Pyridoxal Phosphate: Molecular Species in Solution[1]

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Infrared spectra of aqueous solutions of pyridoxal phosphate at various pD values are reported. Assignments of the (N^+-D) bending mode and $(C-O^-)$ stretching modes are made, and these data, together with pK values obtained for pyridoxal phosphate at ionic strength 2.0, are used to calculate microscopic equilibrium constants for aqueous solutions of pyridoxal phosphate. The degree of hydration of the carbonyl function is also calculated using data obtained from dimethyl sulfoxide spectra of pyridoxal phosphate.

Introduction

The development, in recent years, of cells suitable for use with aqueous solutions has extended the usefulness of infrared spectroscopy so that it is now possible to examine directly species present in aqueous media.[2,3] This technique is particularly useful in examining substances of biochemical interest or other systems having complicated equilibria. Because one can examine these systems directly, problems involving the determination of species present in solution which hitherto were not amenable to previously available analytical techniques can now be solved.[4] This investigation was undertaken to determine the various species of pyridoxal phosphate present in aqueous solution and to determine, if possible, the microscopic equilibrium constants.

Considering pyridoxal phosphate hydrochloride as the parent tetrabasic acid, the equilibrium scheme shown in Fig. 1 shows half of the possible species present in solution; if this scheme were repeated with all of the carbonyl functions in the hydrated form, then it would show all of the species which are possible in aqueous solutions.

Experimental

Pyridoxal hydrochloride and pyridoxal phosphate were obtained from Nutritional Biochemicals and were used without further purification. Deuterium oxide was obtained from Bio-Rad Laboratories and was not less than 99.5% D_2O. The 2-, 3-, and 4-pyridols used as comparison compounds for the spectral work were of reagent quality and were recrystallized when necessary. The dimethyl sulfoxide and pyridine used in the spectral work were distilled prior to use. All other reagents were of analytical quality.

Sodium deuterioxide solution was prepared by treating a carefully weighed piece of sodium metal with D_2O and diluting to the required volume. DCl solution was prepared by decomposing phosphorus pentachloride with D_2O and distilling the DCl solution from the mixture. Solutions for infrared measurements were prepared by weighing the required amounts of reagents into a small flask. For the purpose of calculating concentrations, it was assumed that the ratio of the density of water to an aqueous

TABLE Ia
INFRARED SPECTRA OF PYRIDOXAL PHOSPHATE AND ITS ANALOGS IN AQUEOUS SOLUTION

Compound	Phase	pD	Equiv.	Remarks	$\nu(\mathrm{C{=}O})$	Unassigned[a]	Ring	Ring	Ring	$\delta(\mathrm{N^+{-}D})$	Ring	Ring	$\nu(\mathrm{C{-}O^-})$	$\delta(\mathrm{O{-}D})$
Pyridoxal	D_2O	2.0				1607 ms				1500 s		1469 m		1390 m
		4.2				1605 m	1585 m			1497 s		1470 s		1390 mw
		11.7			1647 m				1566 ms				1411 vs	
Pyridoxal	25 wt. % MeOH		0.00			1605 mw			1565 vw	1495 s		1463 s		1392 mw
			0.42			1603 mw	1585 mw			s~1493		1465 b		1395 vw
			1.24		1640 b		1580 m					1465 b		1390 s
			2.34		1640 b			1568 m				s~1460	1413 s	
Pyridoxal phosphate	D_2O	4.7	1.95		1662 ms		s~1595	1565 w		1490 ms		1450 ms		1392 mw
		5.8	2.49		1663 ms			1567 w		1492 s		1455 s		1389 mw
		6.7	2.91		1662 ms		s~1595	1565 w	s~1530	1485 bs		1455 bs		1390 w
		7.7	3.11		1662 ms			1570 w	1534 vw	1492 s		1457 m		1390 w
		8.1	3.38		1662 ms			1568 w	1537 w	1490 m		1450 vb		
		9.0	3.88		1652 s				s~1575	1535 mw		1450 b	1415 s	
		11.0	5.41		1655 s					1540 mw	1465 m	1445 mw	1418 s	
2-Pyridol	D_2O				1640 vs					1560 vs		1455 vw		1390 mw
3-Pyridol	D_2O	6.9					1593 m	1572 m		1502 ms		1435 w		
		8.6					1593 m	1572 m		1502 ms	1480 w	1435 w		
		10.0						1573 ms	1557 ms		1480 ms		1411 s	
		11.7						1575 ms	1557 ms		1480 s		1411 s	
4-Pyridol					1634 s					1520 s				1393 ms
Pyridine	D_2O			Acid		1595 s				1490 ms	s~1475	1449 s		
				Base		1594 s					s~1475	1446 s		

TABLE Ib
INFRARED SPECTRA OF PYRIDOXAL PHOSPHATE AND ITS ANALOGS IN THE SOLID STATE

Compound	Phase	Remarks	$\nu(\mathrm{C{=}O})$	Unassigned[a]	Ring	Ring	Ring	$\delta(\mathrm{N^+{-}D})$	Ring	Ring	$\nu(\mathrm{C{-}O^-})$	$\delta(\mathrm{O{-}D})$
Pyridoxal	KBr	Deuterated	1637 m	1605 s			1554 bs	1500 bs		1450 bs		1390 m
	KBr		1638 m				1558 bs		1465 m	1449 m		
	Nujol	Deuterated	1635 w	1605 m			1558 ms	1496 s				
	Nujol		1635 w				1558 m					
Pyridoxal phosphate	KBr	Deuterated	1645 m				1555 s	1505 mw			1408 ms	
	KBr		1645 m				1555 s	1498 mw[b]			1409 m	
	Nujol		1645 m				1558 m				1410 mw	
Pyridoxamine	Nujol	Deuterated			1593 mw	1548 m	1510 m					
	Nujol				1593 mw	1548 m						

[a] Unassigned band; involves ionizable D. [b] Anomalous band.

(2) R. C. Gove, R. B. Barnes, and E. Peterson, *Anal. Chem.*, **21**, 382 (1949).

(3) L. H. Jones and R. A. Penneman, *J. Chem. Phys.*, **22**, 965 (1954).

(4) K. Nakamoto, Y. Morimoto, and A. E. Martell, *J. Am. Chem. Soc.*, **84**, 2081 (1962).

Fig. 1.—Equilibrium scheme of pyridoxal phosphate species possible in aqueous solution starting with the hydrochloride as the parent acid. Only those forms which do not have the carbonyl group hydrated are shown.

solution was the same as the ratio of the density of deuterium oxide to a solution of deuterium oxide of the same concentration.

The compounds in the table of solid state spectra which were deuterated were prepared by dissolving the compound in D_2O or DCl solution, placing the samples in a desiccator over P_2O_5 and allowing the solvent to be drawn off. This method is sufficient to deuterate those hydrogens which exchange rapidly; that is, hydroxylic and amino (ammonium) hydrogens complete their exchange in a very short time. Molar ratios of solvent to solute were not calculated, but were approximately 100 or more.

Spectral measurements were made on a Perkin–Elmer Model 21 spectrophotometer equipped with sodium chloride optics. The sample cells used had silver chloride windows and a path length of 0.035 or 0.021 mm. The reference cell used had barium fluoride windows and a similar path length.

pD measurements of solutions used in infrared work were made with a Beckman Model G pH meter equipped with micro saturated calomel and glass electrodes. Potentiometric measurements were made with a Radiometer Model 4 pH meter equipped with standard size saturated calomel and glass electrodes.

The spectrometer was calibrated with polystyrene film and frequencies were found to be accurate to ± 2 cm.$^{-1}$. The Model G pH meter was calibrated with standard buffers, and the Radiometer was calibrated by titration of standard hydrochloric acid with standard sodium hydroxide. All pH values given are hydrogen (or deuterium) ion concentrations, and are not corrected for activities.

Results

An examination of the spectra of pyridoxal phosphate as a function of pD as shown in Fig. 2a and 2b reveals that the only readily identifiable band is that of the carbonyl function at 1650–1660 cm.$^{-1}$. This assignment is undoubtedly correct not only because there are no other functional groups present which absorb in this region, but also because of the agreement with the observed frequencies in similar compounds.[5,6] The assignment of the carbonyl band is further borne out by a comparison with the spectra of 2- and 4-pyridol, both of which are principally in the keto form. These two compounds exhibit the carbonyl band while 3-pyridol, which retains its alcohol function, does not. The fact that the frequencies in Table I

(5) D. Heinert and A. E. Martell, *J. Am. Chem. Soc.*, **81**, 3933 (1959).
(6) D. Heinert and A. E. Martell, *ibid.*, **84**, 3257 (1962).

are somewhat lower than the solid state values previously reported is doubtless due to intermolecular hydrogen bonding with the solvent.

It is interesting to note the pD dependence of the carbonyl band in pyridoxal and pyridoxal phosphate. Presumably, the carbonyl of pyridoxal is hydrated or forms a hemiacetal at lower pD values, but is converted to the free carbonyl to a greater extent as the pD increases. The intermediate pD ranges could not be studied in the case of pyridoxal, since after a small amount of base had been added the substance precipitated. In pyridoxal phosphate the degree of hydration of the carbonyl group is certainly lower as evidenced by the fact that the carbonyl band always appears in the pyridoxal phosphate spectra. The frequency shift of 10 cm.$^{-1}$ toward longer wave length observed at higher pD values in pyridoxal phosphate is presumably due to the neutralization reaction

Upon loss of the phenolic proton and the attainment of a fully conjugated system, the bond order of the carbonyl oxygen is decreased with a consequent shift to longer wave lengths.

Assignment of the (N^+-D) bending mode is based upon the comparison of the solid state spectra of the deuterated and nondeuterated pyridoxals and the appearance of this band in pyridine in acid solution. These spectra are shown in Fig. 3, 4, and 5. From this evidence and the pD dependence of this band, there can be little doubt that this is a correct assignment. The pD dependence of this band can be seen most clearly from an examination of Fig. 2 (pyridoxal phosphate) and Fig. 6 (pyridoxal). In addition, this band appears in the spectra of 3-pyridol (Fig. 7) in acid solution.
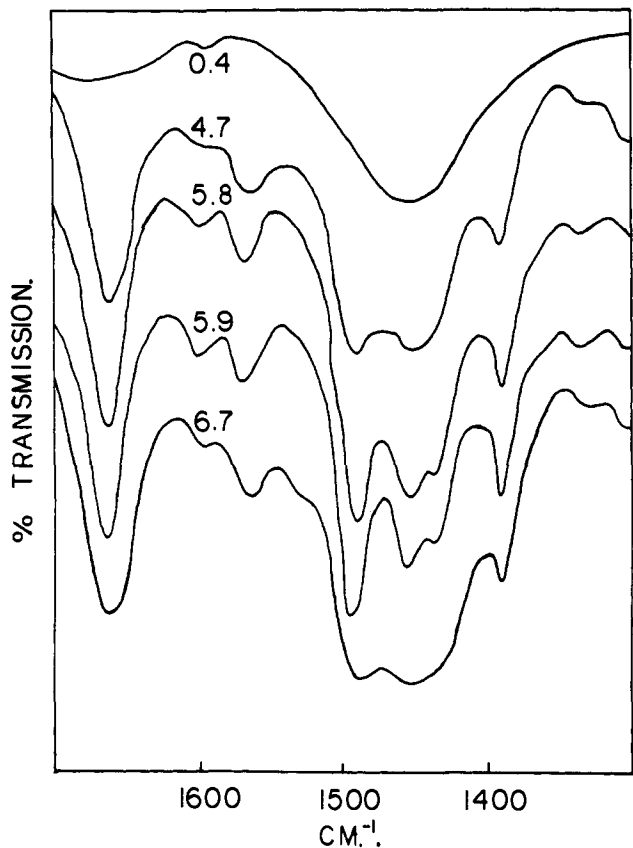
Fig. 2a.—Aqueous infrared spectra of pyridoxal phosphate in the low pH region. Concentrations are approximately 0.5 M, and pD values are shown beside each curve.
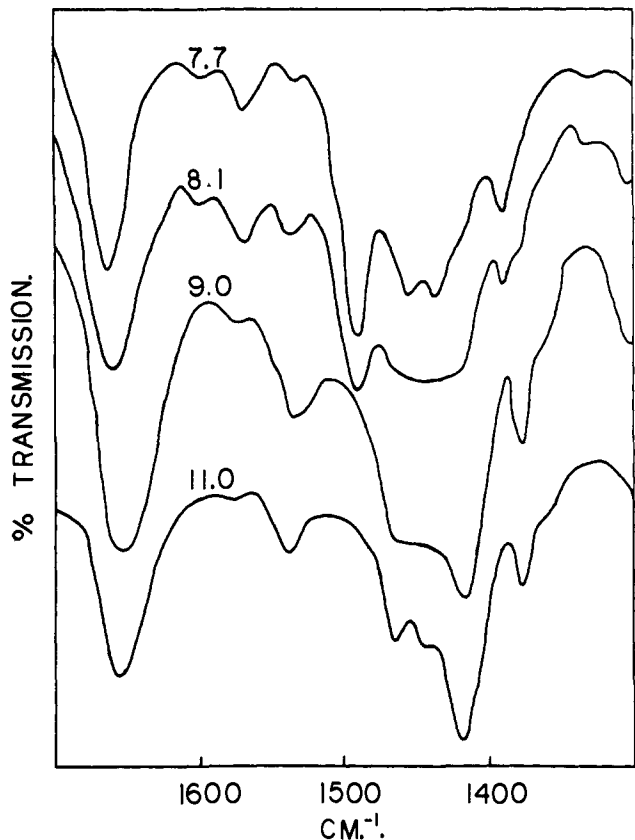

Fig. 2b.—Aqueous infrared spectra of pyridoxal phosphate in the high pH region. Concentrations are approximately 0.5 M, and pD values are shown beside each curve.
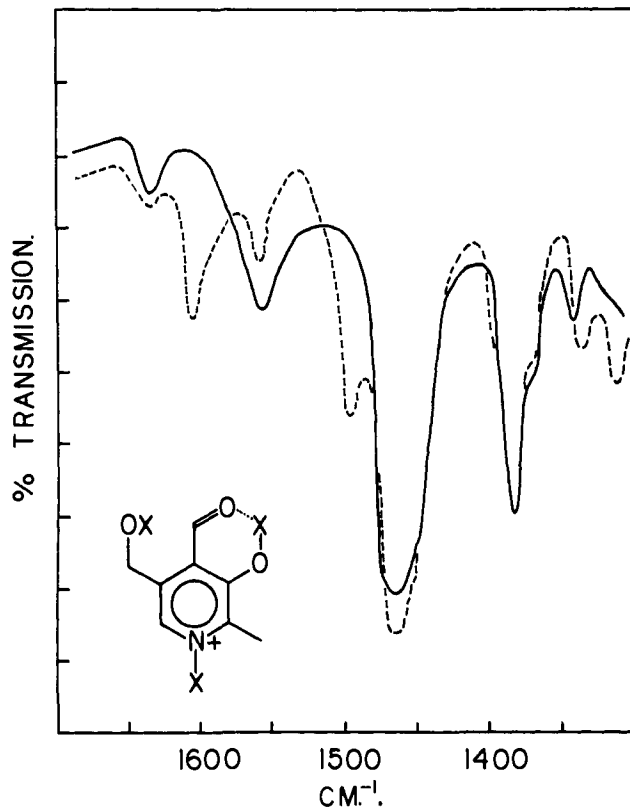

Fig. 3.—Spectra of pyridoxal hydrochloride (————) and deuterated pyridoxal hydrochloride (– – – –) in Nujol mulls.
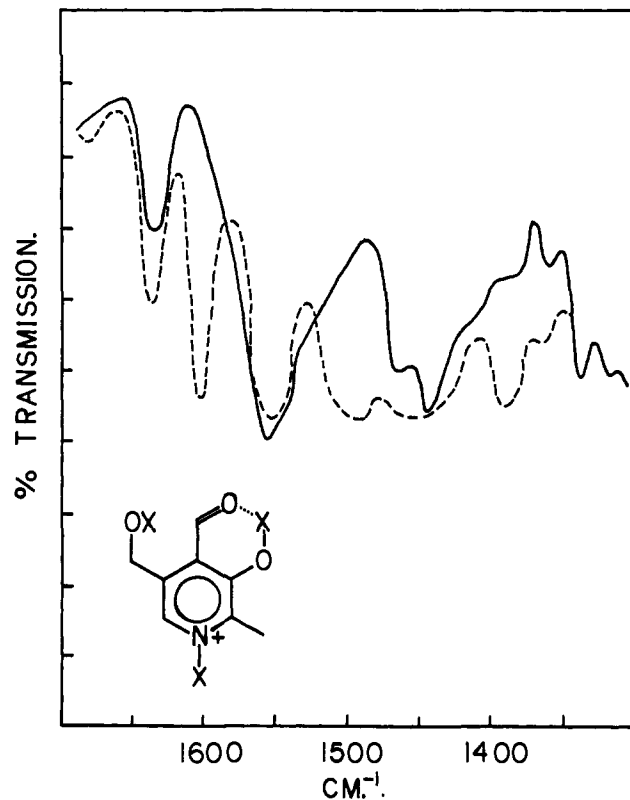

Fig. 4.—Spectra of pyridoxal hydrochloride (————) and deuterated pyridoxal hydrochloride (– – – –) in KBr disks.

The assignments of the (O–D) bending and (C–O⁻) stretching modes were made on the basis of the correlation between the pD dependence of these bands and the effects of deuteration. The (C–O⁻) band appears only at high pD values as would be expected from
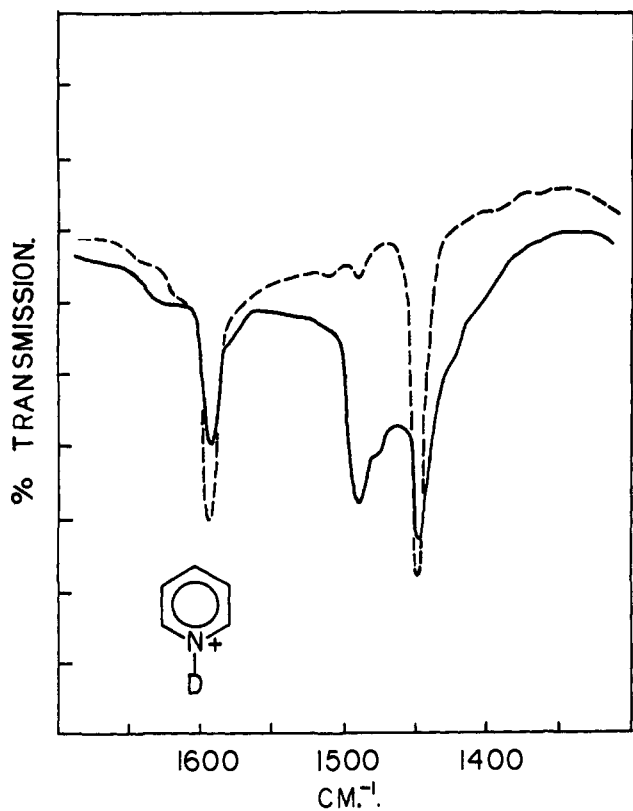
Fig. 5.—Spectra of pyridinium ion (———) and pyridine (– – – –)
in deuterium oxide solution.

Fig. 6.—Spectra of pyridoxal hydrochloride in deuterium oxide
solution: ———, at pD 2.0; – – – –, at pD 3.5; · · · · ·, at pD
11.0.

the loss of the phenolic proton. This band assignment
is also supported by the close correlation of the ap-
pearance of this band with the shift in the carbonyl
frequency from 1662 to 1652 cm.$^{-1}$. The assignment

Fig. 7.—Spectra of 3-pyridol in deuterium oxide solution: ———
pD 6.2; – – – –, pD 7.9; · · · · ·, pD 11.0.

of the (O–D) bending mode is based merely upon
the pH dependence of the band and the fact that the
value of the frequency is quite reasonable for a band of
this type.

The band at *ca.* 1605 cm.$^{-1}$ remains unassigned.
However, certain conclusions may be drawn concern-
ing this band. From its appearance in acid solution
only, it must involve an ionizable proton. This is also
seen from the fact that it appears in the deuterated
pyridoxal.

The remaining frequencies shown in Fig. 2 are as-
signed to skeletal vibrations of the pyridine ring, and
are in agreement with assignments of other investiga-
tions on analogous compounds.[5,6]

In order to determine the microscopic equilibrium
constants for this system, it is first necessary to know
the values of the over-all dissociation constants. There
are determinations in the literature such as that of
Christensen[7] and that of Williams and Neilands.[8]
The latter workers obtained pK values of <2.5, 4.14,
6.20, and 8.69 for the first, second, third, and fourth
dissociation constants, respectively, at 25° and an ionic
strength of 0.16 M. It was decided, however, to de-
termine these constants at a higher ionic strength in
view of the high concentrations required for infrared
measurements of aqueous solutions. Figure 8 shows
the potentiometric titration curve obtained for pyri-
doxal phosphate hydrochloride. The substance was
titrated at μ = 0.1 and 2.0 as the tetrabasic acid by
weighing out the required amount of pyridoxal phos-
phate and adding one equivalent of hydrochloric acid
to the cell before titrating. The determination of μ =
2.0 is shown here, but the two curves were essentially
superimposable. The ionic strengths of the solutions
used in infrared work were approximately 2.0.

(7) H. Christensen, *J. Am. Chem. Soc.*, **80**, 99 (1958).
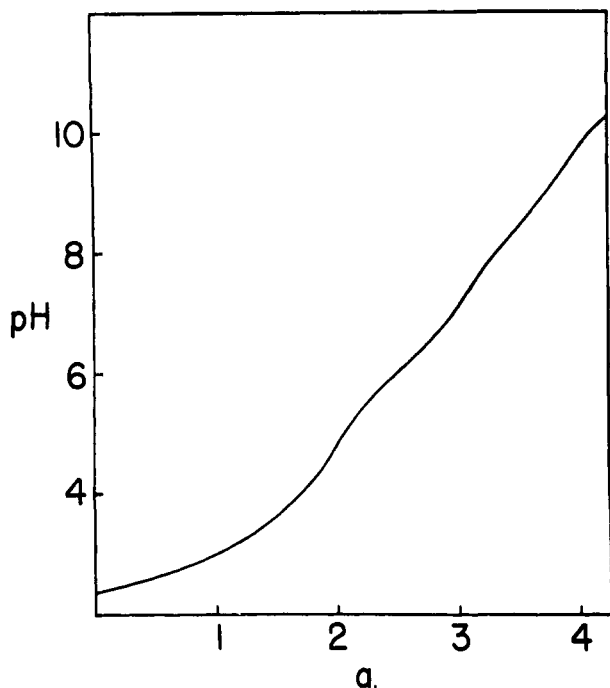(8) V. R. Williams and J. B. Neilands, *Arch. Biochem. Biophys.*, **53**, 56
(1954).

Fig. 8.—Potentiometric titration curve of pyridoxal phosphate hydrochloride.

The first two dissociation steps overlap so it is desirable to solve the usual algebraic relationships by graphical means. Figure 9 shows the plot which was obtained.

In making this determination, it was found that the pyridoxal phosphate was about 94% pure. The presence of impurity shifts the observed inflection to lower equivalents than those calculated. Since the deviation of the observed inflection from the calculated inflection was constant for all equivalence points, it was assumed that the 4% impurity was water or some other inert material. This conclusion was supported by elementary analysis of the sample. An impurity containing titratable hydrogen would be expected to shift one of the equivalence points back toward the calculated value depending upon the pH at which the impurity was neutralized. The results of these determinations are given.

DISSOCIATION CONSTANTS OF PYRIDOXAL PHOSPHATE

$\mu = 0.1$		$\mu = 2.0$
1.4 ± 0.4	pK_1	1.64 ± 0.08
3.44 ± 0.01	pK_2	3.58 ± 0.01
6.007 ± 0.001	pK_3	5.751 ± 0.007
8.45 ± 0.04	pK_4	8.17 ± 0.01

The precision attained in the determination of these dissociation constants is further indication of the inert nature of the impurity present in the pyridoxal phosphate sample. All of the uncertainties given above are standard deviations. For the values which were calculated from a slope–intercept equation, expressions were derived for the standard deviations of the constants using the value of the constant obtained from the least squares fit of the line as the "true" value.

Discussion

Before attempting to make any calculations of microscopic constants, a qualitative examination of the aqueous infrared absorption spectra and equilibrium scheme will be profitable.

The most significant observation is the persistence of the (N$^+$–D) band, the intensity of which remains almost unchanged until the loss of the final proton where
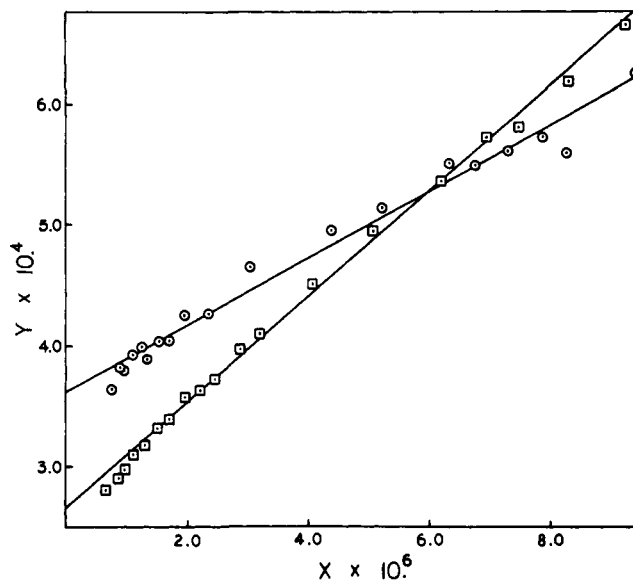

Fig. 9.—Slope–intercept plot for determination of K_1 and K_2; $X = \{[\text{H}]^2 T_{\text{OH}} + [\text{H}]^3\}/\{2T_{\text{A}} - T_{\text{OH}} - [\text{H}]\}$; $Y = \{[\text{H}]T_{\text{OH}} + [\text{H}]^2\}/2\{2T_{\text{A}} - T_{\text{OH}} - [\text{H}]\} - [\text{H}]/2$.
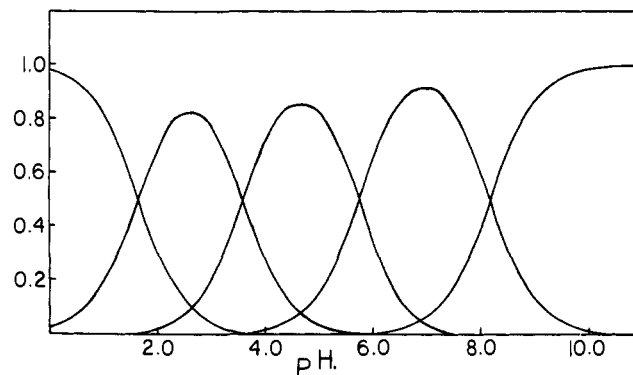

Fig. 10.—Plot of mole fraction vs. pH for pyridoxal phosphate hydrochloride. The curves show, from left to right, respectively' the mole fractions of the species H_4A, H_3A, H_2A, HA, and A.

a dipolar form is impossible. The conclusion to be immediately drawn from this is that the dipolar forms containing the pyridinium proton are highly favored.
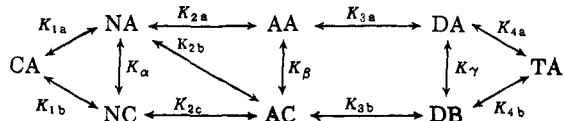
In addition to this, the (C–O$^-$) band at ca. 1420 cm.$^{-1}$ must be due almost entirely to the presence of the trinegative anion, TA. This very strong band is seen very clearly in the spectra at pD 9.0 and 11.0. Although it is somewhat obscured at pD 8.1—due to the use of a cell with greater light loss and a consequent decrease in signal to noise ratio—this band appears again as a small shoulder at pD 7.7. At lower pD the band is not observed. The appearance of this band, and its relative strength, is in good correlation with the mole fraction of TA present at the pD values where it is observed. Also, the shift of the carbonyl band occurs only at the two highest pD values with a very slight shift noticeable at pD 8.1. This evidence seems to indicate that the phenolic proton is not lost until the last neutralization step. On this basis, it is possible to omit all forms in Fig. 1 which have a C–O$^-$ group except that of TA.

Forms such as AD and DC can certainly be eliminated immediately because of their phenoxide structures. However, in forms such as NB, AB, and DA, there is a pyridinium proton on the ring which may influence the effects of the ionization of the phenolic proton on the infrared absorption band. Form NB is of this type, but may be considered negligible since it

represents the highly improbable case of the phenolic proton being ionized in preference to both the primary phosphate and pyridinium protons. If the formation of a pyridinium dipolar form is to be favored, then NA must represent the predominant form. Form NC may also be present to at least a small extent, but no measurements could be made in this pD region owing to solubility difficulties. The solid state spectrum of pyridoxal phosphate does show a band at 1420 cm.$^{-1}$, so presumably NB is present to some extent in the crystalline material.

Forms AB and DA should have a band in the vicinity of the 1420 cm.$^{-1}$ band in TA. While the presence of the pyridinium proton might be expected to exert some influence against the electron enrichment of the ring owing to the ionization of the phenolic proton, no resonance forms can be written for such an interaction, and consequently this effect would be expected to be small. In the case of DA, the band due to the phenoxide form may be merged with that of TA; in the case of AB, however, this band has entirely disappeared. That is, the intensity of the 1420 cm.$^{-1}$ band correlates well with the fraction of TA present with a possibility that DA is contributing, but precludes any possibility of the presence of AB (see Fig. 10).

These considerations lead to the formulation of the equilibrium scheme.

$$K_{1a} \nearrow NA \xrightarrow{K_{2a}} AA \xleftarrow{K_{3a}} DA \searrow K_{4a}$$
$$CA \qquad \Big\updownarrow K_\alpha \quad K_{2b} \quad \Big\updownarrow K_\beta \qquad \Big\updownarrow K_\gamma \qquad TA$$
$$K_{1b} \searrow NC \xrightarrow{K_{2c}} AC \xleftarrow{K_{3b}} DB \nearrow K_{4b}$$

With the exception of TA, it is impossible to find the extinction coefficients of any of these species directly. The extinction coefficient for the C–O$^-$ group of TA may be calculated using the spectrum at pH 11.0, but this value is useless for calculation of other species— such as DA—because the band is obscured. The extinction coefficient of the 3-pyridol cation was used in making calculations for the 1490 cm.$^{-1}$ band. This extinction coefficient is, strictly, only applicable to the N$^+$–D group on other cations. Although some error would be expected if this extinction coefficient is transferred to other charge types, this provides the best approximation thus far available for pyridoxal phosphate in aqueous solution. The value of the extinction coefficient was 35.3 l./mole-mm.

On the basis of the above discussion, the constants for the system may now be calculated.

The microscopic constants are defined as

$$K_\alpha = [NA]/[NC], \quad K_\beta = [AA]/[AC], \quad K_\gamma = [DA]/[DB]$$

In the calculation of the constants, the sum of the concentration of any pair of tautomeric forms is obtained from Fig. 10. If two equations at two similar pH values are solved for the same species, divided by the respective total concentrations, and equated, it is possible to calculate the value of one of the constants. This procedure is based upon the assumption that at two similar pH values the mole fraction of a particular species will be constant. The above constant, together with mole fraction data and over-all dissociation constants, allows one to calculate the remainder of the constants by simple algebraic methods. It was not possible to calculate any values of K_α, but the predominant species in the acid regions, as has been pointed out above, are the pyridinium dipolar forms, such as NA, so this microscopic constant would be expected to be very large. The other equilibrium constants are defined as usual and are shown in the diagram of the equilibrium scheme. The values of these constants are

$$K_\beta = 7.7 \pm 0.8 \qquad\qquad K_\gamma = 1.6 \pm 0.2$$
$$K_{3a} = 1.3 \times 10^{-6} \qquad K_{3b} = 6.5 \times 10^{-6}$$
$$K_{4a} = 1.1 \times 10^{-8} \qquad K_{4b} = 1.9 \times 10^{-8}$$

The estimated errors in the values of these constants is 10–20% ($< \pm 0.1$ log value).

The calculation of the degree of hydration of the carbonyl function is based upon the assumption that the extinction coefficient of the carbonyl group in dimethyl sulfoxide is the same as the extinction coefficient of the unhydrated carbonyl in D$_2$O solution. Considering the highly polar nature of both solvents, this assumption seems reasonable.

The actual degree of hydration of the carbonyl function is almost constant over all pD values from 4.7 to 11.0. The degree of hydration at high pD values seems to be slightly higher than the intermediate pD values, but the scatter of the points was such that these higher values could be attributed to experimental errors.

If K_H is defined as

$$K_H = [\text{hydrated forms}]/[\text{nonhydrated forms}]$$

then K_H is found to be $+0.36 \pm 0.07$.

Direct Condensation of 2-Deoxy-D-ribose with Purines. Structure of the Products

BY JOHN A. CARBON

The direct reaction of 2-deoxy-D-*erythro*-pentose (I) with any of various purines in hot polar solvents leads to the production of a diastereoisomeric pair of compounds with empirical formulas corresponding to the condensation of equimolar quantities of the sugar and purine with elimination of 1 mole of water. The products are thus isomeric with the corresponding 2′-deoxynucleosides. These compounds (IV and V) possess ultraviolet spectra only compatible with 9-substituted purines and were readily hydrolyzed by alkali, but not by acid, to the free purines. A possible formulation of these products as 2,3-dideoxy-3-(9-purinyl)-D-*erythro*- (and *threo*-) pentoses (IV and V) was proved by an unambiguous synthesis of one of the isomeric pairs. The products are thought of as arising from a nonstereospecific Michael addition of the purine to an intermediate α,β-unsaturated aldehyde (II), generated by loss of the C-3 hydroxyl of the 2-deoxy sugar. This sequence constitutes an hitherto unobserved reaction of 2-deoxy sugars.

The chemistry of 2-deoxy-D-*erythro*-pentose (2-deoxy-D-ribose) and the synthesis of 2′-deoxy-D-*erythro*-pentofuranosyl derivatives of purines and pyrimidines (2′-deoxynucleosides) has been a subject of great interest to many workers, not only because of the im-

portance of this group of compounds in biological phenomena, but also because of the challenging synthetic problems encountered in this field.[1] It was

(1) For a recent review see T. L. V. Ulbricht, *Angew. Chem.*, **74**, 767 (1962).