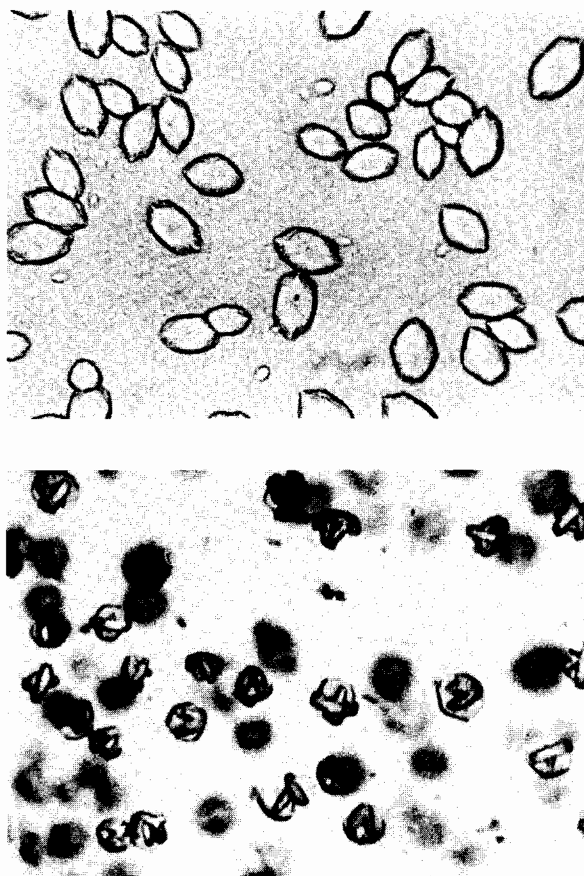

Fig. 1. Crystals of horse heart ferritin grown from MPD (a), and from polyethylene glycol (b). In both cases the longest dimensions of the crystals are approximately 0.15 mm.

The dimensions of the heart ferritin crystals obtained so far are too slight to allow any crystallographic investigation. All the crystals obtained are isotropic under the polarizing microscope, and thus belong to a cubic space group. This observation is in keeping with the molecular symmetry of the ferritin oligomer, which, in the case of the spleen protein, is coincident with crystallographic symmetry. Details of the crystalline forms isolated as well as of their growth solutions will be presented.

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O10

C-13 and P-31 NMR Studies of ^{13}CN-Cyanocobalamin in Sulfuric Acid–Water Mixtures

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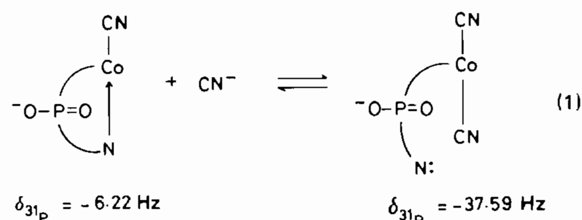
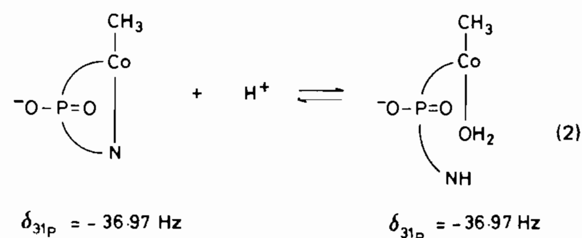
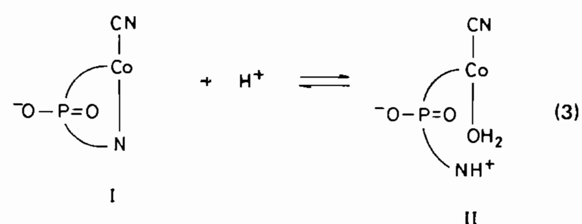
In a previous study [1] we observed, in agreement with Satterlee [2, 3] an upfield shift of 31.4 Hz of the chemical shift of the phosphorus atom of the nucleotide loop of cyanocobalamin (CNCbl) upon displacement of the axial benzimidazole ligand by excess cyanide (Eqn. 1). However, we observed no change in chemical shift for methylcobalamin (CH_3-



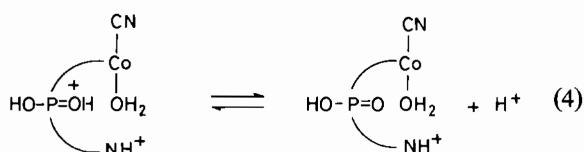
Cbl) upon displacement of axial benzimidazole by protonation (Eqn. 2, pK_a = 2.89) although further

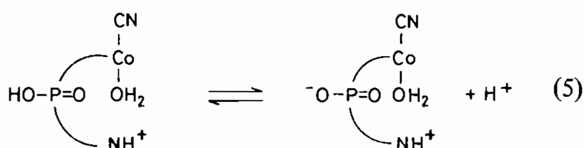


lowering the pH causes an upfield shift of the phosphorus resonance due to protonation of the phosphodiester [1]. We consequently have attempted to determine if a change in phosphorus chemical shift occurs upon displacement of the axial base of CNCbl by protonation (Eqn. 3, pK_a = 0.1 [4]).



In sulfuric acid–water mixtures the ^{13}C NMR spectrum of ^{13}CNCbl shows two resonances at all H_o values [5–7] between +1.0 ($[H_2SO_4]$ = 0.0732 *M*) and –0.5 ($[H_2SO_4]$ = 1.196 *M*), one at 111.77 ppm ($w_{1/2}$ = 10.4 Hz) and one at 121.40 ppm ($w_{1/2}$ = 22.2 Hz). As the intensity of the former increases while that of the latter decreases with increasing acidity, they are assigned to the base-off and base-on species, respectively. Similarly, the ^{31}P NMR spectrum of CNCbl shows two well defined resonances ($w_{1/2}$ *ca.* 2.0 Hz) separated by about 30 Hz with the downfield member decreasing in intensity (base-on) while the upfield member increases in intensity (base-off) with increasing acidity in this acidity range. However, in this case, the chemical shifts of both resonances move upfield with increasing acidity due to phosphodiester protonation. Plots of the ^{31}P-chemical shift of the base-off species *vs.* H_o clearly show evidence of two sequential protonations which must be assigned to the first (Eqn. 4) and second (Eqn. 5) phosphodiester pK_a's. The complete ionization scheme for CNCbl thus includes eight microscopic species and 12 microscopic pK_a's from





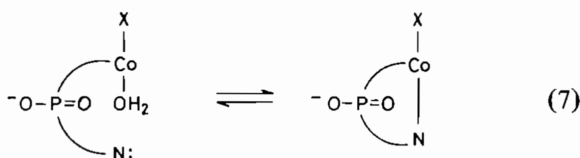
which analytical equations for the dependence of the ^{31}P-chemical shifts of the base-off and base-on species as well as the dependence of the fraction of base-on species present (*i.e.*, $\alpha_{base\text{-}on}$, evaluated from the relative integrals of the ^{31}P and/or ^{13}C resonances) upon acidity may be derived. A non-linear least squares fit of the base-off ^{31}P-chemical shifts to such an equation using H_o gives a poor fit, particularly at higher acidities. Although the fit is considerably improved by use of the H_A (amide) acidity function [8–10] which is known to be applicable to many compounds which protonate at doubly bonded oxygen [11–17], an exact fit can be obtained by use of the Cox and Yates [18] generalized acidity function treatment (Eqn. 6, where C_{H^+} is the concentration of hydrogen ion and X is the 'excess acidity')

$$-H = m^*X + \log C_{H^+} \quad (6)$$

using m^* = 0.217. This treatment gives values for the macroscopic pK_a's for phosphodiester deprotonation of the base-off species of –1.57 and –0.04 and chemical shifts of –335.81 Hz and –37.40 Hz for the base-off phosphodiester protonated and deprotonated species (II in Eqn. 3), respectively. A similar treatment of the ^{31}P chemical shifts of the base-on species does not yield reliable values for the phosphodiester pK_a's as this species can only be observed at acidities less than 3.14 *M* in H_2SO_4 ($\alpha_{base\text{-}on}$ is 0.033 at this acidity). Only the chemical shift of the base-on, phosphodiester deprotonated species (I in Eqn. 3) is reliably determined to be –1.72 Hz. Hence an upfield shift of 35.7 Hz is seen for the ^{31}P-chemical shift upon displacement of the axial base of CNCbl by protonation (Eqn. 3).

A similar attempt to correlate $\alpha_{base\text{-}on}$ with acidity to determine the three macroscopic pK_a's for the overall system fails as the standard deviation of the fit shows little or no variation with m^* (Eqn. 6) and the final fit parameters at a given m^* are sensitive to the inital guesses indicating that they are poorly determined by the data. Interestingly, at m^* = 0.919 (Eqn. 6) the $\alpha_{base\text{-}on}$ data strongly resembles the titration of a single protonable group, a plot of log $(\alpha/1 - \alpha)$ *vs.* H (m^* = 0.919) giving a good straight line with a slope of 0.993 ± 0.013 and an apparent pK_a of 0.11 ± 0.01. This may indicate that the base-on-base-off pK_a is fairly insensitive to the state of protonation of the phosphodiester and that the literature value of 0.1 [4] is a good estimate for this pK_a.

The lack of an upfield shift of the phosphorus resonance of CH_3Cbl upon displacement of the axial base by protonation, in contrast to CNCbl, must surely be due to differences in the magnetic environments of the phosphorus atoms of the base-on species, as the chemical shifts of the two base-off species are essentially identical. There is a considerable difference in affinity of the free-base benzimidazole ligand for the cobalt atom in these two cobalamins. Using a value of 5.56 for the pK_a of α-ribazole (the detached benzimidazole ribonucleoside) [19] the equilibrium constants for formation of the base-on species (Eqn. 7) may be calculated to be 467 for CH_3Cbl and 2.88 × 10^5 for CNCbl. As phosphodiester ^{31}P-chemical shifts are known to be sensitive



to O–P–O bond angles and C–O–P–O torsion angles [20–22] it seems likely that the geometry about the phosphodiester phosphorus atom is distorted, relative to the base-off species in the tightly coordinated base-on CNCbl but it is not distorted in the much more loosely coordinated base-on CH_3Cbl. Existing

X-ray crystal structures of base-on CNCbl [23] and 5'-deoxyadenosylcobalamin [24] (the CH_3Cbl structure has not been determined) support a difference in phosphodiester geometry between CNCbl and an alkylcobalamin.

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O11

Inhibition of Human Carbonic Anhydrase II by Anions and some 'Neutral' Compounds

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Anions inhibit carbonic anhydrase-catalyzed reactions by binding to the zinc ion in the active center. The pH dependence of the inhibition of the esterase activity shows that anions predominantly bind to enzymes having a protonated catalytic group. Formally, anion binding can be described as a competition with OH^- for a coordination site on the metal ion [1].

Pocker and Deits [2] recently showed that anions inhibit the CO_2 hydration catalyzed by bovine carbonic anhydrase at high pH values in an uncompetitive fashion, and they presented a kinetic scheme to explain this phenomenon.

We have studied the anion inhibition of human carbonic anhydrase II (or C). We have confirmed the

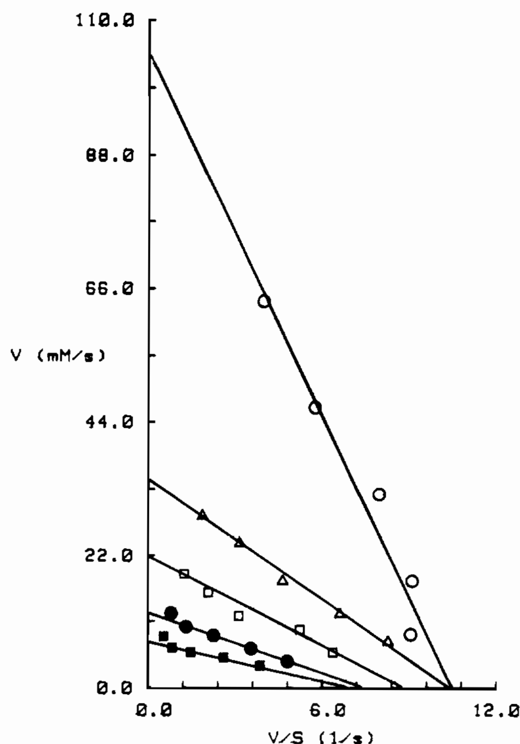

Fig. 1. Inhibition by N_3^- of CO_2 hydration catalyzed by human carbonic anhydrase II at pH 8.9, 25 °C. Buffer: 50 m*M* 1,2-dimethylimidazole-H_2SO_4 with Na_2SO_4 to yield an ionic strength of 0.2 *M*. Inhibitor concentrations: (○), 0 m*M*; (△), 1 m*M*; (□), 5 m*M*; (●), 10 m*M*; (■), 30 m*M*.