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UTILIZING AN INTRINSIC ^{31}P NMR PROBE IN STUDIES OF COBALAMIN LIGATION

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SUMMARY: Phosphorous 31 nuclear magnetic resonance spectroscopy has been employed to observe changes in axial ligation in hydroxycobalamin solutions. The naturally occurring phosphorous nucleus in the benzimidazole side "arm" of the corrin is shown to exhibit different chemical shifts depending upon whether the benzimidazole is coordinated to the cobalt(III) ion or whether it has been displaced. The phosphorous resonance's linewidth at half maximum peak height seems also to be an indicator of the cobalt oxidation state, exhibiting a twenty fold larger width in the cobalt(II) complex. The implications of these findings for future studies on certain coenzyme B_{12} dependent enzymes is discussed.

Nuclear magnetic resonance (nmr) studies of enzymes and ligand binding proteins with specific chemical functions (hemoglobin, myoglobin) rely upon the presence of a spectroscopically detectable reporter group at their active sites (1-4,6,7). Although many classes of enzymes have come under scrutiny by nmr at least one important group has not; those enzymes which depend upon adenosyl cobalamin (coenzyme B_{12}) as a cofactor necessary for fulfilling their enzymatic function. Adenosyl cobalamin, Figure 1, is a cobalt-corrin macrocycle complex in which the cobalt normally appears in the diamagnetic +3 oxidation state (low spin d^6), with both the paramagnetic +2 (d^7) and diamagnetic +1 (low spin d^8) states accessible under moderate conditions (8). The paucity of nmr data on B_{12} enzymes results, in part, from the high molecular weights encountered in most members of the group and this, in turn, renders attainment of satisfactory concentrations suitable for current low field nmr sensitivity levels quite difficult to achieve.

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Moreover, until now such studies were further hampered by a lack of active site reporter groups. There does exist, however, at least one subgroup of the B_{12} dependent enzymes which possess sufficiently low molecular weights (approximately 76,000 Daltons) so as to be amenable to nmr studies provided that a suitable nmr probe is found. These enzymes are the coenzyme B_{12} dependent ribonucleotide reductases (9). This communication presents the initial aspects of an inquiry into the potential for employing ^{31}P nmr as a probe of events occurring at the corrin site in such enzymes.

METHODS AND MATERIALS: Hydroxycobalamin was purchased from Sigma and in general was used without further purification. As an index of sample purity portions of each 1.0 gram sample obtained from the supplier were chromatographed (Biogel) and samples showing a high degree of inhomogeneity were further purified by column chromatography. All samples exhibited single resonance ^{31}P and ^{13}C (for $^{13}CN^-$ complexes) nmr spectra. Deuterium oxide (Merck) was once distilled under nitrogen, then stored under nitrogen in sealed bottles prior to its use as the solvent.

Reduction of hydroxycobalamin (Co(III)) to Cob(II)alamin was carried out anaerobically, under a nitrogen atmosphere employing d,l-penicillamine (Aldrich). These samples were transferred to sealed nmr tubes and maintained under a nitrogen atmosphere throughout the nmr experiment. The presence of cobalt +2 was inferred from optical spectra taken from aliquots of the nmr samples which were carefully transferred into degassed optical cells (Figure 3). Optical spectra were obtained using a Cary 219 spectrometer.

NMR spectra were obtained employing a Nicolet NTC 150, widebore, superconducting spectrometer system operating at 3.523T (60.7444 MHz). Twelve millimeter sample tubes were used and pHs were carefully monitored before and after each spectrum with a Corning 112 meter. In general between 500 and 10,000 transients were collected over a 2.5 KHz spectral width consisting of 8192 data points employing quadrature phase detection and two level proton decoupling. 19 usec 90° pulses with a total recycle time of 3.0 sec were employed, and unless otherwise specified, all data were collected at 23°C. 85% phosphoric acid was used as an external chemical shift reference.

RESULTS AND DISCUSSION: Cobalamin derivatives, Figure 1, all possessing the nucleotide (benzimidazole) side arm, which covalently links the axially coordinated benzimidazole to the corrin, are known to function as coenzymes for a bacterial ribonucleotide reductase in Lactobacillus leichmannii. This enzyme has been well characterized

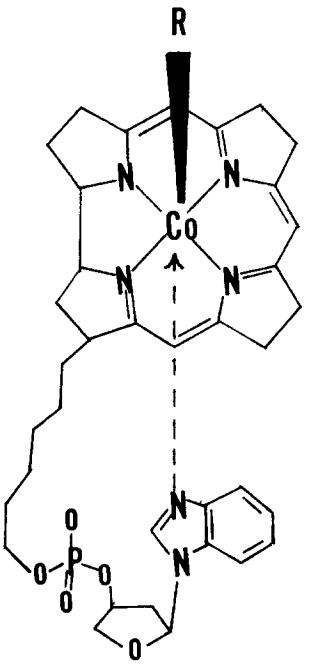

Figure 1. Skeletal figure of a cobalamin showing the corrin macrocycle and the phosphorous containing benzimidazole side "arm" coordinated to cobalt (III). In coenzyme B_{12} R=ribonucleotidyl moiety; in hydroxycobalamin $R=OH^-$.

and studied in some detail (9,10). The finding that the benzimidazole side arm is probably required for proper catalytic functioning (9) suggested that the naturally occurring phosphorous nucleus contained in the side arm would be a potential non-invasive nmr probe of the corrin site. Figure 2 shows a proton decoupled ^{31}P nmr spectrum of a 1.2 mM solution of hydroxycobalamin, a concentration which should be easily attainable with these enzymes. This spectrum demonstrates that the sensitivity characteristics of state-of-the-art spectrometers will allow data accumulation on these types of proteins in reasonable time periods. Although this spectrum was obtained in water, identical spectra have been observed in buffer systems at similar pHs.

The results of initial ligation studies with hydroxycobalamin are shown in Table 1, where it is demonstrated that the ^{31}P

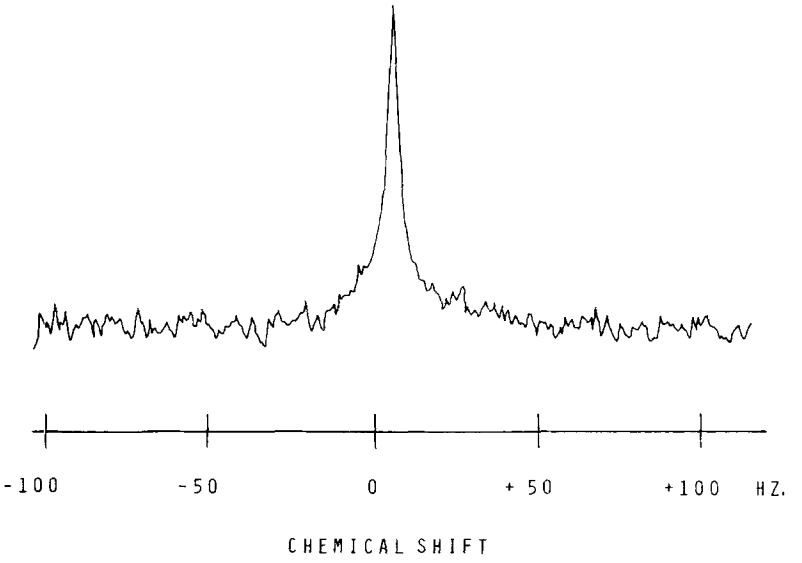

Figure 2. A ^{31}P nmr spectrum of a 1.2 mM solution of hydroxycobalamin, pH = 8.3, unbuffered. This spectrum required 3000 transients collected at 23°C employing a NTC 150 spectrometer. On the chemical shift scale 0.0 Hz is the frequency of external 85% H_3PO_4.

Table 1. Phosphorous 31 nmr data for several cobalamin complexes.

COMPOUND	CONCENTRATION (mM)	CHEMICAL[1] SHIFT (Hz)	OBSERVED[2] LINE WIDTH (Hz)	pH[3]
Hydroxycobalamin	1.2	+6.12	5.0	8.30
^{13}C-Cyanocobalamin[4]	1.2	+4.79	5.8	8.09
^{13}C-Dicyanocobalamin	1.2	+29.9	5.6	8.18
Hydroxycobalamin + d,l-penicillamine	1.2	+4.48	140.0	8.28

1. The chemical shift is reported in Hz relative to external 85% phosphoric acid with an accuracy of ± 1.0 Hz. Positive signs indicate a shift upfield from the reference. Complete proton decoupling was employed throughout. 2. The observed linewidths are uncorrected for broadening due to field inhomogeneity, although this contribution is smaller than 1 Hz in all cases as determined by linewidth measurements on the phosphoric acid reference. 3. PH's were measured before and after data accumulation and found to agree within 0.05 pH units. 4. The state of cyanide ligation in the cyano- complexes was unequivocally established by direct observation of the ^{13}C nmr employing $^{13}CN^-$.

resonance is sensitive to the cobalt ligation and oxidation state. In the former case it is found that the ^{31}P resonance exhibits different chemical shifts depending upon whether the benzimidazole is coordinated to the cobalt, as in hydroxycobalamin and monocyanocobalamin, or dissociated from the cobalt as in dicyanocobalamin. This correlates with two environments being available to the side arm: "arm on" (benzimidazole coordinated to Co(III)) and "arm off" (benzimidazole dissociated).

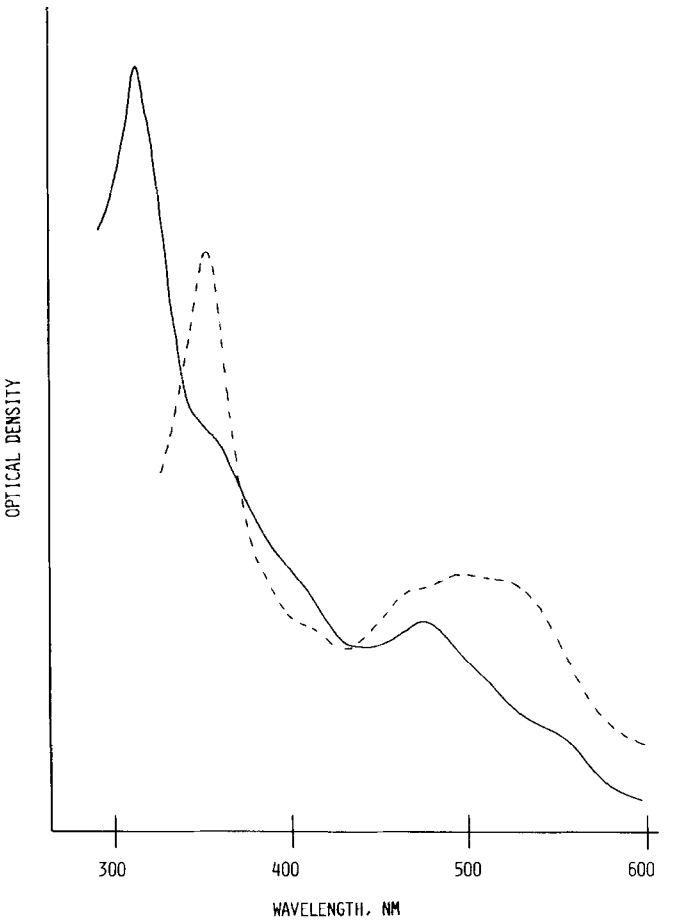

Figure 3. The optical spectrum of oxygen free aqueous solutions of hydroxycobalamin before (- - -) and after (——) addition of d,l-penicillamine demonstrating the production of the reduced Co(II) cobalamin spectrum. The spectrum of the reduced form presented here (——) is that of an aliquot taken from an nmr sample.

Besides demonstrating chemical shift differences this study reveals that the reaction between hydroxycobalamin and the thiol d,l-penicillamine results in production of a much broadened ^{31}P resonance. It is known that thiols function as reducing agents for cob(III)alamins, producing cob(II)alamins, and because paramagnetic cobalt(II) complexes have been shown to possess relatively broad resonances (11) it is inferred that the broadening of the ^{31}P resonance is indicative of cob(II)alamin.

This conclusion is supported by the optical spectra which are presented in Figure 3. This figure reveals that when aliquots of the nmr samples were diluted in degassed solutions and maintained under anaerobic conditions the characteristic cob(II)alamin optical spectra were obtained.

Further studies of cobalamin ligation incorporating many different ligands are now being carried out utilizing both ^{31}P and ^{13}C nmr and optical spectroscopy. These will be reported in the near future.

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