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# AQUEOUS SOLUTION EQUILIBRIA OF PYRIDOXAMINE, PYRIDOXAL, 3-HYDROXYPYRIDINE-4-ALDEHYDE, AND 3-HYDROXYPYRIDINE-2-ALDEHYDE AS STUDIED BY PROTON RESONANCE

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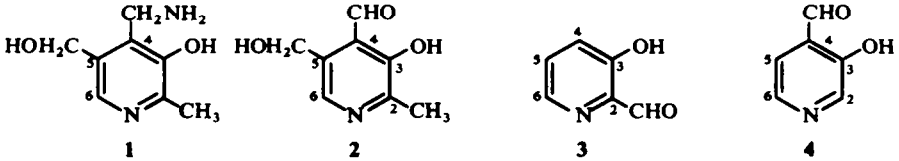
**Abstract**—The acid-base equilibria of two members of the vitamin $B_6$ group of compounds, pyridoxamine and pyridoxal, and two simple analogs of the latter, 3-hydroxypyridine-4-aldehyde, and 3-hydroxypyridine-2-aldehyde, have been studied by PMR in $D_2O$ solutions over the pD range 1–13. The pD dependence of the chemical shifts has been used to obtain pK values for the individual acid dissociation steps of each compound, and to deduce the structures of the principal species involved in each step. pK values so determined correlate well with those obtained in aqueous ($H_2O$) solution by direct titrimetry or spectrophotometry. Deprotonation sequences of the four compounds derived from the PMR results corroborate the essential features of these sequences proposed previously from spectrophotometric investigations.

## INTRODUCTION

NUMEROUS studies have been made of nonenzymatic transamination and related reactions of amino acids which are catalyzed by pyridoxal and whose rates are increased by the addition of metal ions. Results of work involving model systems have recently been summarized.[1–3] We are currently investigating the widely accepted mechanism for transamination involving pyridoxal cofactors and metal ions[4] by the application of high resolution NMR techniques to aqueous systems of pyridoxal:alanine:Zn (II) or Al (III) and pyridoxamine:pyruvate:Zn (II) or Al (III). The results of these investigations will be presented subsequently.[5] In order to identify the solute species in these systems and to follow their reactions during the entire transamination sequence, it became necessary to obtain rather complete interpretations of the PMR spectra of pyridoxal and pyridoxamine over the pH range 1–12 in the presence and absence of alanine or pyruvate.

Herein are reported the results of a detailed investigation of the PMR spectra in $D_2O$ solutions (pD ~ 1–13) of pyridoxamine, pyridoxal, and two simple analogs of the latter, 3-hydroxypyridine-2-aldehyde and 3-hydroxypyridine-4-aldehyde. In their simplest forms these compounds may be represented as **1–4**, respectively.




**1** **2** **3** **4**

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PMR spectroscopy has not been previously applied as a means of studying pyridoxal-catalyzed transformations of amino acids despite favorable reaction rates in some instances. PMR spectra of pyridoxal and pyridoxamine in $D_2O$ have been determined by Korytnyk and Singh,[6] but detailed observations were reported only at very acidic and basic pD's and in neutral solution. The multiplicity of possible solute species within the entire pD range requires that a complete PMR investigation include observations at numerous intermediate pD values as well. Such observations have been made in this investigation and have led to definitions of solute structures which are in some cases independent of but always complementary to conclusions from earlier spectrophotometric studies[7–11] of aqueous and alcoholic solution equilibria of these compounds, including those from the careful and extensive work of Martell *et al.*[10,11] Solute structures proposed or determined from the two methods are compared and the strong pD dependences of chemical shifts are correlated with the apparent acid dissociation constants determined indirectly by spectrophotometry[8–10] or directly by titrimetry.[9,12,13]

## RESULTS AND DISCUSSION

The pD dependence of the proton chemical shifts of compounds **1–4** has been measured in $D_2O$ solutions and the results are presented in Figs 1, 4, and 6. The plots reveal the resemblance to acid-base titration curves. Values of pK for the various acid-base equilibria may be determined directly from these plots and are related to those measured titrimetrically in $H_2O$ solution by the relation $pK(D_2O) = pK(H_2O)$

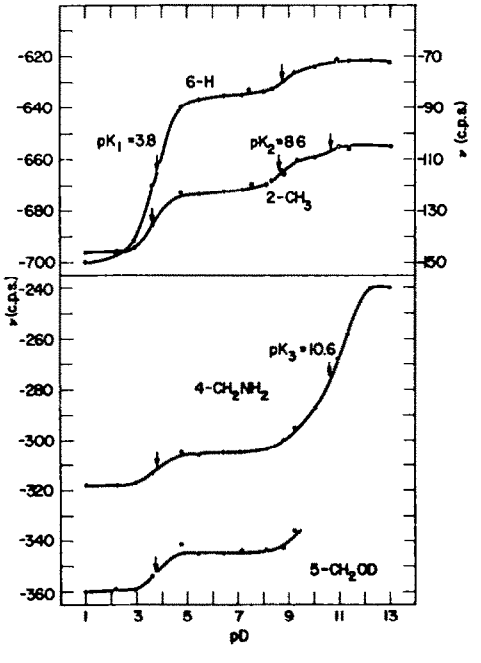


FIG. 1 Chemical shifts of pyridoxamine at 100 Mc/s as a function of pD in $D_2O$ solution. Shifts of 6-H and 2-$CH_3$ are referred to the left- and right-hand scales, respectively.

+ 0·40. The most accurate pK values determined from chemical shift data for the individual acid dissociation steps of each compound are given in the figures. PMR spectra relevant to the discussion are given in Figs 2 and 5. In the following discussion

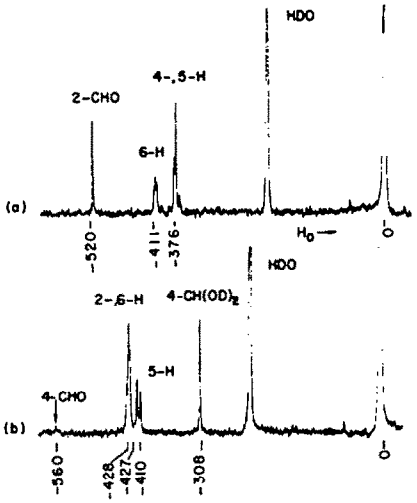


FIG. 2 60 Mc/s PMR spectra in $D_2O$ solution: (a) 3-hydroxypyridine-2-aldehyde at pD 7·01; (b) 3-hydroxypyridine-4-aldehyde at pD 1·45. Chemical shifts are measured in c/s relative to a t-butanol internal standard.

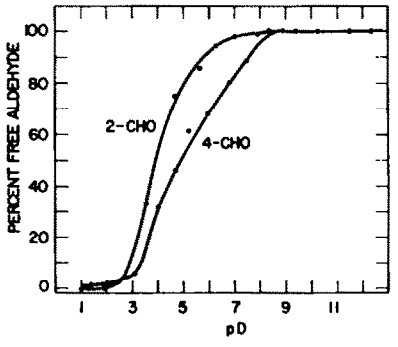


FIG. 3 Percent free aldehyde species as a function of pD in $D_2O$ solution for 3-hydroxypyridine-4-aldehyde and 3-hydroxypyridine-2-aldehyde.

considerable emphasis is placed on the identification of species involved in the acid-base equilibria of the four compounds. In those pD ranges in which one or more species are present in rapid equilibrium, the PMR method can, of course, sense only the average chemical shift of a given ring substituent and is thus incapable of *separate* detection of all species present in substantial concentration. In particular, the presence of species potentially involved in tautomeric equilibria can only be inferred. In such cases the most polar form is assumed to prevail in aqueous solution and reference should be made to the UV spectral studies of Martell *et al.*[10,11] for a more complete definition of these equilibria.

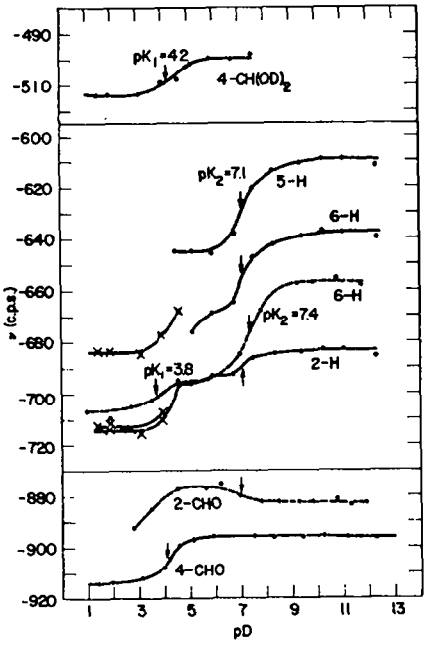


FIG. 4 Chemical shifts at 100 Mc/s as a function of pD in $D_2O$ solution. ———, 3-hydroxypyridine-4-aldehyde; X hydrated aldehyde, ● free aldehyde. – – – –, 3-hydroxypyridine-2-aldehyde.

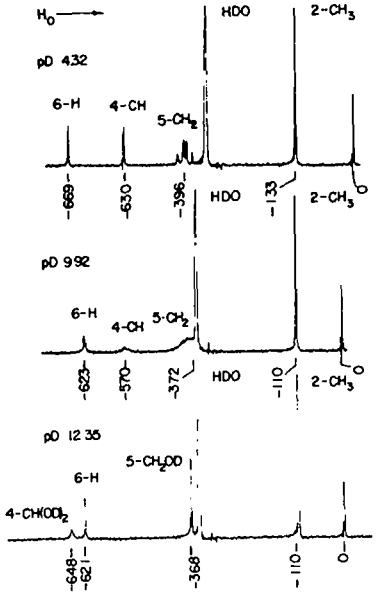


FIG. 5 100 Mc/s PMR spectra of pyridoxal in $D_2O$ solution at different pD values. Chemical shifts are measured in c/s relative to a t-butanol internal standard.

*Pyridoxamine.* Plots of the pD dependence of chemical shifts are shown in Fig. 1. Spectra of pyridoxamine and pyridoxamine 5-phosphate in acid solution have been published previously.[6] Over the pD range of measurement the spectrum of pyridoxamine consists of four sharp, featureless lines of relative intensities 1:2:2:3 from low to high field. The order of these signals is invariant to pD. Assignment of the 2-Me and 6-H signals follows from the relative intensities. The lower field methylene signal

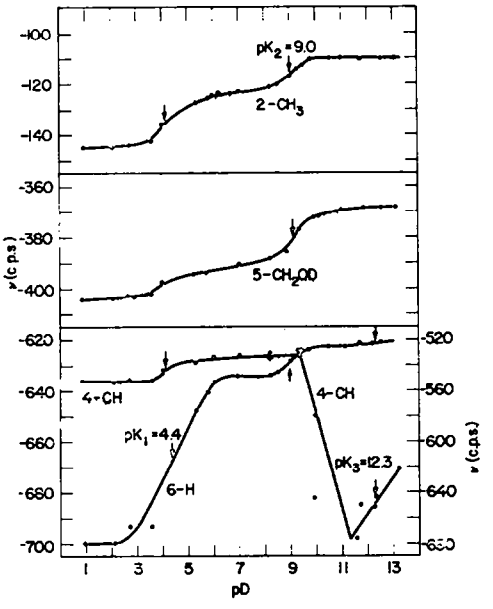


FIG. 6 Chemical shifts of pyridoxal at 100 Mc/s as a function of pD in $D_2O$ solution. Shifts of 6-H and 4-CH are referred to the left- and right-hand scales, respectively.

has been assigned by Korytnyk and Singh[6] to 5-$CH_2OD$ on the basis of the $^{31}P$-$^1H$ spin doublet observed for the analogous methylene resonance in the phosphate. This assignment is substantiated by the marked pD dependence of the chemical shift of the higher field methylene signal (*vide infra*).

Pyridoxamine in acidic solution (pD $\lesssim$ 2) exists in the diprotonated* form **5** (cf. Chart I). Upon addition of base to aqueous or methanol solutions three protons are removed in discrete steps.[8,11–13] In aqueous ($H_2O$) solution acid dissociation constants have been determined by direct titrimetry to be the following:[13] $pK_1$ 3·37, $pK_2$ 8·01, $pK_3$ 10·13. As seen in Fig. 1, the first deprotonation produces a marked change in the chemical shifts of all ring substituents. Points of inflection closely correlate with the titrimetric $pK_1$ value. Similar observations are made for the second and third deprotonations, although these reactions do not produce substantial chemical shift changes for every ring substituent. The three deprotonations are most clearly manifested in the pD dependence of the 2-Me shifts.

It is observed that the three deprotonations cause increases in shielding of all ring

* Terms such as "protonated", "deprotonation", etc., are used throughout for convenient descriptive purposes when discussing processes in $D_2O$ solution.

substituents, indicating a net electron release to the ring in each process. The first deprotonation creates the largest increase in shielding for all substituents except 4-$CH_2ND_2$, which is most affected by the third deprotonation. The second deprotonation causes a much smaller shielding increase for all substituents than the first. These observations are consistent with loss of phenolic and pyridinium[14] protons in the first two steps, but cannot distinguish which proton is lost in each. When taken in conjunction with conclusions from titrimetric measurements in aqueous solution[12,13] and UV spectral observations in aqueous methanol,[11] the chemical shift data can be interpreted satisfactorily in terms of the cationic species **6** as the predominant product of the first deprotonation in aqueous solution, although other tautomeric forms in rapid equilibrium with **6** are possible.[11] Similarly, the second deprotonation is inferred to produce the dipolar neutral species **7a**. The extremely large chemical shift change of the 4-methylene protons in the region of

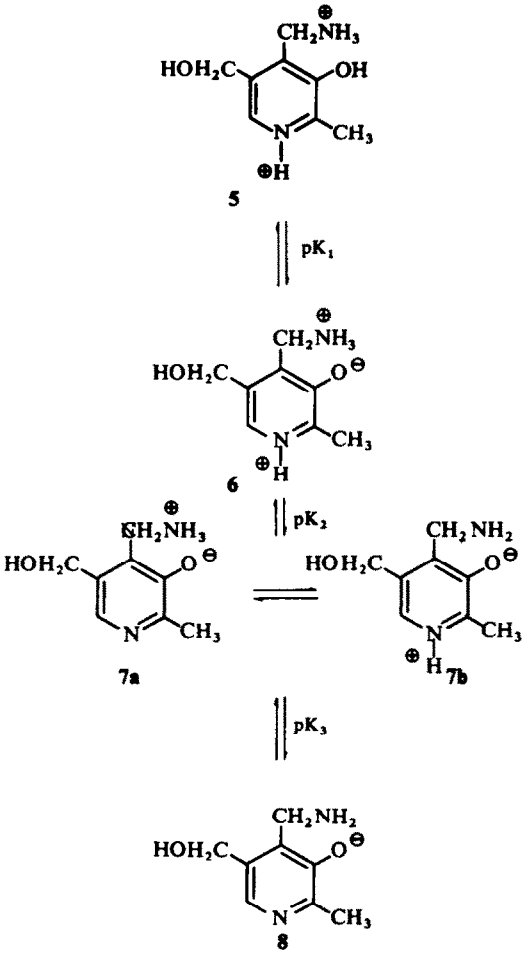


CHART I. Acid-base equilibria of pyridoxamine in aqueous solution.

pD ≈ $pK_3$ + 0·40 clearly demonstrates the final deprotonation product to be the anion **8**. In this process it is also observed that 6-H undergoes a substantial shift to higher field, a behavior which is not expected if only 4-$CH_2ND_3^{\oplus}$ is deprotonated. From this observation it is concluded that an additional dipolar form **7b** arises from the second step and that its deprotonation in the final step results in the increased shielding of 6-H in the pD range ~9–11 where the observed shifts are averaged over **7a**, **7b** and **8**. The deprotonation scheme for pyridoxamine in aqueous solution is summarized in Chart I.

3-*Hydroxypyridine-4-aldehyde.* A PMR spectrum of this compound in acidic solution is presented in Fig. 2. The AB pattern of 5-H and 6-H centered at −417 c/s ($J$ = 5·8 c/s) and the 2-H signal at −428 c/s are clearly resolved. The remaining features are a sharp singlet at −308 c/s and a somewhat broader low intensity signal at −548 c/s. The latter signal is in the characteristic low field region of pyridine aldehydes.[15] The former signal is assigned to the 4-$CH(OD)_2$ group of the hydrated aldehyde whose existence in acidic solutions of hydroxypyridinealdehydes,[10] pyridinealdehydes,[16] and aliphatic aldehydes[16] has been deduced previously from other measurements.

The relative intensities of the 4-CHO and 4-$CH(OD)_2$ signals and the chemical shifts of all signals are markedly pD dependent. The ratio of intensities of the free and hydrated aldehyde signals increases with increasing pD until at pD 8·9 that of the hydrated form can no longer be detected. The relative amounts of the two forms over the pD range 1–12 are readily determined by signal integration and are set out graphically in Fig. 3. These results correlate well with those from UV spectral studies in aqueous solution[10] which revealed a decrease in the hydrated aldehyde form with increasing pH in the 1·1–8·7 range.

The pD dependence of the chemical shifts is shown in Fig. 4; all protons become more shielded as the pD of the solution is raised. In strongly acidic solution the hydrated cation **9** (cf. Chart II) is the only species present in significant concentration. As the pD is increased to ~5 the shifts of all protons increase appreciably, the changes for 4-CHO and 2-H being particularly well defined. The inflection points correspond closely to $pK_1$ determinations in $H_2O$ solution by titrimetry (3·97) and spectrophotometry[10] (4·05). Because both hydrated and free aldehyde are present in appreciable concentrations at pD ~ 5, the first deprotonation results in the formation of the two dipolar neutral species **10** and **11**. In addition to the relative intensity changes noted above for the 4-$CH(OD)_2$ and 4-CHO signals, the increasing concentration of free aldehyde as the pD is raised is evidenced by the appearance of a second AB pattern occurring at higher fields than that which is initially observed under more acidic conditions. The intensity of this pattern becomes sufficient above pD ~ 4·5 that its chemical shifts can be accurately measured. As the pD is increased above this value the low field pair of signals of the AB feature become progressively broadened compared to the upfield pair. Inasmuch as the α-protons have been observed to be broadened in pyridine by coupling with the nitrogen,[17] a similar observation here indicates ring deprotonation of **10** and **11** and identifies 6-H as the proton from which the low field pair of signals is mainly derived. Inflection points of the 2-H, 5-H and 6-H shifts of the free aldehyde species are in good agreement with the $pK_2$ values of 6·74 and 6·77 obtained in aqueous solution by direct titration and spectrophotometry,[10] respectively. The 4-CHO shift proved to be insensitive to the second deprotonation.

The deprotonation scheme for 3-hydroxypyridine-4-aldehyde in aqueous solution which is in full accord with PMR and ultraviolet spectral[10] results is given in Chart II.

*3-Hydroxypyridine-2-aldehyde.* A PMR spectrum in neutral solution which illustrates the ABX pattern of 4-H, 5-H and 6-H and the presence of hydrated and free aldehyde species is presented in Fig. 2. The percentage of free aldehyde form in the pD range 1–9·5 as determined from signal intensities is given in Fig. 3. The X proton

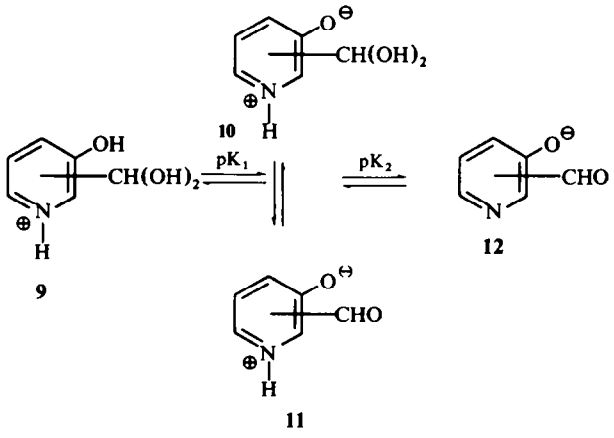


CHART II. Acid-base equilibria of 3-hydroxypyridine-2-and -4-aldehydes in aqueous solution.

in the ABX pattern was established to be 6-H on the basis of spectral comparisons with a series of 2,3-disubstituted pyridines. A plot of chemical shifts *vs* pD is set out in Fig. 4. No attempt was made to analyze quantitatively the overlapping ABX patterns of the free and hydrated aldehyde species in the pD region in which both are present. The two deprotonations are clearly evident in the pD dependence of the 6-H and 2-CHO signals. The values of $pK_1$ and $pK_2$ obtained from the chemical shift data are in fair agreement with those determined by titrimetry (3·27, 6·86) and spectrophotometry[10] (3·40, 6·95) in $H_2O$ solution. Enhanced shielding of ring substituents attendant to the first deprotonation and progressive broadening of 6-H in the pD 7–10 region lead to a description of the solution equilibria of 3-hydroxypyridine-2-aldehyde, shown in Chart II, which parallels that of the 4-aldehyde.

*Pyridoxal.* The solution equilibria of this important member of the vitamin $B_6$ group of compounds have been previously investigated in detail by UV spectroscopy in aqueous[7–10] and methanol[11] solution and by acid-base titrimetry in aqueous solution.[9,12] PMR spectra of pyridoxal and a number of its derivatives and analogs have been reported.[6,18] The pD dependence of the spectrum of pyridoxal in $D_2O$ solution has been described[6] in less detail than in this investigation and the published spectra are indicative of lower resolution in certain spectral regions than that reported here.

Three types of PMR spectra, illustrated in Fig. 5, were observed for pyridoxal. Aside from chemical shift changes observed for all signals, the major differences observed are in the signals of the ring substituents in the 4- and 5-positions. In the

simplest form (**2**) of the molecule these signals should arise from the —CHO and —$CH_2OD$ groups, respectively. At pD ~ 1 through 9·7 no free aldehyde signal is observable. In acidic solution the signal associated with the 4-position (−546 c/s) is split into a doublet ($J$ = 2 c/s) with unit intensity. The quartet of intensity two at −401 c/s must derive from the 5-substituent. Both of these features are consistent with the cationic hemiacetal species **13** (cf. Chart III). The two methylene protons are rendered inequivalent by the asymmetric center and their signals appear as a simple AB pattern ($J$ = 14 c/s). One of these protons is observably spin-coupled with that at the asymmetric center producing the doublet at −546 c/s.

In moderately basic solution (pD 9·7) the 5-methylene signal becomes very broad and that of the condensed aldehyde proton broadens and shifts downfield. Finally,

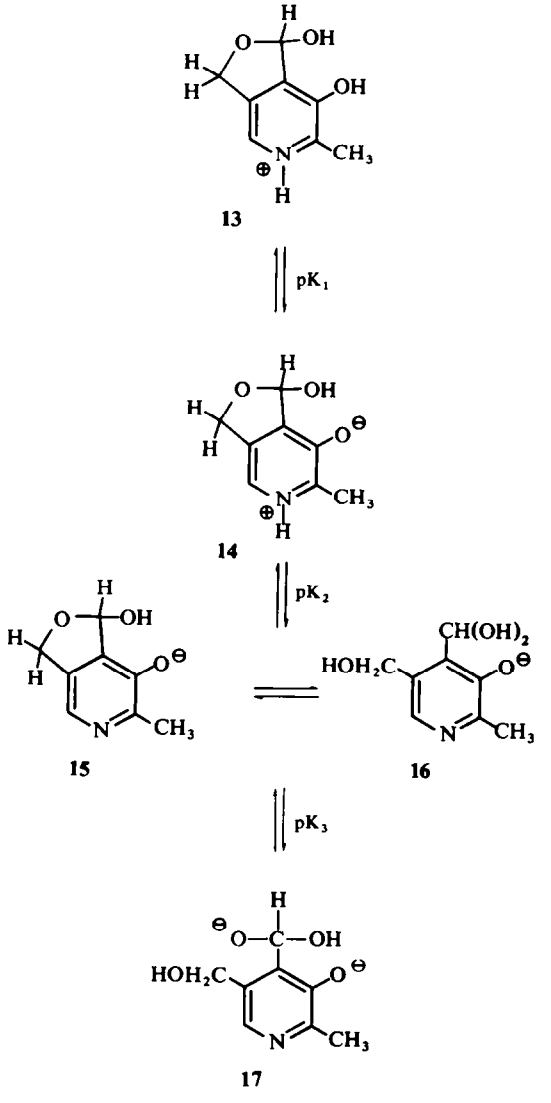


CHART III. Acid-base equilibria of pyridoxal in aqueous solution.

in strongly basic solution (pD 12) the "aldehyde" proton signal has shifted to even lower field and both it and the 5-methylene signal have become considerably sharper. Reference to the pD dependence of the chemical shifts shown in Fig. 6 reveals that all signals are appreciably shifted to higher field upon deprotonation of **13** and that in $D_2O$ $pK_1 = 4{\cdot}4$, in fair agreement with the titrimetric value of 4·23[12] obtained in $H_2O$ solution. Because the spectra indicate retention of the hemiacetal ring and the absence of free aldehyde up to pD 8, the principal product of the first deprotonation is concluded to be the dipolar neutral species **14**. In the pD range 8–10 the 4- and 5-substituent signals broaden and the chemical shifts of 6-H, 5-methylene, and 2-$CH_3$ are again shifted to high field. The $pK_2$ value determined from the shifts correlates well with the result (8·70) from titrimetry.[12] In this range no free aldehyde signal is observed. The products of the second deprotonation are concluded to be the hemiacetal anion **15** and the hydrated aldehyde species **16**. The slow interconversion **15** $\rightleftharpoons$ **16** accounts for the line broadening effects noted above.

Under strongly alkaline conditions (pD 10–13·3) a third deprotonation is evidenced by the small upfield shift of 6-H. However, the scatter in the experimental data makes assigning an accurate $pK_3$ value impossible. Nevertheless, from the 6-H and 4-CH curves, this quantity may be estimated to be 12·2–12·4. In this pD region it is observed that the shift of 4-CH sharply decreases and then increases with the maximum low field shift occurring at pD 11–11·5. This behavior is interpreted in terms of a shift of the equilibrium **15** $\rightleftharpoons$ **16** to the right as the pD is increased, followed by deprotonation of the hydrated aldehyde to yield the anion **17**. No free aldehyde was detected up to pD 13. Deprotonation of 5-$CH_2OD$ rather than 4-$CH(OD)_2$ is considered less likely because the shift of the 5-methylene protons is essentially constant from pD 11–13. The deprotonation sequence for pyridoxal deduced from the PMR results is shown in Chart III. Previous conclusions from UV spectral observations[9,10] that certain fractions of free aldehyde species exist in alkaline solutions should now be re-examined in light of the PMR results.

## EXPERIMENTAL

Pyridoxal monohydrochloride and pyridoxamine dihydrochloride monohydrate were purchased from Calbiochem Corporation and used as received. The 3-hydroxypyridine-2- and 4-aldehydes were prepared in these laboratories by Dr. M. J. O'Connor using published procedures,[19] and were purified by sublimation before use. Solns of NaOD were prepared by dissolving clean Na in $D_2O$. $D_2SO_4$ was available from commercial sources.

Solns used in the PMR measurements were prepared in 99·5% $D_2O$ and the desired pD value achieved by addition of $D_2SO_4$ or NaOD solns. Total concentration of solute in all forms was ~0·1 M over the pD range ~1–13 except in the case of pyridoxamine. The low solubility of this compound required the use of ~0·01 M solutions at pD 7–10; ~0·1 M solns were used otherwise. True pD values of the solns were obtained from the relation pD = pH + 0·40;[20] pH values were measured using a Beckman model G meter.

PMR spectra were determined using Varian HA/HR-100 or HA/HR-60 spectrometers. Using the usual sideband technique, chemical shifts were measured to ±1 c/s at ~35° relative to t-BuOH, present to the extent of ~1% v/v, as an internal standard. For the dilute pyridoxamine solns, a Varian C-1024 CAT was used to obtain time-averaged spectra.

Determination of the acid dissociation constants of 3-hydroxypyridine-2- and -4-aldehydes was performed by direct titration of 0·01 M solns containing 0·1M NaCl using a Radiometer titration assembly. The solns were titrated from strongly acidic to strongly alkaline conditions using standard NaOH soln.

Two distinct titration breaks were observed for each compound. The following $pK_a$ values were obtained at 24°.

3-Hydroxypyridine-2-aldehyde: $pK_1 = 3{\cdot}27 \pm 0{\cdot}02$; $pK_2 = 6{\cdot}86 \pm 0{\cdot}02$
3-Hydroxypyridine-4-aldehyde: $pK_1 = 3{\cdot}97 \pm 0{\cdot}01$; $pK_2 = 6{\cdot}74 \pm 0{\cdot}01$

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