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Metal binding to pyridoxal derivatives. An NMR study of the interaction of Eu(III) with pyridoxal phosphate and pyridoxamine phosphate†

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Abstract. The solution conformations of pyridoxal-5'-phosphate and pyridoxamine-5'-phosphate have been investigated using Eu(III) as a nuclear magnetic resonance shift probe. Binding of Eu(III) to pyridoxal phosphate results in the formation of two complexes, at the phosphate group and the *o*-hydroxy-aldehyde moiety, which are in slow exchange on the nuclear magnetic resonance time-scale. The lanthanide-induced pseudo contact shifts calculated using the McConnell-Robertson equation (*J. Chem. Soc.* (1950), 22, 1561) are in good agreement with the experimentally observed values for both pyridoxal phosphate and pyridoxamine phosphate and lead to a family of closely related conformations.

Keywords. Conformation; lanthanide; nuclear magnetic resonance; pyridoxal-5'-phosphate; pyridoxamine-5'-phosphate.

Introduction

Vitamin B₆ in its coenzyme form as pyridoxal phosphate (PALP)¹ is a cofactor for a variety of enzymatic reactions (Snell, 1958; Braunstein, 1960; Snell *et al.*, 1968). Studies on model systems (Metzler *et al.*, 1954; Longenecker and Snell, 1957; Blum and Thanassi, 1977) have shown that presence of metal ion plays an important role in PALP catalysed, nonenzymatic transformations of amino acids. While Schiff's base formation between amino acids and PALP is well known (Metzler *et al.*, 1954), a considerable amount of information is also available from crystal structure studies (Cutfield *et al.*, 1967; Bentley *et al.*, 1968; Capasso *et al.*, 1974), on the mode of binding of metal ions to Schiff bases.

Recently, lanthanide ions like Eu(III) and Gd(III) have been used to determine the solution conformations of biologically important molecules (Barry *et al.* 1971; 1974; Glasel, 1973). This paper describes attempts at determining the structures of PALP-metal complexes in solution, using Eu(III) as a paramagnetic, nuclear magnetic resonance (NMR) shift probe. The purpose of this study is two-fold : (i) to establish the sites of interaction of the metal ion with PALP,

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¹ Abbreviations used: Pyridoxal-5'-phosphate (PALP); Pyridoxamine-5'-phosphate (PAMP) Lanthanide induced shift (LIS).

in aqueous solution; (ii) to determine the solution conformations of the metal complexes of PALP and the related molecule, pyridoxamine-5'-phosphate (PAMP), using the lanthanide-induced shift (LIS) method.

Materials and methods

Materials

PALP and PAMP were obtained from Sigma Chemical Company, and were used without further purification. Europium chloride was the kind gift of Dr. J. A. Glasel.

NMR studies

PALP solutions (66 mM) in D₂O at pH ~ 2 were used in the ¹H NMR experiments. A stock solution of EuCl₃ (1 M) containing 66 mM PALP was prepared in D₂O and adjusted to pH ~ 2. Titrations varying the Eu(III) concentrations were carried out by addition of aliquots of this stock solution, to the PALP solution. Metal concentrations were varied between 0 and 480 mM. pH values reported are uncorrected for the deuterium isotope effect.

Solutions of PAMP (74 mM) in D₂O at pH ~ 2 were used for the ¹H NMR experiments. A stock solution of 984 mM EuCl₃ containing 74 mM PAMP was prepared in D₂O and the pH was adjusted to ~ 2. Titrations were carried out as for PALP, by varying the metal concentrations from 0 to 380 mM.

The spectra were recorded using a Varian HA-100 spectrometer. The residual HDO resonance served as the internal lock. The methyl resonance of *t*-butanol was used as the internal reference. Induced shift ratios at various Eu(III) concentrations were calculated, using the induced shift of the most affected resonance as the standard. Extrapolation to zero Eu(III) concentration yielded experimental shift ratio values, which were then used for comparison with the values, calculated theoretically as outlined below.

Theoretical calculations of shift ratios

The method suggested by Barry *et al.*, (1971) uses the equation for pseudocontact shifts for an axially symmetric field suggested by McConnell and Robertson, (1958),

$$\frac{\Delta \nu_i}{\nu_0} = K \frac{(3 \cos^2 \theta_i - 1)}{r_i^3}$$

where r_i is the distance from the metal to the i th proton, θ_i is the angle between the vector, r_i and the principal symmetry axis of the complexed paramagnetic ion, averaged over motions rapid on the NMR time scale. K , a function of temperature and magnetic properties of the complex, is a constant for a given metal and is independent of the nuclear resonance observed and ν_0 is the spectrometer frequency. The use of shift ratios, viz.,

$$(SR)_i = \frac{\Delta \nu_i}{\Delta \nu_{std}} = \frac{(3 \cos^2 \theta_i - 1)}{(3 \cos^2 \theta_{std} - 1)} \cdot \frac{r_{std}^3}{r_i^3}$$

removes the unknown constant K from the relationship.

In the case of PAMP, the Eu(III) ion binds at the phosphate group. For computational purposes, Eu(III) was fixed using an Eu-O distance of 2.4 Å with the magnetic symmetry axis lying along the Eu-P vector as shown in figure 1a. This is similar to the binding geometry found for the AMP-Eu(III) (Barry *et al.*, 1971; Glasel, 1973) and UMP-Eu(III) complexes (Inagaki *et al.*, 1978).

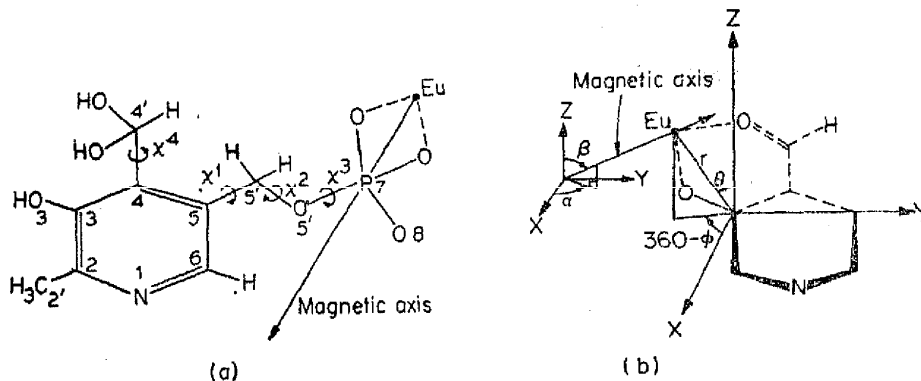


Figure 1a. Scheme indicating the position of metal binding at the phosphate group of PALP; the O-Eu distances are 2.4 Å and the magnetic axis is along the Eu-P vector; applies to both PALP-Eu(III) complex I and PAMP-Eu(III) complex.

Figure 1b. Scheme showing the position of metal binding at the *o*-hydroxyaldehyde group; applies to PALP-Eu(III) complex II; The pyridine ring is in a plane perpendicular to plane of the paper

For PALP, two complexes were detected corresponding to metal binding at the phosphate and the *o*-hydroxyaldehyde moieties. For the former, the choice of magnetic axis is the same as shown in figure 1 a. For the latter, the metal ion is fixed using spherical polar coordinates (r, θ, ϕ) and the direction of the magnetic axis is fixed by two more parameters α and β , as shown in figure 1b. The method followed is that of Barry *et al.* (1973). Calculations of shift ratios were carried out using an IBM 360/44 computer

Conformational search

The flexible side chain at C(5) in both PALP and PAMP can adopt a variety of conformations characterized by different values of the dihedral angles χ^1, χ^2, χ^3 (see figure 1a). In addition, one more rotation is possible about the C(4)-C(4') bond, denoted by χ^4 , for both PALP and PAMP. The dihedral angles are defined as follows: χ^1 , C(4)-C(5)-C(5')-O(5'); χ^2 , C(5)-C(5')-O(5')-P; χ^3 , C(5)-O(5')-P-O(8'); χ^4 , C(3)-C(4)-C(4')-H(4') for PALP and χ^4 , C(3)-C(4)-C(4')-N(4') for PAMP. The crystal structure data for PALP (Fujiwara, 1973) and PAMP (Giordano and Mazzarella, 1971) were adopted to generate the initial conformation of these molecules. For PALP (complex I) and PAMP metal complexes with Eu(III) bound at the phosphate group, the metal position was fixed, as described earlier. Initially, for PALP, the dihedral angle χ^4 was fixed at the crystal structure value of 0° and the remaining three dihedral angles were varied from

0 to 360° at 20° intervals. This requires the calculation of shift ratios at 18³ (= 5148) points in conformational space. Later, the value of x^4 was allowed to vary from 0 to 360°, whereas, χ^1 , χ^2 and χ^3 were confined to only those values, for which the calculated shift ratios of CH₃(2')/CH₂(5') and CH(6)/CH₂(5') were in agreement with the experimental results. For PAMP, however, stereochemical constraints using 'contact criteria' as a filter for disallowed structures were introduced to reduce the computation time. The contact distances as suggested by Barry *et al.* (1971) were used to filter out the disallowed structures.

Metal search

For the PALP complex binding Eu(III) at the *o*-hydroxyaldehyde grouping (complex II), the metal position and the direction of magnetic axis were fixed as mentioned earlier. Initially, a coarse search was carried out in which the metal position was changed in space by varying r , C(3)-Eu distance (see figure 1b), from 2.8–3.3 Å, θ from 0 to 180° and ϕ from 30 to 90°. The value of r was thus restricted, so as to maintain the metal approximately symmetrically placed between O(3) and O(4') oxygen atoms. For each metal atom position, a search for the direction of the magnetic axis was made by varying the values of α and β (see figure 1b). Since a rotation is possible about the C(5)–C(5') bond, the dihedral angle χ^1 was simultaneously varied from 0 to 360° at 20° intervals. A fine search in a limited region, where agreement with experiment was obtained, was carried out at intervals of 2° for the angular parameters θ, ϕ, α and β , and at an interval of 20° for χ^1 .

The agreement between the calculated and the experimental values is expressed by the agreement factor, R

$$R = \left[\frac{\sum_i (\text{obs}_i - \text{cal}_i)^2}{\sum_i (\text{obs}_i)^2} \right]^{1/2}$$

Results and discussion

Proton NMR

Figure 2 shows the effect of adding increasing concentrations of EuCl₃ to a 66 mM solution of PALP at pH ~ 2. The resonances at -141 Hz, -390 Hz and -700 Hz from internal *t*-butanol are assigned to CH₃(2'), CH₂(5') and CH(6), respectively. The resonance at -532 Hz is assigned to the aldehyde proton which exists predominantly in the hydrated form, at acid pH (Metzler and Snell, 1955; Korytnyk and Singh, 1963; Harris *et al.*, 1976). Complexing of Eu(III) results in upfield shifts of all the resonances, except the CH₃(2') peak. Further, additional resonances appear on addition of Eu(III), suggesting the presence of two metal-PALP complexes, which exchange slowly on the NMR time scale. In figure 2c, the resonances at +154 Hz, -132 Hz and -494 Hz are assigned to the CH₃(2'), CH₂(5') and CH(6) protons of complex II. The effect of increasing Eu(III) concentration on the chemical shifts of the PALP protons is summarised in figure 3. The order of metal induced perturbations is CH₂(5') > CH(6) > CH(4') > CH₃(2') for complex I and CH₃(2') > CH₂(5') > CH(6) for complex

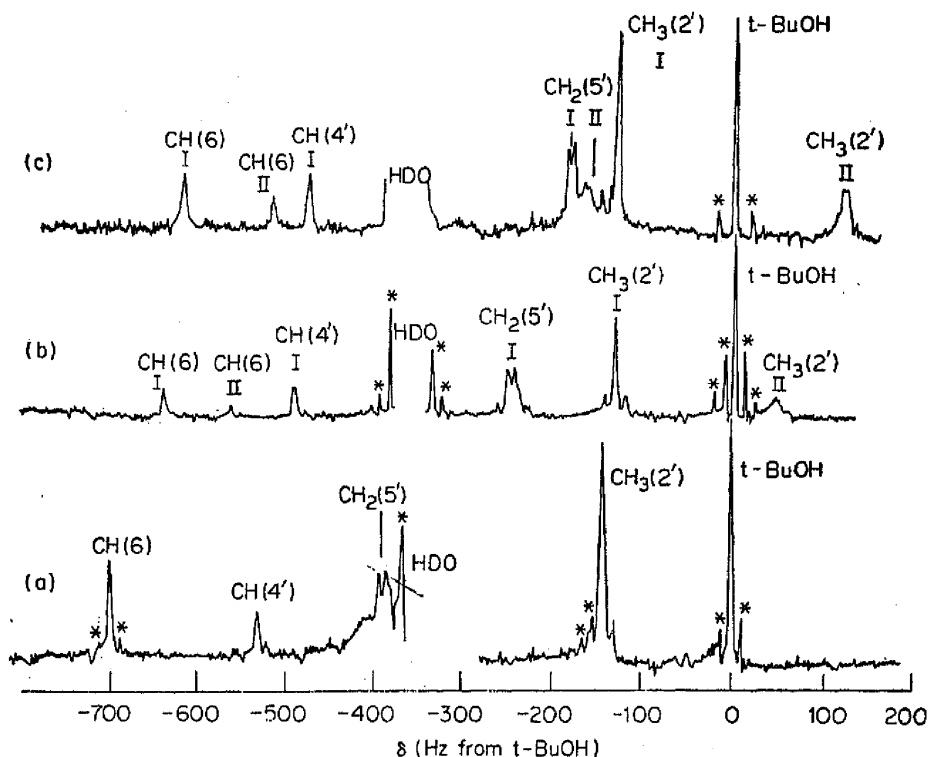


Figure 2. NMR spectra (100 MHz) of (a) 66 mM PALP, (b) 66 mM PALP with addition of 244 mM Eu(III), (c) 66 mM PALP with addition of 344 mM Eu(III) at pH ~ 2.

II. No resonance assignable to CH (4') of complex II could be detected, suggesting a marked broadening of this peak due to proximity of the metal.

In figure 4, a reaction scheme to account for these results is presented. Complexes formed by metal binding to the phosphate and the *o*-hydroxyaldehyde

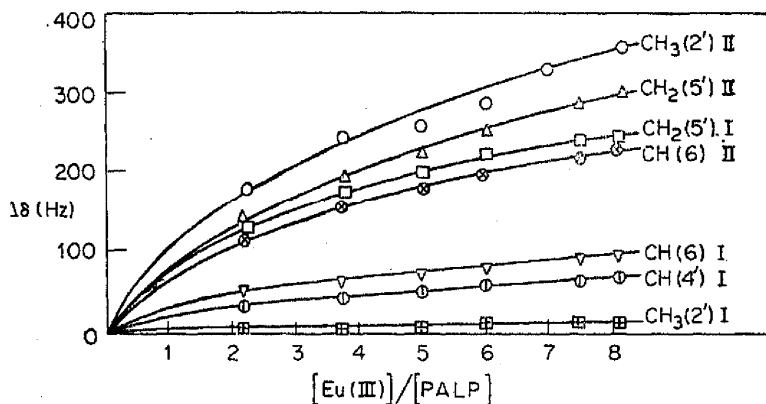


Figure 3. A plot of induced shifts of PALP protons on addition of Eu(III) against $[Eu(III)]/[PALP]$.

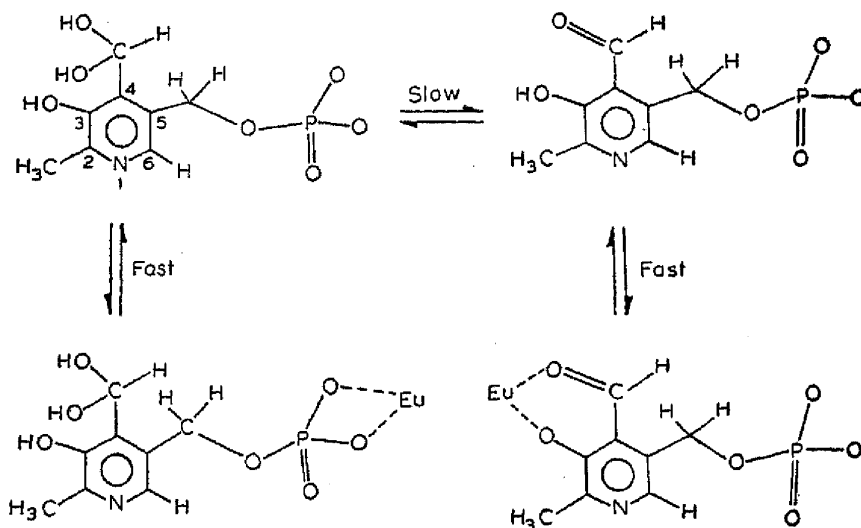


Figure 4. Reaction scheme for the interconversion of Eu(III)-PALP complexes.

moieties are present and slow exchange results from the hydration-dehydration step, which is rate determining. The concentration of complex II increases with increasing Eu(III), suggesting that the free aldehyde is summarized by metal binding. The observations are of particular interest as both enzymatic and nonenzymatic reactions of PALP require Schiff's base formation via the free aldehyde. Bell *et al.*, (1956) have studied the aldehyde-diol equilibrium for $\text{CH}_3\text{CHO} \rightleftharpoons \text{CH}_3\text{CH}(\text{OH})_2$ and have obtained rate constants of $0.1-1 \text{ s}^{-1}$.

The effect, of adding increasing concentrations of EuCl_3 to an 80 mM solution of PAMP, is shown in figure 5. In this case, only the complex corresponding to binding at the phosphate group is possible, and only one set of resonances is observed. The resonances at -151Hz , -327Hz , -394Hz and -714Hz , which are assigned to $\text{CH}_3(2)$, $\text{CH}_2(4)$, $\text{CH}_2(5)$, $\text{CH}(6)$ respectively, have induced shifts in the order $\text{CH}_2(5) > \text{CH}_2(4) > \text{CH}(6) > \text{CH}_3(2)$ as shown in figures 5b and 5c. The effect of increasing Eu(III) concentration on the PAMP resonances are summarized in figure 6.

Calculation of shift ratios

Figures 7 and 8 show the plots of experimental ^1H shift ratios against Eu(III) concentration for PALP and PAMP respectively. Theoretical computations of shift ratios were carried out for PALP-Eu(III) (complex I) and PAMP-Eu(III) complexes fixing the metal at the phosphate group and using the conformational search method (see methods). For the PALP-Eu(III) complex II, the metal search method was adopted to determine the metal position and the magnetic axis and shift ratios were calculated varying χ^1 .

The results of computations for PALP-Eu(III) complex I, are tabulated in table 1. The angles (χ 's) given in the table are typical of the family of conformations for which the computed shift ratios are in good agreement with the experimental values within the tolerance limits. Tolerance limits of 20% $\text{CH}_3(2)$, 7.5 % $\text{CH}_2(4)$ and 6% $\text{CH}(6)$ have been used, The dihedral angles χ^1 , χ^2 and

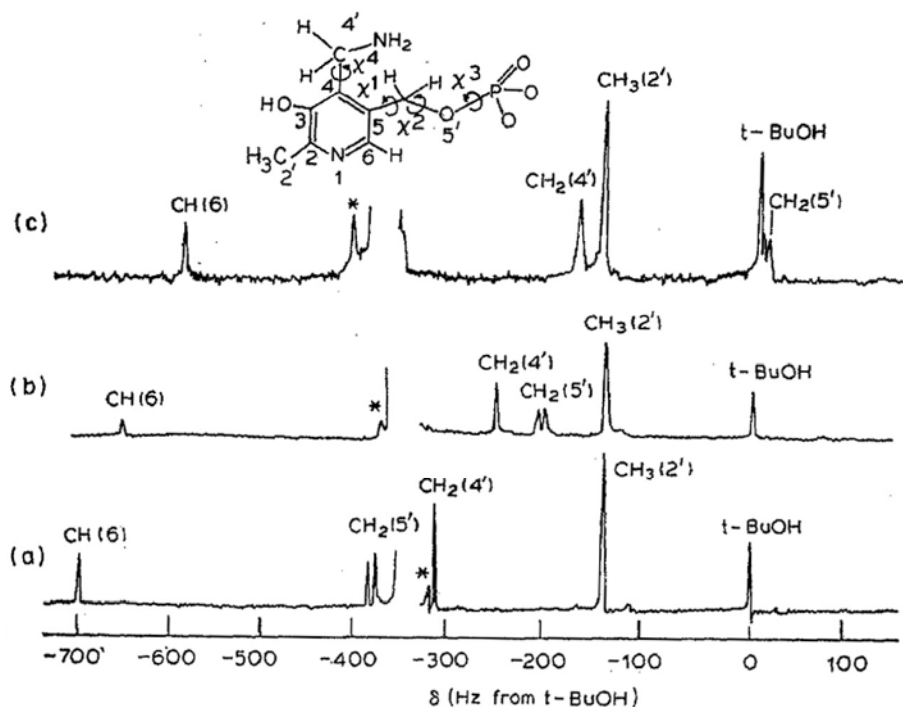


Figure 5. ^1H NMR spectra (100 MHz) of (a) 80 mM PAMP, (b) 80 mM PAMP and 240 mM Eu(III), (c) 80 mM PAMP and 984 mM Eu(III) at pH \sim 2.

χ^4 have an angular spread of $\pm 30^\circ$ about the mean values of 200° , 180° and 90° respectively. The value of χ^3 has a spread of about 30° around the staggered conformations. However, the definition of χ^3 is arbitrary and for convenience, the oxygen atom of the phosphate not bound to the metal is used. It should be noted that in the absence of metal the three oxygens are equivalent. While the

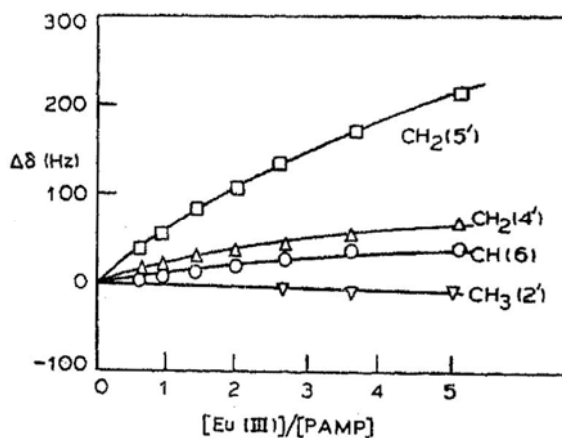


Figure 6. A plot of induced shift for PAMP protons on addition of Eu(III) against $[\text{Eu(III)}]/[\text{PAMP}]$

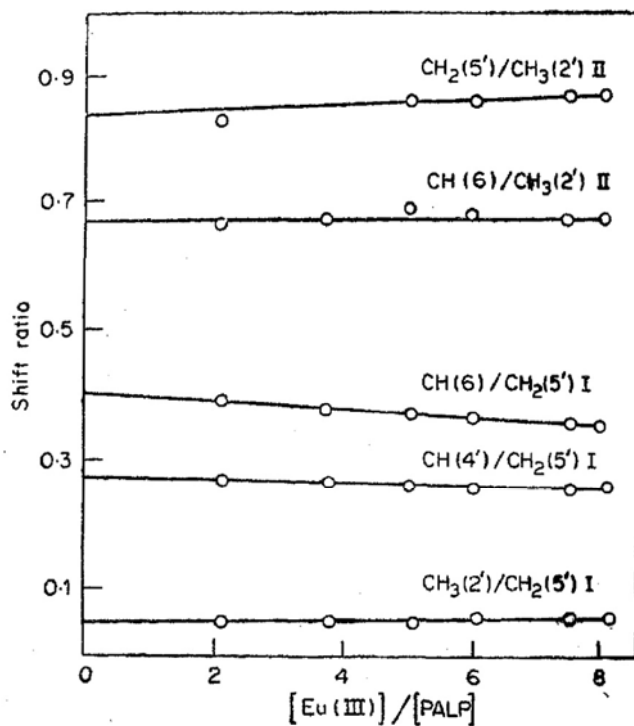


Figure 7. Shift ratios obtained from experimental data for various protons of PALP, for both complexes I and II, as a function of $[\text{Eu(III)}]/[\text{PALP}]$.

angles χ^1 and χ^2 are in good agreement with the crystal structure values, however, χ^4 has a value of 90° instead, of the observed 0° in the crystal structure (Fujiwara, 1973). Since the NMR method yields only a dynamically averaged structure, it is likely that the observed shift ratios may represent average values.

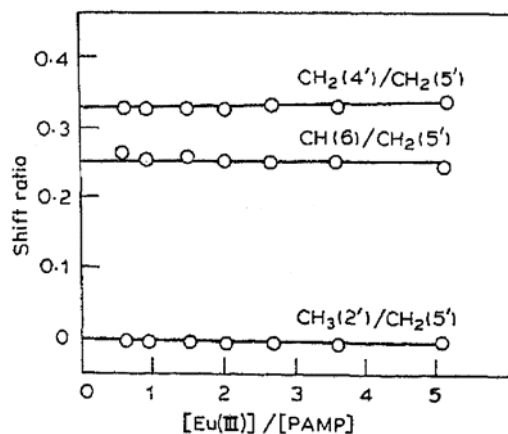


Figure 8. Shift ratios obtained from experimental data for various protons of PAMP as a function of $[\text{Eu(III)}]/[\text{PAMP}]$.

Table 1. Calculated shift ratios^a of ¹H resonances for PALP-Eu(III) complex I by conformational search method.

Conformational angles ^b (in degrees)				Shift ratios ^c			R%
χ^1	χ^2	χ^3	χ^4	CH ₃ (2')/ CH ₂ (5')	CH(4')/ CH ₂ (5')	CH(6')/ CH ₂ (5')	
170	170	30	80	0.046	0.273	0.409	0.9
160	200	340	90	0.042	0.283	0.417	2.7
200	180	300	120	0.057	0.275	0.413	1.5
260	170	30	110	0.051	0.275	0.405	1.0

^a The experimental shift ratios are CH₃(2') 0.05, CH(4') 0.275, and CH(6') 0.41, normalized with respect to CH₂(5').

^b Angles quoted here are representatives of the family of conformations for which the calculated shift ratios are in agreement with experimental values; conformations with $\pm 20^\circ$ around these values also satisfy the shift ratio criteria within the tolerance limits.

^c Tolerance limits used were : CH₃(2') 20%, CH(4') 7.5% and CH(6') 5%

In table 2, the computed shift ratios for PAMP and the conformational parameters are presented. In this case, the side chain conformational angles show relatively smaller variations about their mean values of $80^\circ (\pm 40)$ for χ^1 , $145^\circ (\pm 5)$ for χ^2 and $50^\circ (\pm 5)$ for χ^3 , respectively. Agreement with observed shifts are obtained for χ^4 values close to 20° and 100° as against the crystal structure value of -60° . Tolerance values of 5% for CH₂(4') and 7.5% for CH(6) were used. The 2'(CH₃) showed negligible shifts in the presence of metal. It may be noted that for both PALP complex I and PAMP-Eu(III) complex, the value of χ^2 is close to that expected for a *trans* arrangement of C(5) and P about the C(5')-(5') bond.

The shift ratio calculations for the PALP-Eu(III) complex II was carried out by the metal search method (see methods). The results are presented in table 3. There is a good agreement between the calculated and the observed shift ratios for two positions of the metal atom with respect to the plane of the pyridine ring. In both the positions the metal is displaced by about 1.5 Å. Correspondingly, there are two values of χ^1 ($\chi^1 = 140^\circ$ and 220°) for which the calculated shift ratios of CH₂(5') protons are in good agreement. It is interesting to note that when the metal was fixed in the plane of the pyridine ring, agreement could not be obtained despite the fact that there are only two shift ratios available for fitting the data. Good agreement between the calculated and the experimental shift ratios could be obtained when the metal was displaced from the plane containing the atoms O(3)-C(3)-C(4)-C(4)-O(4) and for a value of $\chi^1 = 140^\circ$ or 220° .

While these calculations for complex II consider only a 1 : 1 PALP-Eu(III) complex, it is possible that 1 : 2 PALP-Eu(III) complexes, with the metal binding at both sites, need be considered. However, the good agreement obtained for

Table 2. Calculated shift ratios^a of ¹H resonances of PAMP-Eu(III) complex by conformational search method.

Conformational angles ^b (in degree)				Shift ratios ^c			
χ^1	χ^2	χ^3	χ^4	CH ₃ (2')/ CH ₂ (5')	CH ₂ (4')/ CH ₂ (5')	CH(6)/ CH ₃ (5')	R%
125	145	50	20	0	0.339	0.254	0.9
80	140	50	90	0	0.323	0.264	3.5
45	140	50	110	0	0.343	0.249	2.3

a The experimentally observed shift ratios are : CH₃(2') 0.0, CH₂(4') 0.335 and CH(6) 0.255, normalised with respect to CH₂(5').

b Conformational angles of $\chi^1 \pm 20^\circ$ and $\chi^4 \pm 20^\circ$ also satisfy the experimental shift ratios: χ^2 and χ^3 show variations of only $\pm 5^\circ$.

c Tolerance limits used: 6% for CH₂(4') and 7.5% for CH(6). Note that induced shift for CH₃(2') is zero.

calculated and experimental shift ratios suggests that at the concentrations of metal used, the formation of 1 : 2 complexes is unlikely.

In fitting the observed pseudocontact shifts for PALP-Eu(III) complex I, effectively only three shift ratios have been considered. This raises the question as to whether the NMR data are sufficiently sensitive to changes in the side chain orientation and whether it would be possible to fit the observed data to stereochemically unfavourable structures. Consequently, the hard sphere contact

Table 3. Calculated shift ratios^a of ¹H resonances for PALP-Eu(III) complex II by metal search method.

Parameters for metal position ^b						Shift ratios ^c		
<i>r</i> (Å)	θ (°)	ϕ (°)	α (°)	β (°)	χ^1 (°)	CH(6)/ CH ₃ (2')	CH ₂ (5')/ CH ₃ (2')	R%
2.8	110	138	214	10	220	0.707	0.835	2.0
2.8	110	42	214	170	140	0.713	0.859	2.2

a The experimental shift ratios are : CH(6) 0.69 CH₂(5') 0.85, normalised with respect to CH₃(2') of complex .

b The values of *r*, θ , ϕ , α and β are typical of the values of corresponding to a family of metal positions for which the calculated shift ratios are in agreement with experiment.

c Tolerance limits used were 4% for both CH(6) and CH₂(5') shift ratios.

model (see methods) was used to test the "stereochemical allowedness" of the conformations. It was found that the conformations that were acceptable by the NMR criterion were also stereochemically acceptable. Further, regions of conformational space that yielded unfavourable interatomic contacts also did not give good agreement with experimental findings. This provides convincing evidence for the ability of the NMR shift ratios to filter out unfavourable conformations of PALP. Similar results were obtained for PAMP. Despite the conformational flexibility of the side chains, inherent in these molecules, only a limited range of values yielded shift ratios in agreement with experiment.

Similar calculations for complex II of PALP-Eu(III) yielded good agreement with observed values only when the metal was displaced from the plane of the atoms O(3)-C(3)-C(4)-C(4)-O(4), by as much as 1.0-1.5 Å. It is interesting to compare the results with the planarity of the Schiff's bases chelated to different metal ions like Cu²⁺, Zn²⁺, Ni²⁺ and Mn²⁺, whose crystal structures have been determined. Copper ion having a mean coordination bond of <2Å occupies the centre of a nearly planar tetradentate ligand (Cutfield *et al.*, 1967; Bentley *et al.*, 1968). The increased coordination bond lengths in Ni²⁺, Zn²⁺ and Mn²⁺ (Capasso *et al.*, 1974; Willstadter *et al.*, 1963) results in increased ring strain which determines the puckering of the Schiff's bases. In the crystal structure of pyridoxylidene-valine-manganese(II), with a mean coordination length of 2.16 Å, the mean planes of pyridine and valinate rings formed on chelation of the metal by the Schiff base have a dihedral angle of about 150° (Willstadter *et al.*, 1963). The calculated shift ratios for PALP-Eu(III) complex II were in agreement with the experimental values for χ^2 values of 140° and 220°. These two values correspond to the two positions of Eu(III) atom with respect to the plane of the pyridine ring. For agreement of the CH₂(5') shift ratio, the oxygen atom O(5') of the side chain has to be on the opposite side of the plane of the pyridine ring, as compared to the metal atom. This orientation results in the hydrogens at C(5') being placed on the same side as the metal atom. While, the difficulty in observing CH(4') resulted in the availability of only two shift ratios, the sensitivity of these two values to metal position suggest that the structures derived may, indeed, be reliable.

In both PALP and PAMP, the lanthanide induced shift (LIS) method leads to families of closely related conformations of flexible side chains at C₅. Interestingly, the conformations which are allowed by the NMR method are also stereochemically acceptable. The values of χ^1 and χ^2 obtained from crystal structures for PALP and PAMP are in good agreement with the values obtained by the LIS method, in solution. This, is particularly significant as no assumptions were made in this study regarding the conformations of the side chain, in the calculations of the NMR shift ratios.

Recently, the limitations of the LIS method in conformational analysis have been the subject of some discussion. Sullivan (1976) has shown that the LIS method fails to discriminate the diastereomeric derivatives of isoborneol which differ in configuration, in the freely rotating portion of the molecule. Gerald and Williams (1978) in their study on 5'-AMP and Inagaki *et al.* (1978) in their study on 5'-UMP have shown that the conformations obtained from the LIS method are not consistent with those derived experimentally, from NMR-spin-spin coupling constants and Nuclear Overhauser Effect measurements. A reasonable

agreement between the shift data and other experimental data are obtained only by combining different conformers with a suitable weighting scheme.

Despite these limitations, preliminary shift ratio calculations for PALP, at 20° intervals for χ^1 , χ^2 and χ^3 with large tolerances did neither yield stereochemically unfavourable structures for complex I, nor, agreement for complex II when the metal was fixed in the plane of the pyridine ring.

The work on PALP and PAMP described in this paper establish the presence of two PALP-Eu(III) complexes which are in slow exchange on the NMR time scale. Increasing concentrations of Eu(III) shifts the equilibrium towards the free aldehyde form, which is essential for metal binding. The slow step in the exchange between the two complexes is likely to be the hydration-dehydration reaction at the aldehyde carbonyl group. The pseudocontact shift ratio calculations for complex I lead to a family of solutions, which are stereochemically favourable. In complex II with the metal coordinated to the hydroxyl and aldehyde carbonyl groups, the best fit to NMR data is obtained when the metal is displaced from the plane of the pyridine ring. PAMP has only one metal binding site at the phosphate group and yields a family of closely related solutions, which are similar to those obtained for the related PALP-Eu(III) complex-

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