_2\dot{C}OH$ with PPH_2^+ at pH 1.0.

The reaction of e_{aq}^- with PPH at pH 5.5 produces, immediately after the pulse, a transient (T_1) absorption with maxima at ~ 395 and ~ 620 nm; see Figure 3b and Table II. At $\sim 10 \mu sec$ later, the spectrum (T_2) changes and one observes maxima at 385, 450, and 620 nm. It was not possible to obtain good kinetic information for the rate of the proton-catalyzed reaction $T_1 + H^+ \rightarrow T_2$. The spectra observed at pH 10.0 and 13.3 are the same but are different from that obtained at pH 5.5; see Figure 3b. From the change in absorbance with pH monitored at 460 nm and at 540 nm, two titration curves are obtained from which pK_a values of 3.7 and 6.9 are derived. The increase in absorbance at pH > 11 (insert in Figure 3b) is due to the conversion of H atoms into e_{aq}^- , which in turn react with PP^- .



Based on the above results, the following scheme (reactions 8-10) is tentatively suggested. Other equilibria between various forms of the radicals may also be present.



The one-electron reduction of pyridoxal phosphate in acid, near neutral and alkaline solutions, is indicated by reactions 8-10, respectively. The redox potential⁶ of PPH is much higher (more positive) than that of PH, indicating that the formyl group has a strong effect on the electron affinity of the molecule. It follows, therefore, that one-electron reduction of the formyl group may be occurring. Alternate structures for the $\cdot\text{PPH}_2$ and $\cdot\text{PPH}^-$ radical could be written based on the ionization of the $-\dot{C}OH$ radical. The pK_a of the $\text{Ph}\dot{C}OH$ radical formed from benzaldehyde has been reported to be 8.4²¹ or 10.5.²²

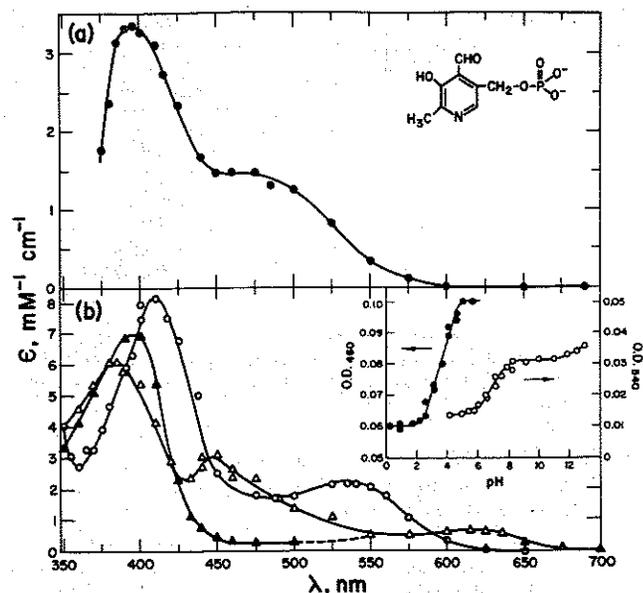
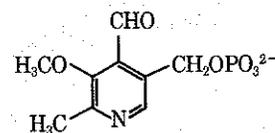


Figure 3. Absorption spectra of intermediates produced from the one-electron reduction of pyridoxal phosphate ($2 \times 10^{-4} M$) in: (a) 1.0 M isopropyl alcohol, 1 atm argon, pH 1.0, total dose ~ 3 krad/pulse; and (b) 1.0 M *tert*-butyl alcohol, 1 atm argon at pH 5.5 (transients T_1 (\blacktriangle) and T_2 (\triangle) read at "zero" time and $\sim 10 \mu sec$, respectively, after the pulse), and pH 13.3 (O). Insert: change in absorbance at 460 nm (using *i*-PrOH) and at 540 nm (using *t*-BuOH) with pH.

Support for the scheme of reactions represented by reactions 8-10 was, however, obtained from the reaction of e_{aq}^- with 3-methoxy pyridoxal phosphate. At pH 5.6 (under ex-



perimental conditions similar to those used for PPH), the transient species observed has an absorption spectrum similar to that observed for PPH at pH 5.5. Between pH 5.5 and 10.2 no change in the absorption spectrum of the transient species produced from 3-methoxy pyridoxal phosphate could be observed. This finding would seem to support the mechanism suggested above for the ionization of $\cdot\text{PPH}_2$ to $\cdot\text{PPH}^-$ with a $pK_a = 6.9$, namely the ionization of the phenolic hydroxyl group.

Conclusions

The one-electron reduction of pyridoxine and pyridoxal phosphate by e_{aq}^- and $(CH_3)_2\dot{C}OH$ radicals in water has been found to be efficient and to form free radicals which have marked acid-base properties. Pyridoxine shows two pK_a values of 4.8 and 11.4, while the values for pyridoxal phosphate are 3.7 and 6.9. The 11.4 and 6.9 values fall in good agreement with the correlation^{15,24} of pK_a (radical) vs. redox potential of the parent compound for the ionization of ketyl radicals $-\dot{C}(OH)- \rightleftharpoons -\dot{C}(O^-)- + H^+$. This agreement not only supports the suggested assignment given above for the radical intermediates observed, but provides information on the nature and reactivity of these intermediates. For example, the kinetic potentials^{11,25} of $-\dot{C}(O^-)-$ radicals are generally much lower (i.e. more negative) than those of the corresponding $-\dot{C}(OH)-$ radicals. This makes $-\dot{C}(O^-)-$ radicals much more powerful reducing agents in electron transfer processes.

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