Nuclear Magnetic Resonance Spectra of Pyridoxylidene(amino acido)aluminum(III) Complexes and Detection of a Possible General Intermediate in Metal-Catalyzed Reactions of Vitamin B_6[1]

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Abstract: The nmr spectra of a series of pyridoxal–amino acid Schiff base complexes of aluminum(III) in D_2O solution in the pD range 3–5.5 have been studied. Under these conditions the complexes formed contain a 1:1 molar ratio of Schiff base to metal ion. The spectra are similar to those reported previously by others for the 1:1 pyridoxylidenealanatoaluminum(III) system, with the exception that in the present work no evidence is found for a complex species containing an uncoordinated carboxylate group. The observation of rapid changes in the spectra provides evidence for the formation of a previously unreported complex during the transamination process. The rates of growth and disappearance of this new substance relative to the rate of formation of transamination products suggest that it may be a general intermediate for metal-catalyzed model reactions of vitamin B_6. The observed nmr spectrum of the intermediate is employed as a basis for deducing its probable bonding and electronic structure.

In the studies of pyridoxal-catalyzed reactions of amino acids, considerable attention has centered about the formation and characterization of an intermediate resulting from dissociation of the α proton of the amino acid residue in the imine. Experimental verification of such intermediates has been limited thus far to non-metal-catalyzed systems. Characterization of intermediates obtained by dissociation of the α proton from the amino acid in the aldimine has been described for model systems by Maley and Bruice.[2] In this case, it was found that abstraction of a proton from the Schiff bases formed by condensation of glycine or alanine with 1-methyl-4-formylpyridinium iodide produces a species absorbing in the visible region of the spectrum (500 and 600 nm), suggesting the formation of dihydropyridine-type tautomeric structures of the carbanion zwitterion.

A number of investigators have reported enzyme–substrate complexes of B_6 enzymes absorbing near 500 nm,[3–6] which similarly seem to be formed by the dissociation of the α proton of the amino acid of the aldimine Schiff base. Also, the complex formed from diethyl aminomalonate and pyridoxal, detected in the course of decarboxylation studies with aminomalonic decarboxylase, absorbs at 460 nm.[7] The similarities of the D-alanine–enzyme complex absorbing at 505 nm, and the compound formed between pyridoxal *N*-methyl chloride and diethyl aminomalonate absorbing at 480 nm, have been recently pointed out.[8]

Thus far in model systems containing catalytic metal ion complexes of the pyridoxal–amino acid Schiff bases, this intermediate has not been reported. Its detection would be of interest since the proposal by Metzler, Ikawa, and Snell,[9] that it is the key intermediate in nonenzymic transamination, has received general acceptance. In this paper, nmr evidence for the formation of such an intermediate is now presented.

Experimental Section

Nmr spectra of D_2O solutions of the compounds under investigation were taken with a Varian HA-100 spectrometer. The probe temperature was 30 ± 2°. Chemical shifts are reported with respect to an internal capillary of hexamethyldisiloxane (HMDS) whose proton resonance was also used as an internal locking signal.

In this paper, pD is the negative logarithm of the deuterium ion concentration. The deuterium ion activity was measured by the method of Covington, *et al.*,[10] and was converted to concentration by means of tabulated activity coefficients.[11] The ionic strength was maintained at unity by the addition of the appropriate amount of sodium chloride.

Pyridoxal hydrochloride, pyridoxamine dihydrochloride, and the amino acids were obtained from Mann Laboratories. The D_2O was 99.5 mol % of Matheson Coleman and Bell. The NaOD was prepared as previously described.[12] The Al(III) solutions were prepared by adding the hydrated sulfate to D_2O and evaporating to dryness several times. The resulting D_2O solutions were filtered and were standardized by Schwarzenbach's method of back titration with zinc(II).[13]

Results and Discussion

At pD values between 3.0 and 6.5, the nmr spectrum of solutions of 1:2:2 molar ratios of aluminum(III) ion, pyridoxal, and an α-amino acid shows the slow, incomplete formation of complexes such as I, containing one Schiff base ligand per Al(III) ion. At pD values above 5, bis complexes are also formed and their contribution becomes greater with increasing pD so that the 1:1

(1) This work was supported by a research grant (AM-11694) from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service.

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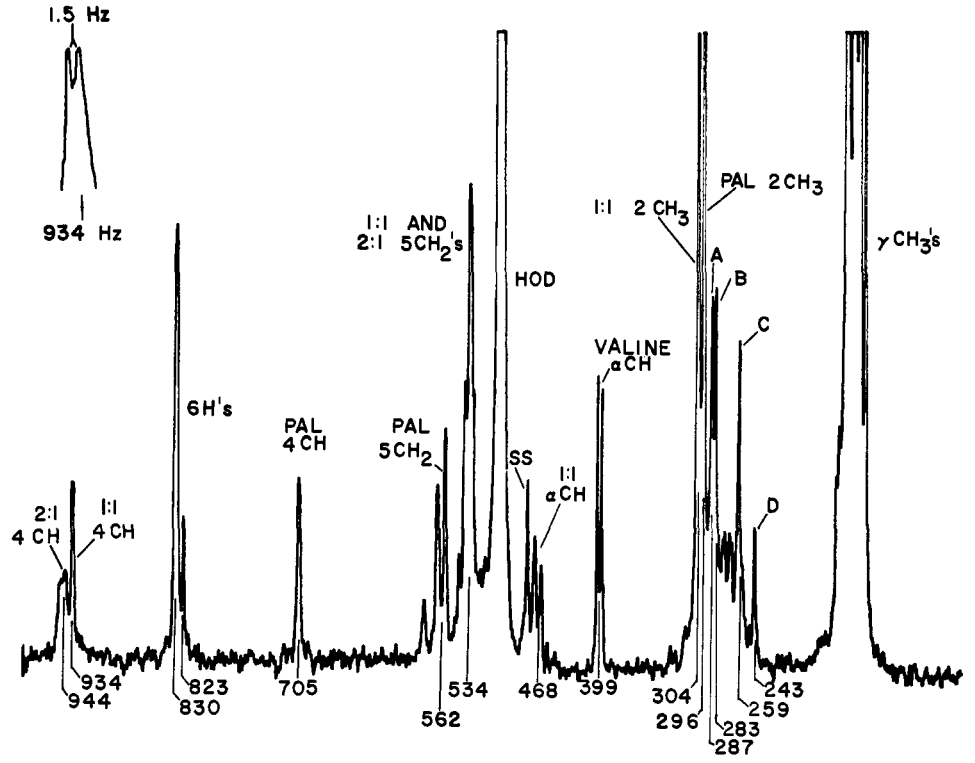
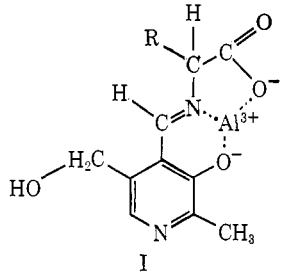


Figure 1. The 100-MHz spectrum of a D_2O solution containing a 1:2:2 molar ratio of Al(III), pyridoxal, and valine at pD 5.5 immediately after mixing. Resonance frequencies are in hertz *vs.* internal capillary of hexamethyldisiloxane; 1:1 and 2:1 denote molar ratios of Schiff base ligand to metal ion; PAL = pyridoxal; numbers refer to substitutuents on pyridine ring; letters A, B, C, and D refer to 2-CH_3 resonances of stereoisomers of bis Schiff base–Al(III) complex (ref 14).



I

1:1 Pyridoxylidene(amino acido)aluminum complex (I)

complex is unobservable above pD 6.5. The bis (2:1) complexes have already been discussed in detail.[14]

The nmr spectra of the 1:1 complexes are exemplified by Figure 1. (In this case a D_2O solution of 0.05 *M* Al(III), 0.10 *M* valine, and 0.10 *M* pyridoxal was adjusted to pD 5.5 and its nmr spectrum was run within 0.5 hr.) Free pyridoxal and free valine are observed as well as the spectra of the diasterioisomers of the bis complex[14] (labeled 2:1 and A, B, C, and D). In addition, resonances in accord with structure I are observed and these are labeled 1:1. These resonances have chemical shifts quite similar to those of the bis complexes with the exception of the pyridine 2-methyl (2-CH_3) region where the bis complex resonances are influenced by the magnetic anisotropy of the other coordinated Schiff base. The 4-CH resonance (resonance of proton at the azomethine carbon) of the 1:1 complex is a doublet due to the spin–spin coupling to the α CH of the valine moiety.

The 1:1 complexes are rather inert with respect to acid decomposition. Solutions whose pD was rapidly lowered to 0–1 with DCl or D_2SO_4 show a decrease in intensity of 1:1 complex resonances which is consistent with a half-life of about 0.5 hr at room temperature. In contrast, solutions of the bis complex are very rapidly hydrolyzed to the 1:1 complex and free pyridoxal and amino acid. The reaction is so rapid that no trace of the bis complex can be found by nmr within 20–30 sec of addition of the mineral acid. This kinetic phenomenon (slow dissociation of the 1:1 complexes) also manifests itself in the slow formation of 1:1 complexes.

In addition to the resonances found for the aldimine complexes and for their components, other resonances are observed to appear slowly between pD 3.5 and 5.5. These new resonances are labeled M in Figure 2. Previous workers assigned these resonances to uncomplexed Schiff base;[15] however, it has been established[16] that at these concentrations free Schiff base is not observable as a separate entity below pD ~7 in the absence of metal ion. Thus, the laws of equilibrium require that the peaks at M cannot arise from free Schiff base in the presence of metal ion.

In systems containing resonances M, some transamination can be shown to have occurred by lowering the pD to about 1 so as to hydrolyze the complexes. The acidified solutions show substantial pyridoxamine, whose resonances were assigned by the addition of further pyridoxamine. Signal integration before and after acidification showed that the appearance of the resonances at M parallels the appearance of pyridoxamine but that the disappearance of the pyridoxal 4-CH signal was not entirely accounted for by formation of the pyridoxamine.

(14) E. H. Abbott and A. E. Martell, *J. Amer. Chem. Soc.*, **92**, 5845 (1970).

(15) O. A. Gansow and R. H. Holm, *ibid.*, **91**, 573 (1969).

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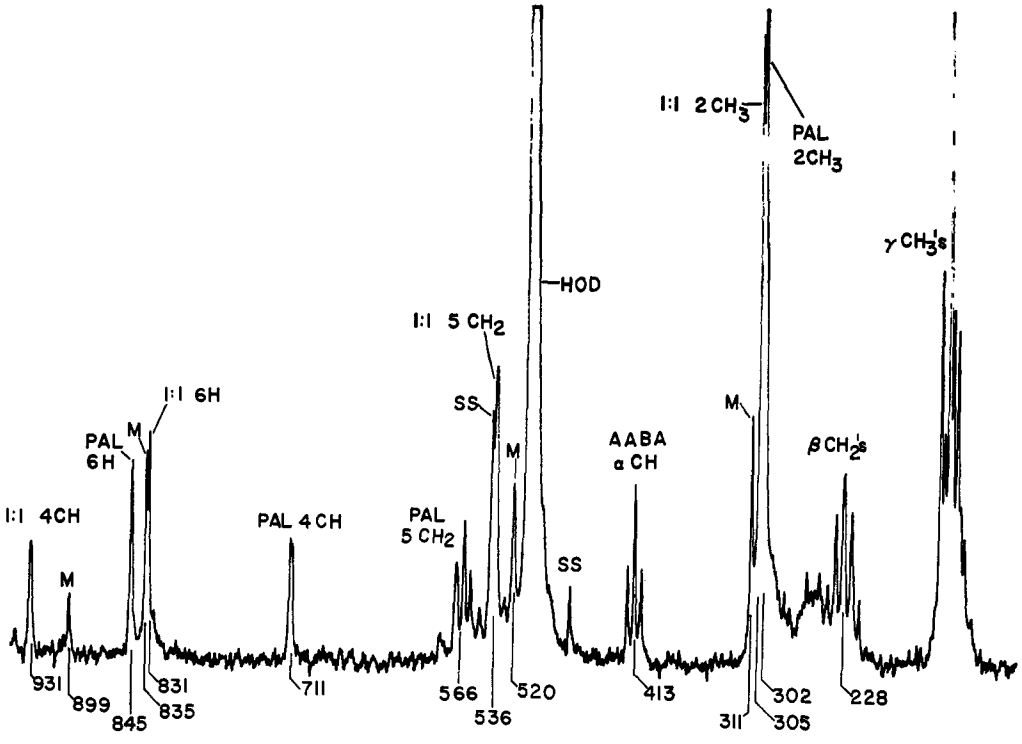


Figure 2. The 100-MHz nmr spectrum of a D_2O solution containing a 1:2:2 molar ratio of Al(III), pyridoxal, and α-aminobutyric acid at pD 4.7 30 min after mixing; M designates resonances of intermediate listed in Table I; AABA = α-aminobutyric acid; other symbols and frequency scale are as defined for Figure 1.

With the amino acids α-aminobutyric acid, alanine, and norvaline, the peaks at M are detectable within 0.5 hr in solutions between pD 4.5 and 5.0. After 3–4 hr resonances due to free pyridoxamine are detectable without acidification. Substantial free pyridoxamine is probably present earlier in the reaction; however, its resonances cannot be resolved because they are too close to the relatively intense pyridoxal and aldimine complex resonances. When the free pyridoxamine concentration exceeds about 0.01 *M*, its 2-CH_3 resonance is observed between or near those of the 1:1 aldimine complex and the free pyridoxal. The resonance of the 6-H (proton at the position of the pyridine ring) is observed at slightly higher field than the free pyridoxal 6-H. The 4-CH_2 resonance is not detectable with certainty though it appears in a region free of interference. This is probably due to the fact that it must be at least half deuterated, thus lowering the intensity and also making it a 1:1:1 triplet or at least a broadened singlet.

In the valine case, the appearance of resonances at M is far slower than with α-aminobutyric acid, alanine, or norvaline. These resonances are barely detectable even after 6 hr. Valine also is transaminated much more slowly than are these other amino acids. These semiquantitative observations indicate that the appearance of the peaks at M is associated with transamination and/or with the appearance of pyridoxamine in these systems.

The possibility that the resonances arise from the Schiff base formed from the reaction of pyridoxal with pyridoxamine or its aluminum(III) complexes was investigated. Although such a Schiff base forms readily, as does its mono- and bisaluminum(III) complexes, in no case were 4-CH resonances found below 910 Hz nor were there 2-CH_3 resonances above 308 Hz. The possibility that the resonances in question arise from a mixed ligand complex involving the aldimine and pyridoxylidenepyridoxamine was rejected since it should have more than one set of resonances and since it does not explain the high field shift of the 4-CH relative to the 4-CH's of the 1:1 and 2:1 complexes. It would also be difficult to explain the existence of this complex in a pD region below that in which the bisaldimine chelates exist.

The structure of the species giving rise to resonances labeled M should be similar to that of the aldimine since it has resonances near the 4-CH, 6-H, 5-CH_2, and 2-CH_3 resonances of the free Schiff bases, the 1:1 complex, and the 2:1 complexes. We shall therefore identify the sources of its resonances with those of known aldimine structures giving rise to the resonances in their respective regions of the spectrum. Its 4-CH is found at higher field than any of the pyridoxal aldimines yet investigated, lying some 10 Hz to higher field than the coordinated aldimine. The 6-H and 5-CH_2 resonances are well within the regions found for either the coordinated or free aldimine and the 2-CH_3 resonance occurs at 5 Hz lower field than any 2-CH_3 resonances yet investigated. Table I presents a comparison of the resonances of M with those of 1:1 aldimine complex and with those of a similar ketimine complex, pyruvilidenepyridoxaminoaluminum(III), whose nmr spectrum has recently been reported.[17]

Although the gross structures of M and of the 1:1 aldimine complex should be similar, the electronic configurations may be quite different. Ultraviolet and nmr spectra both show that the *o*-hydroxy Schiff

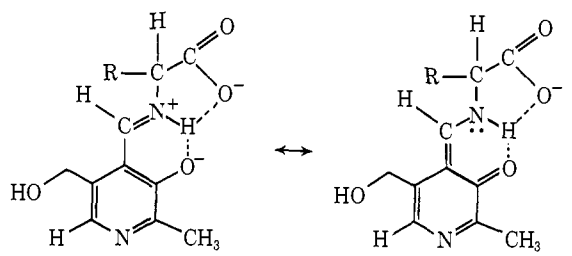
(17) O. A. Gansow and R. H, Holm, *J. Amer. Chem. Soc.*, 91, 5984 (1969).

Table I. A Comparison of the Chemical Shifts with Respect to HMDS of Several 1:1 Aluminum(III) Complexes

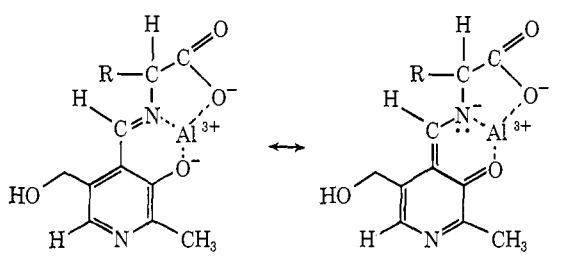
	1:1 aldimine complex[a]	M[a]	1:1 ketimine complex[b]
2-CH_3	305	311	284
5-CH_2	536	520	*c*
6-H	831	835	815
4-CH	931	899	516

[a] From Figure 2, this work. [b] From Figure 6, ref 17. The ketimine resonances were reported with respect to *tert*-butyl alcohol and have been made comparable to data obtained in this research by addition of 154 Hz. [c] Obscured by the HDO isotopic impurity of the solvent.

bases, which in the protonated form are frequently drawn as IIa, have important resonance contributions from bond arrangements such as that in IIb.[18,19] If, however, one considers the metal complexes IIIa and IIIb, the resonance form IIIb becomes a



IIa ⟷ IIb

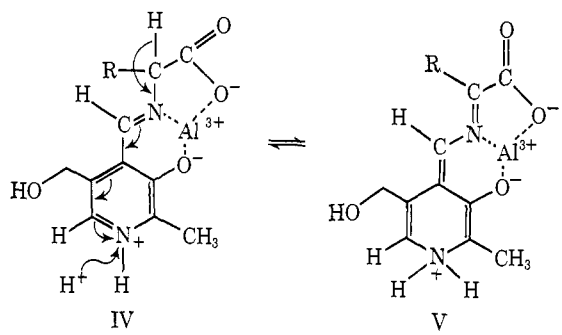


IIIa ⟷ IIIb

much poorer representation than the corresponding form IIb is for the metal free case. Thus, it is observed that the Schiff bases have an absorption maximum near 414 mμ[20] while their metal chelates have a maximum near 380 mμ.[21] Despite this considerable difference in electronic configuration, the nmr spectrum of the 1:1 complex is very similar to that of the free, azomethine nitrogen-protonated Schiff base.

Thus, we shall consider a very different explanation for the structure of M. It has long been assumed that the structure of the intermediate in nonenzymic pyridoxal reactions has a conformation similar to that of V and is formed from IV by tautomerization of the type shown.[9] Our rendering of this reaction differs from the usual structures in that a proton has been displaced from the α position of the amino acid, but because of the strong electropositive nature of the metal ion, the proton remains dissociated and is not tautomerized to the pyridine nitrogen. The negative charge of the group consisting of the carbon and nitrogen of the azomethine group and the α carbon of the amino acid is stabilized by delocalization over the three atom grouping, by the proximity of the tripositive metal ion, and by delocalization with the positive pyridine nitrogen. The fact that the solution is colorless is not surprising since the amount of charge delocalization and the length of the conjugated system are less than those in the analogous metal-free Schiff base systems that were observed to have absorption bands at about 500 nm.[22] The attraction of the metal ion for the negative charge, tending to localize it near the azomethine group, is considered an important factor in determining the electronic structure of the intermediate, indicated by VIa and VIb.

In spite of the differences in structure indicated by formulas I and VIa and VIb, the environment of the protons at each position is not greatly different, so that the chemical shifts should be similar. The shift to higher field for the 4-CH resonance (Table I) is understandable because of the localization of negative charge at the three-atom center involving the azomethine group and the α carbon of the amino acid. The magnitudes and signs of the remaining chemical shifts can be rationalized in several ways; however, since the shifts are small, this would not represent reliable supporting evidence for the structure.



IV ⇌ V

In view of the above arguments, it is proposed that the intermediate M is involved in the metal ion catalyzed transamination mechanism in accordance with the reaction sequence indicated by I → VIa, VIb → VII (Scheme I). The formation of the mixed ligand complex of the transamination products seems likely but is not an essential part of the proposed mechanism. As indicated above, the presence of the intermediate VI in appreciable concentrations in the reaction mixture is ascribed to its resonance stabilization and to the stabilization of negative charge in the ligand by coordination of the metal ion.

Another possible source for resonances M would be the formation of binuclear 1:1 complexes such as VIII and IX. The tendency of highly charged metal ions to polymerize is well known; however, several lines of reasoning argue against this explanation. Resonances M are all sharp singlets but VIII and IX are respectively cis and trans isomers and would be expected to have slightly different nmr spectra. Models show that steric effects are about equal for the two isomers. It might, of course, be that the spectra are unresolvable one from the other, but this seems unlikely. Steric interaction of the 2-CH_3 groups excludes

(18) A. E. Martell in "Chemical and Biological Aspects of Pyridoxal Catalysis," E. E. Snell, *et al.*, Ed., Pergamon Press, New York, N. Y., 1963, p 13.
(19) G. O. Dudek and R. H. Holm, *J. Amer. Chem. Soc.*, **83**, 3814 (1961).
(20) D. E. Metzler, *ibid.*, **79**, 485 (1957).
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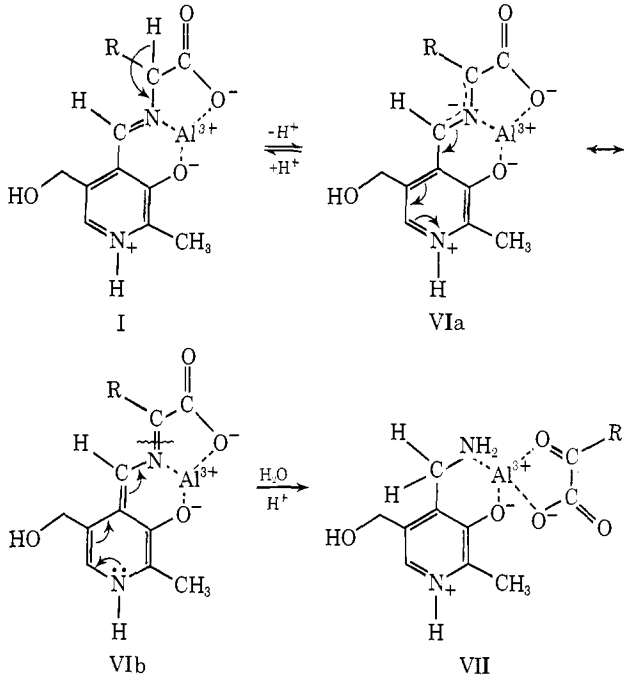
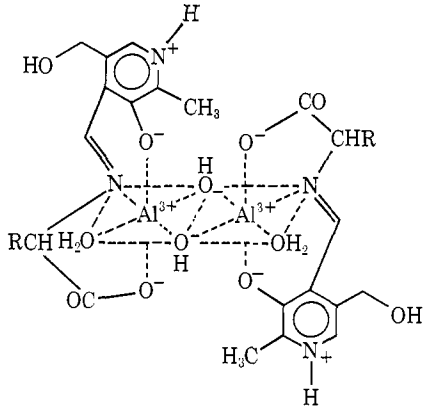
Scheme I. Formation of Metal Complex Intermediate in Transamination

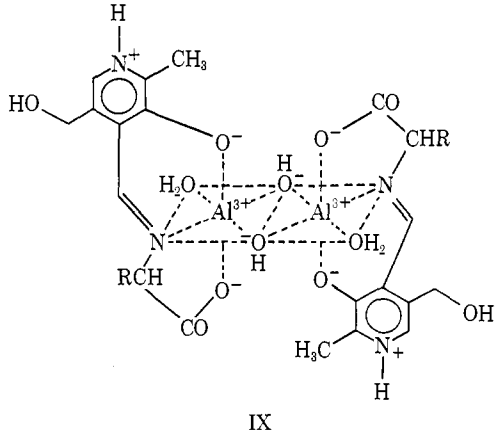


I VIa VIb VII

binuclear species with both the heterocyclic rings on the same side of the olated metal ion plane.



VIII

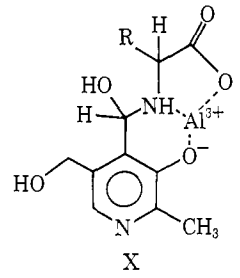


IX

A second point against such a structure is the pD range in which they are observed. It is unlikely that hydroxide ion at 10^{-10} *M* can compete with a second ligand for coordination when at much higher pD's; the second ligand is known to be coordinated to form the bis complex. The fact that in the pD range where resonances M are observed the heterocyclic nitrogen is protonated might offer resolution of the paradox because the proton would destabilize the complex both by electrostatic repulsion of the metal ion and by withdrawal of charge from the phenoxide-type oxygen and azomethine nitrogen. At higher pD's, the heterocyclic nitrogen is deprotonated and a second ligand could then force the hydroxide ions out of the coordination sphere. Such an explanation, however, would be inconsistent with the observed rapid equilibrium between the bis complex and the 1:1 complex, I.

Another argument against polynuclear species is the small amplitude of resonances M and the slowness with which they appear in the case where the amino acid is valine. None of the possible polynuclear structures seem to have sufficient steric effects of the amino acid alkyl group to rationalize this observation.

The possibility that resonances M arise from a coordinated carbinolamine such as X was discarded



X

because the carbinolamine is considered to be an intermediate in the formation of the Schiff base, suggesting that resonances M would be present from the outset in their maximum concentration, and that aldimine resonances would appear more slowly than those of the carbinolamine. Observations reported in this research demonstrate that the reverse is true. Further, if X were responsible, five resonances corresponding to the intermediate would be expected, rather than four that were actually observed. Also, the 4-CH resonance would be shielded by hydroxyl rather than by an unsaturated carbon linkage (—CH=), resulting in a shift to lower field.

To summarize the reasoning given above concerning the nature of the intermediate, the following points are considered significant. (1) Resonances M appear slowly, roughly paralleling the appearance of pyridoxamine. This is the case both for the relatively rapidly reacting alanine and slowly reacting valine. (2) Resonances M decrease in intensity as transamination of aldimine to ketimine nears completion. (3) The structure of M must be similar to that of the aldimines because of similarity of nmr spectra. (4) The 4-CH resonance of M is found at higher field than that of any pyridoxal aldimine yet reported; however, it is still far too low to permit M to be identified with any species with a saturated 4-CH such as the ketimine. (5) Polynuclear complexes and mixed ligand complexes seem to be excludable as sources of M. (6) The intermediate is formed in appreciable concentrations and is not an unstable intermediate representing an activated state of the metal complex.

It seems likely that the presumed intermediate for vitamin B_6 catalysis may indeed be fairly stable in the form of its metal complexes. The evidence in support

of this statement (6) is largely circumstantial and by no means complete. However, if the species giving rise to resonances M does not have the structure suggested, it is very difficult to explain its presence in these systems on the basis of what is now known. Lastly, it would be a strange coincidence indeed if the appearance of M were unrelated to the transamination model reaction, in view of its apparent kinetic relationship to pyridoxamine formation, and its structural similarity to the aldimine as detected by nmr.

Carbon-13 Nuclear Magnetic Resonance Spectroscopy of Cobalamins and Cobinamides Selectively Enriched with Carbon-13[1a]

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Abstract: The cmr spectra of aqueous (D_2O) methylcobalamin, methylcobinamide, and cyanocobinamide, selectively enriched with ^{13}C in the ligands attached to cobalt, were recorded at 25.2 MHz and 32°. The cmr spectrum of [^{13}C]cyanocobinamide exhibits two well-resolved Co–^{13}CN resonances (chemical shift difference, 1.1 ppm) corresponding to the isomers: cyanoaquocobinamide and aquocyanocobinamide. The spectrum of di[^{13}C]cyanocobinamide at 32° shows only one rather broad Co–^{13}CN resonance, indicating that the chemical shift difference between the two sites is small. At 10°, two ^{13}CN resonances can be resolved partially (chemical shift difference <0.7 ppm) for di[^{13}C]cyanocobinamide. In contrast, the cmr spectrum of methylcobalamin exhibits one relatively sharp Co–$^{13}CH_3$ resonance broadened only slightly by the ^{59}Co nucleus, whereas the spectrum of methylcobinamide exhibits two Co–$^{13}CH_3$ resonances of approximately equal intensity. The chemical shift of the $^{13}CH_3$ moiety as well as the ^{13}C–H coupling constant are markedly affected by the nature of the trans ligand and a linear correlation has been established between the shift or the coupling constant and the β band of the visible spectrum. The electronegativity of the cobalt atom of methylcobalamin, estimated from the ^{13}C–H coupling constant and the carbon–cobalt bond length, varies from approximately 2.2 to 2.6 in the "base-on" and "base-off" form.

Deoxyadenosylcobalamin and methylcobalamin function as coenzymes in enzymatic reactions involving the transfer of hydrogen or of a methyl moiety, respectively. The most striking feature of these corrinoid coenzymes is the carbon–cobalt bond which is alternately cleaved and re-formed during the catalytic process.[2] The chemical and physical properties of the organometallic bond (X) are markedly influenced by axial ligands (Y) trans to it[3] (Figure 1). The electronic rearrangements in the carbon–cobalt bond that accompany coordination in the trans axial position are of interest both to the inorganic chemist concerned with ground state trans effects which are particularly large in these complexes, and to the biochemist concerned with the chemical changes that occur in the coenzyme during catalysis.

Because carbon-13 chemical shifts are remarkably sensitive to the electronic environment of the ^{13}C nucleus,[4] large trans effects should be evident in the ^{13}C nuclear magnetic resonance (cmr) spectra of the axial ligands in the cobalamins and cobinamides. This paper describes the cmr spectra of methylcobalamins, methylcobinamides, and cyanocobinamides selectively enriched with carbon-13 in the axial ligands.

Experimental Section

Materials. Cyanocobalamin was purchased from Sigma Chemical Co. Other corrinoids were prepared from cyanocobalamin by published procedures: aquocobalamin,[5] methylcobalamin,[6] diaquocobinamide,[3a] cyanoaquocobinamide,[7] and methylcobinamide.[3a] Carbon-13 methyl iodide, 61.8% enriched, was purchased from Prochem; [^{13}C]methyl iodide, 90% enriched, was prepared from [^{13}C]methanol (a gift from D. G. Ott of LASL) according to the method of Murray and Ronzio.[8]

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