The Chemistry of Vitamin B_{12}. Part 20.[1] Diaquocobinamide: pK Values and Evidence for Conformational Isomers

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An improved method for preparing aqueous solutions of diaquocobinamide without hydrolysis products is described. The pK_a values have been determined in 0.2 mol dm^{-3} $NaClO_4$ at 25 °C as pK_1 = 5.9 ± 0.1 and pK_2 = 10.3 ± 0.2 and are shown to involve one proton each. Evidence is presented that diaquo-, aquohydroxo-, and dihydroxo-cobinamide exist in solution as isomers depending on hydrogen bonding between the axial ligands and different amide side-chains.

Diaquocobinamide (dac) can be regarded as the 'parent' complex of the corrinoids and the determination of equilibrium constants for the binding of ligands to dac is important both for analysing metal–ligand interactions within the corrinoid family and for comparing corrinoids with other non-corrinoid Co^{III} complexes. However, because the only commercially available corrinoids are cobalamins and the preparation of dac is rather inconvenient, very little work has been reported on dac, ahc, and dhc,† which are connected by equilibrium (1) (only axial ligands are shown). The pK_1 of dac has been determined as 6.0 but only an approximate value has been reported for pK_2 and the number of protons has not been determined.[2,3]

$$H_2O\text{-}Co\text{-}OH_2 \rightleftharpoons HO\text{-}Co\text{-}OH_2 + H^+ \rightleftharpoons HO\text{-}Co\text{-}OH + 2H^+ \quad (1)$$

$$H_2O\text{-}Co\text{-}CN + H_2O \rightleftharpoons H_2O\text{-}Co\text{-}OH_2 + CN^- \quad (2)$$

The main problem in the preparation of dac stems from the fact that removing the cobalamin side-chain from vitamin B_{12}, whether by hydrolysis in concentrated HCl [4] or with cerium(III) hydroxide in the presence of cyanide [5] yields cyanoaquocobinamide (Factor B) in which the cyanide is very firmly held; for equilibrium (2), $\log_{10}K \leqslant -14$.[2] The cyanide can be displaced by photolysis but, in order to prevent rapid recombination and to displace equilibrium (2) to the right, the photolysis must be carried out in acid in a stream of N_2 gas to remove the HCN formed; [2] we have found that acid conditions always cause some hydrolysis of the amide side-chains, as shown by t.l.c. The cyanide can also be displaced from Factor B by reduction (*e.g.* with $NaBH_4$) to the Co^I complex which reacts rapidly with MeI to give methylcobinamide; this can easily be purified and readily photolysed in the presence of air to give dac.[5,6] Unfortunately, photolysis involves free radicals which, in the presence of air, produce stable yellow corrinoids, as shown by increased optical density at *ca.* 455 nm.[7a,8] Attempts to avoid acid conditions by photolysing Factor B in the presence of $AgNO_3$ or precipitated Ag_2O were also unsuccessful. We have, therefore, devised a simple apparatus (see Experimental section) for simultaneously increasing the rate of photolysis of Factor B with an ordinary tungsten lamp while reducing the rate of hydrolysis; this converts aqueous solutions of Factor B into dac in a single step without detectable levels of hydrolysis products.

We have previously termed those equilibria of corrinoids which involve changes in the nature of the axial ligands (including their relative orientation) 'major' equilibria, in contrast to the 'minor' equilibria involving steric, hydrogen-bonding, and hydrophobic interactions of the corrin ring and the axial ligands, and we have pointed out that the major equilibria may be split into a set of minor equilibria involving variants or sub-species of the main complexes which differ in, for example, their hydrogen-bonding interactions.[9] Good examples of the effect of minor equilibria on the major equilibria are provided by (*a*) the cyanoaquocorrinoids possessing carboxylic acid side-chains which have been studied by Friedrich and co-workers,[7b] where the relative stability of the two isomers (with cyanide in the upper and lower co-ordination positions) depends on the number of ionised carboxylate side-chains, and (*b*) the organocobalamins, where the neutral five-co-ordinate 'base-off' form includes a variant in which the heterocyclic base is apparently held against the corrin ring by hydrophobic and/or charge-transfer interactions.[10]

The major equilibria involving dac, ahc, and dhc are represented by equilibrium (1), together with the possible inversion of the axial ligands in the case of ahc. It turns out that these complexes also provide good examples of minor equilibria which, we shall conclude, depend on hydrogen bonding between the axial ligands (H_2O and/or OH^-) and different amide side-chains, *i.e.* they are similar to the minor equilibria investigated by Friedrich and co-workers.[7b] The main evidence for the additional equilibria is provided by kinetic studies of the co-ordination of the ligands CN^-, I^-, and $[Co(CN)_6]^{3-}$.

We have been studying the kinetics of the substitution of co-ordinated H_2O by cyanide in corrinoids where the *trans* ligand is H_2O/OH^-, dbzm (vitamin B_{12a}), CN^-, $HC{\equiv}C^-$, $CH_2{=}CH^-$, and CH_3^- over the range of pH 4—14 and have found that all the observed kinetic plots and rate constants can be interpreted in terms of (*a*) the known pK values of HCN and the corrinoid complexes, (*b*) labile ahc and kinetically inert dhc, (*c*) simple pseudo-first-order kinetics under each set of experimental conditions, and (*d*) the scheme used by Reenstra and Jencks [11] in their study of the reaction of B_{12a} with cyanide (*i.e.* reaction of both CN^- and HCN, followed by loss of a proton and isomerisation in the latter case), *except* that the kinetic traces observed with ahc are bi- or even tri-phasic, *i.e.* show the occurrence of two or three parallel or consecutive reactions. Full details of all these kinetic studies and their interpretation will be published later; here we are concerned only with the unusual kinetics observed with ahc. To check whether these anomalous kinetics are observed only with the combination of ahc + CN^-, we have also studied the kinetics of co-ordination of I^- and $[Co(CN)_6]^{3-}$ by dac and of the latter by ahc.

The main aims of this paper are, therefore, to develop an improved method for the preparation of solutions of dac, to

† Abbreviations: dac, ahc, and dhc = diaquo-, aquohydroxo-, and dihydroxo-cobinamide, respectively. Cobinamides lack the nucleotide side-chain ending in 5,6-dimethylbenzimidazole (dbzm) which is present in the cobalamins. Factor B is cyanoaquo/cyanohydroxocobinamide.

determine accurate values for pK_1 and pK_2 for use in subsequent work on equilibrium constants involving dac, ahc, and dhc, and to provide evidence for the existence and nature of the additional minor equilibria in these cobinamides.

Experimental

Materials.—Samples of vitamins B_{12} and B_{12a} were kindly given by Mr. Domleo of Glaxo-Allenbury (Pty) Ltd, South Africa. Cyanoaquocobinamide (Factor B) was prepared as previously described [12] and converted into dac as described below. AnalaR reagents were used wherever possible ($HClO_4$, $NaClO_4$, NaOH, NaCN, NaI, 0.880 ammonia); exceptions included $K_3[Co(CN)_6]$ (B.D.H., reagent grade) and Bu^sOH (Merck, chemically pure).

U.v.–Visible Spectra.—These were recorded with a JASCO UVIDEC-1 spectrophotometer using 1-cm cells and, unless otherwise stated, at 25 °C. The kinetics of ligand substitution were studied with a Durrum D-110 stopped-flow spectrophotometer.

Thin-layer Chromatography.—T.l.c. was carried out at room temperature on cellulose (Merck, 0.1 mm, pre-coated), using the following three solvents (numbered as in refs. 5 and 13): I, Bu^sOH–water (9.5 : 4); III, Bu^sOH–0.88 ammonia–water (9.5 : 0.675 : 4); V, Bu^sOH–0.88 ammonia–water–KCN (1 mol dm^{-3}) (250 : 0.4 : 100 : 0.3). Vitamin B_{12} was used as a marker in order to obtain values of R_{B12} (*i.e.* R_f values relative to that of vitamin B_{12}).[5,13]

pH Determinations.—These were made with a Metrohm EM 147 micro glass electrode.

Preparation of dac.—The apparatus designed for photolysis consisted of an annular glass container with an outer diameter of 8.5 cm, a width of 3 mm between the two walls, and a height of 10 cm, open at the top. The hollow centre accommodated a 60-W tungsten lamp. The annular space contained approximately 40 cm^3 of solution, which was stirred and flushed with a fine stream of nitrogen bubbles emanating from the ends of four plastic capillary tubes. The container and bulb were all placed in a water-bath held at 0 °C. The higher surface area normal to the light and the small depth to which the light had to penetrate through the intensely coloured solution, together with the vigorous flushing to keep [HCN] low, all served to increase the rate of photolysis; while the large contact area with the cooling water and vigorous stirring of the solution served to increase the rate of removal of the heat generated by absorption of the light, and hence to reduce the rate of hydrolysis.

In a typical experiment Factor B (30 mg) was dissolved in water (40 cm^3), $HClO_4$ (1 mol dm^{-3}) added to give pH 2—3, and the resultant solution photolysed in the annular cell until the reaction was complete (*ca.* 5 h). The extent of reaction was monitored by withdrawing a small sample of solution, diluting to the necessary concentration in NaOH (0.1 mol dm^{-3}), and examining the spectrum in the region of the γ-band. At this pH any unphotolysed Factor B ($pK = 11.0$) [7c] is present as the hydroxocyano-complex which has a sharp γ-band at 362 nm ($\varepsilon_{molar} = 2.3 \times 10^4$ dm^3 mol^{-1} cm^{-1}), whose presence can readily be detected in the presence of photolysed dhc, the spectrum of which (see Figure 3) includes a broad shoulder at *ca.* 356 nm (with ε_{362} *ca.* 1.7×10^4 dm^3 mol^{-1} cm^{-1}). The final analysis was carried out by t.l.c. using solvent III; for almost all preparations this revealed no products other than dac.

After photolysis was complete, the solution of dac was carefully neutralised with NaOH (0.1 mol dm^{-3}), degassed by evacuation with a water-pump for 30 min, and usually stored without further treatment as a frozen solution at −20 °C; no changes (spectra, t.l.c., or kinetics) were observed on storage for over two months. The cobinamide dac cannot be separated from the low concentration of electrolyte by extraction through either phenol–chloroform or benzyl alcohol, since this causes partial reduction; however, separation can be accomplished, if necessary, by using an Amicon 52 ultra-filtration unit with a Diaflo UM 2 ultra-filter. Solid dac (or, more probably, ahc) can be prepared by freeze-drying, but partial reduction sometimes occurred (as seen from the spectrum after dissolution); the cause of this reduction is not known.

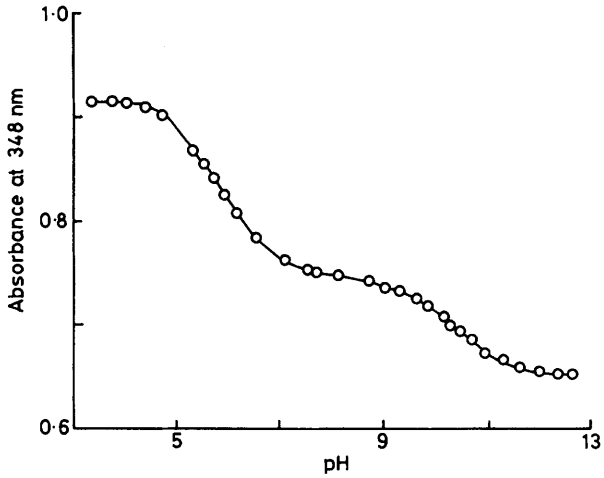

Figure 1. Spectrophotometric pH-titration of dac (3.3×10^{-5} mol dm^{-3})

Results

Determination of p*K Values.*—Initial experiments over the range of pH 2—14 confirmed (*cf.* ref. 2) that aqueous solutions of dac show two reversible pH-dependent equilibria with pK_1 *ca.* 6 and pK_2 *ca.* 10.5. Recording changes in the spectrum over the range 300—400 nm as the pH was increased showed that the two equilibria overlapped, but good isosbestic points were observed at 341 and 357 nm for the first part of the change corresponding to pK_1. The latter part of the change corresponding to pK_2 also gave a good isosbestic point at 377 nm and a reasonable one at 341 nm, but superposition of the spectra produced caustic curves around 320 and 358 nm; the spectral changes in the 300—360 nm region were less regular than might be expected, but nevertheless appeared to be reversible.

The p*K* values were determined quantitatively at 25 °C in $NaClO_4$ (0.2 mol dm^{-3}) by spectrophotometric titration of dac (3.3×10^{-5} mol dm^{-3}) from pH *ca.* 3 to *ca.* 13, following the optical density at 348 nm (the wavelength of greatest overall change); see Figure 1. Analysis of the results gave excellent linear plots (see Figure 2) corresponding to one proton; $pK_1 = 5.9 \pm 0.1$ and $pK_2 = 10.3 \pm 0.2$. The reverse titration from pH 13 to 3 (now at higher ionic strength) gave the same values within experimental error.

Spectra.—The spectra at pH 3.94 (dac), 8.24 (mainly ahc with a little dac), and 12.64 (dhc), all at 25 °C with $I = 0.2$ mol dm^{-3} (perchlorate), are shown in Figure 3. Solutions of all three complexes obeyed Beer's law under these conditions from 3×10^{-6} to 6×10^{-5} mol dm^{-3}. A solution of dhc in

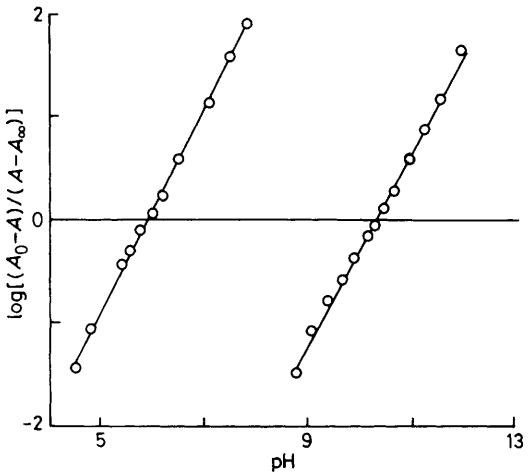

Figure 2. Evaluation of data of Figure 1 to give pK_1 and pK_2; both slopes correspond to 1.0 proton

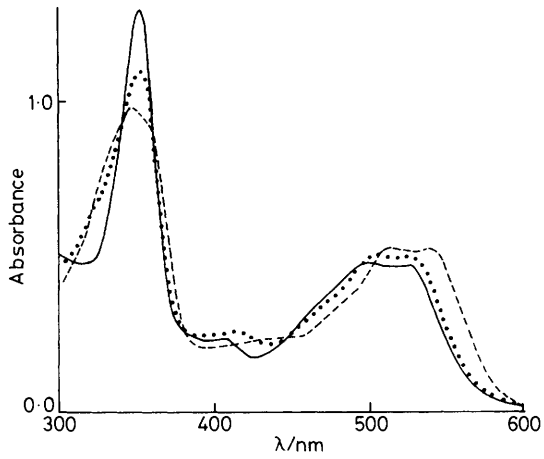

Figure 3. Spectra of 4.8×10^{-5} mol dm^{-3} solutions of dac (——), ahc (· · · ·), and dhc (— — —)

NaOH (1 mol dm^{-3}) showed no significant change over the range 300—600 nm on varying the temperature from 23 to 43 °C; higher temperatures caused irreversible changes. The wavelengths of the main bands and shoulders (nm) and their absorption coefficients ($10^{-4}\varepsilon_{molar}$ in parentheses, determined by conversion to the dicyanide with $\lambda_{max.} = 367$ nm and $\varepsilon_{367} = 3.04 \times 10^4$ dm^3 mol^{-1} cm^{-1}) [7d] for the three species were as follows (Soret bands italicised): dac, 520 (0.98), 494 (1.00), *349* (*2.73*), *ca.* 310 (*ca.* 0.9); ahc, 519 (1.04), 499 (1.05), *349* (*2.27*), *ca.* 318 (sh) (*ca.* 1.3); dhc, 531 (1.10), 508 (1.10), *356* (*sh*) (*ca. 1.9*), *344* (*2.04*), *ca.* 328 (sh) (*ca.* 1.6). The previously reported data for the Soret bands of dac (347.5 nm, 2.8×10^4 dm^3 mol^{-1} cm^{-1}) and dhc (342, 2.1×10^4) can be compared with the present data.[14]

Thin-layer Chromatography.—A comparison of the t.l.c. of five corrinoids using solvent I showed that the relative diffuseness D of their spots (very approximate values based on $D = 1.0$ for methylcobalamin) increased in the following order: methylcobalamin ($R_{B_{12}} = 1.37$, very sharp spot $D = 1.0$) < B_{12} (1.00, $D = 1.2$) < Factor B (slow isomer 0.89, $D = 2.3$; fast isomer 1.03, $D = 2.3$) < B_{12a} (noticeable streak 0.05—0.29, D *ca.* 3.3) < ahc (very marked streak 0.05—1.3, D *ca.* 15). The streaking of the last two has been noted before.[13] Hydroxocobalamin, ahc, and hydroxocyanocobinamide were identified from their spectra as the main species present in the pure solvent in a spectrophotometer cell. Similar streaking was observed with three different samples of ahc. The streaks from B_{12a} and ahc showed no obvious signs of fronting or tailing or of concentrating into separate spots. When B_{12a} and ahc were each run alone in solvent I until a long streak had formed and then run at right angles in solvent V, the streaks ran as thin purple lines (of the dicyanide) parallel to the solvent front, *i.e.* the various species responsible for forming the streak had been converted into a single dicyanide complex.

Equilibrium Constants for Ligand Substitution.—As a preliminary to the kinetic studies, equilibrium constants for the substitution of co-ordinated H_2O in dac by I^-, $[Co(CN)_6]^{3-}$, and acetate (since these studies were carried out in acetate buffer) were determined spectrophotometrically. There was no detectable change in the spectrum of dac in $HClO_4$ (10^{-3} mol dm^{-3}) on adding up to 2 mol dm^{-3} $NaClO_4$, *i.e.* ClO_4^- does not appear to co-ordinate. All the equilibria investigated were established ' instantaneously '. For the two smallest equilibrium constants, where the end-point was unattainable (namely acetate and the second iodide), the data were evaluated by the method of Newton and Arcand.[15]

The co-ordination of acetate hardly affects the wavelength of the γ-band (349 nm in both cases); the only noticeable effect over the whole range 300—600 nm was an increase in optical density around 320 nm. The equilibrium constant was determined at pH 4.25 by titrating 5.5×10^{-5} mol dm^{-3} dac in 0.2 mol dm^{-3} $NaClO_4$ (adjusted to pH 4.25 with $HClO_4$) with acetate buffer pH 4.25 (I variable, up to 0.30 mol dm^{-3}) and following the changes in A_{320}. Correction for the pK_1 of dac (5.9) and the pK_a of $MeCO_2H$ (4.75) gave $K_1 = 13.1 \pm 0.4$ dm^3 mol^{-1} for the substitution of one H_2O by $MeCO_2^-$. There was no evidence for co-ordination of a second acetate by dac at pH 4.25 or for even one acetate by ahc at pH 8.0; but no maximum values can be quoted for either constant, because the likely change in the spectrum is not known and would probably be rather small.

Qualitative experiments showed that dac catalysed the oxidation of I^- by O_2 to I_3^- and that the rate increased with acidity; *cf.* the analogous catalytic activity of aquocobalamin in acid solution.[7e] The equilibrium constants were therefore determined in deoxygenated solutions at the highest pH possible (*ca.* 4) by following changes in optical density in the αβ region (to avoid interference from the intense band of I_3^- at 350 nm).[16] Two well separated equilibria are exhibited by dac, both of which were shown to involve one iodide. The value of $K_1 = (1.76 \pm 0.03) \times 10^3$ dm^3 mol^{-1} was obtained with 2.5×10^{-5} mol dm^{-3} dac at pH 4.0 and $I = 0.2$ mol dm^{-3} ($NaClO_4/I^-$) following changes in A_{522} and after correction for bound acetate. The value of $K_2 = 2.2 \pm 0.2$ dm^3 mol^{-1} was obtained with 3×10^{-4} mol dm^{-3} dac at pH 4.0 by following changes in A_{580} but, because of the low value of K_2, the ionic strength could not be kept constant (up to 2 mol dm^{-3} NaI). The binding constant for acetate *trans* to iodide has not been determined but, because of the *trans* effect of I^-, is expected to be significantly lower than 13; K_2 has, therefore, not been corrected for any bound acetate. The very unusual spectra of these two iodide complexes will be discussed later. The low value of K_1 means that I^- will not readily react with ahc to displace OH^- and that the co-ordination of I^- can be conveniently studied only with dac itself in the acid region. HI is a very strong acid (pK *ca.* −10).[17]

Equilibrium constants for the co-ordination of $[Co(CN)_6]^{3-}$ by dac were determined semi-quantitatively (the exact stoi-

Table. Kinetics of reaction of dac and ahc with CN^-, $[Co(CN)_6]^{3-}$, and I^-

Reagent [a]	pH	Reagent concentration [b] (10^{-3} mol dm^{-3})	Second-order rate constants [c] (dm^3 mol^{-1} s^{-1}) k_f	k_m	k_s	k_m/k_s
(*a*) Varying pH and [reagent]; [Co] = *ca.* 8×10^{-5} mol dm^{-3}						
CN^-	4.50	4—40		6.5	1.8	4
CN^-	5.00	4—40	1.5×10^2	16	3.6	4
CN^-	6.00	4—40	1.1×10^3	2.2×10^2	53	4
CN^-	7.02	2—20	1.4×10^4	2.4×10^3	6.2×10^2	4
CN^-	8.09	1.5—15		2.8×10^4	4.9×10^3	6
CN^-	9.01	0.1—1.0		3.9×10^4	1.5×10^4	3
CN^-	10.00	0.1—1.0		4.2×10^4	1.4×10^4	3
CN^-	11.03	0.2—2.0		1.3×10^4	3.1×10^3	4
CN^-	12.01	0.4—4.0		2.2×10^3	4.2×10^2	5
$[Co(CN)_6]^{3-}$	1.00	1—10		2.1×10^3	6.1×10^2	3
$[Co(CN)_6]^{3-}$	2.00	1—10		1.9×10^3	7.4×10^2	3
$[Co(CN)_6]^{3-}$	5.00	1		7.8×10^3	2.8×10^3	3
$[Co(CN)_6]^{3-}$	5.93	0.1—1.0		9.8×10^3	3.5×10^3	3
$[Co(CN)_6]^{3-}$	7.05	1	4×10^4	1.0×10^4	3.8×10^3	3
$[Co(CN)_6]^{3-}$	8.08	10		5.2×10^3	1.1×10^3	5
$[Co(CN)_6]^{3-}$	11.03	10		28	6.6	4
$[Co(CN)_6]^{3-}$	11.98	10		6.2	2.4	3
I^-	1.0	0.5—5		2.4×10^3	2.9×10^2	8
I^-	2.0	0.5—5		1.9×10^3	2.4×10^2	8
(*b*) Varying [Co]; [NaCN] = 10^{-2} mol dm^{-3}						
CN^-	6.12	1.9×10^{-6} [d]	2.4×10^3	2.3×10^2	61	4
CN^-	6.12	1.9×10^{-5} [d]	2.8×10^3	2.2×10^2	62	4
CN^-	6.12	1.9×10^{-4} [d]	2.2×10^3	2.4×10^2	60	4

[a] Salts used: NaCN, NaI, $K_3[Co(CN)_6]$. [b] Where studied, varying [reagent] had no significant effect on the rate constants. [c] Two or three rate constants obtained by 'curve-stripping' of the kinetic trace (see text). In every case the reaction represented by k_m appeared to account for 50—70% of the total reaction. [d] Value is [Co].

cheiometry was not established) with 1.1×10^{-6} mol dm^{-3} dac in 0.2 mol dm^{-3} acetate buffer at pH 4.0 in a 10-cm cell, following changes in A_{541}, to give $K_1 > 2 \times 10^6$ dm^3 mol^{-1} and $K_2 = (1.5 \pm 0.3) \times 10^5$ dm^3 mol^{-1}. The spectrum of the first product had bands at 352, 499, and 529 nm, that of the second at 357, 515, and 546 nm. The pK of the mono-adduct was not determined. The relatively high binding constants allow the kinetics of co-ordination of $[Co(CN)_6]^{3-}$ to be followed well into the alkaline region. The hexacyanide can apparently only be protonated below pH 1; [18,19] we failed to observe any change in the *d–d* spectra of the complex on acidification even with 3 mol dm^{-3} H_2SO_4.

Relevant equilibrium constants involving cyanide are as follows: dac + CN^-, *i.e.* reverse of equilibrium (2), $\log_{10} K \geqslant 14$; [2] cyanoaquocobinamide + CN^- to give the dicyanide, $\log_{10} K = 8$;[7f] cyanoaquocobinamide, p$K = 11.0$;[7c] HCN, p$K_a = 9.3$.[17]

Kinetics of Ligand Substitution.—The kinetics of co-ordination of CN^-, I^-, and $[Co(CN)_6]^{3-}$ by *ca.* 8×10^{-5} mol dm^{-3} solutions of dac, *etc.*, have been studied by stopped-flow spectrophotometry at 25 °C and $I = 0.2$ mol dm^{-3}. The range of pH and ligand concentration used are given in the Table. The concentration of dac was varied in only one case, namely with cyanide at pH 6 (see Table). Several different preparations of dac (including one prepared by the photolysis of methylcobinamide) were directly compared at pH 6.0 with cyanide; all gave the same absolute and relative values (within experimental error) of k_m and k_s (k_f not evaluated in this case; see below). Several different preparations were used to complete the pH profile for cyanide.

For every experiment the kinetic traces were followed for at least four half-lives and the nature of the starting species and products checked on a conventional spectrophotometer using identical solutions. With cyanide the product was usually the dicyanide; other experiments established that cyanoaquocobinamide reacts with cyanide much faster than either dac or ahc (due to the *trans* labilising effect of CN^-), so that the rate-determining step is the co-ordination of the first cyanide. The only product observed with I^- was the mono-iodide complex. The reaction with the hexacyanide gave a mixture of the mono- and/or bis-adducts, depending on conditions; we assume that here too the rate-determining step is the co-ordination of the first molecule of hexacyanide.

In every case complete formation of the product was attained and the end-point was steady, yet all the kinetic traces were distinctly bi- or even tri-phasic and could be resolved by 'curve-stripping' [20] into two or three pseudo-first-order rate constants (see Figure 4). The second-order rate constants (from fastest k_f, through k_m, to slowest k_s) are listed in the Table; the rates of reaction with CN^- are too fast to ascertain whether k_f still persists above pH 7. These rate constants are independent of cobalt concentration, at least with cyanide at pH 6 (see Table). The variation with pH of k_m and k_s for CN^- and $[Co(CN)_6]^{3-}$ is shown in Figure 5.

Discussion

Our results show that the photolysis of cyanoaquocobinamide (Factor B) in the annular container described in the Experimental section provides a convenient method for preparing solutions of dac without any significant formation of hydrolysis products.

The pK values of dac have been determined in $NaClO_4$ (0.2 mol dm^{-3}) at 25 °C as p$K_1 = 5.9 \pm 0.1$ and p$K_2 = 10.3 \pm 0.2$ and are shown to involve one proton each, in agreement with

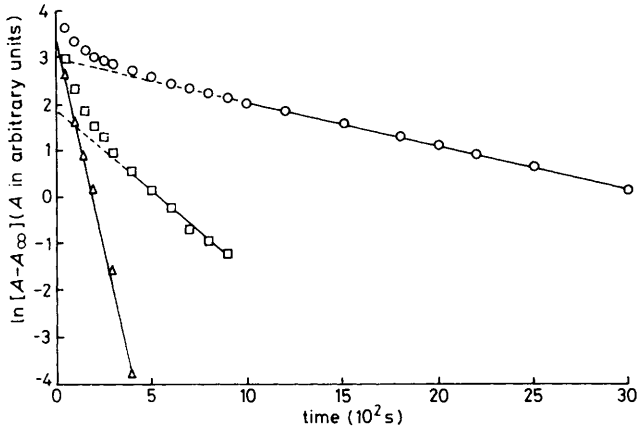

Figure 4. Example of ' curve-stripping ' of a kinetic trace (reaction of ahc at pH 7.0 with 1×10^{-2} mol dm^{-3} NaCN) to give k_f (△), k_m (□), and k_s (○)

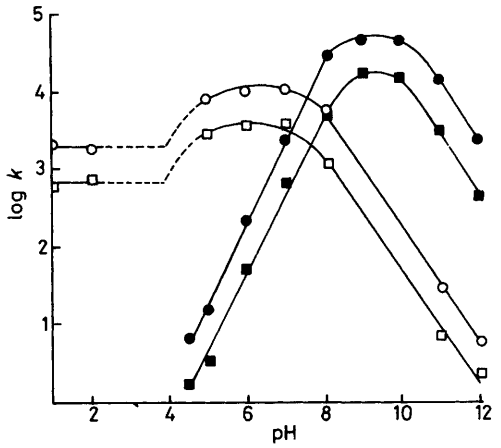

Figure 5. pH-Dependence of k_m (○, ●) and k_s (□, ■) with $[Co(CN)_6]^{3-}$ (open circles/squares) and CN^- (filled)

previous values of $pK_1 = 6.0$ and $pK_2 = ca.\ 10.5$ at lower ionic strength.[2]

The highly unusual spectrum of dhc (see Figure 3) suggested the possibility that it might be a monomeric oxo-complex (with or without H_2O as the sixth ligand) or a dimeric μ-oxo-complex, or even consist of two or more species related according to the pH-independent equilibria (3) and (4) (axial ligands only given). The oxide ligand (O^{2-}) would be expected to have a much greater *trans* labilising effect than OH^-; dhc is, however, kinetically inert towards cyanide compared to ahc (see Table and Figure 5). Solutions of dac, ahc, and dhc all obey Beer's law and no temperature-sensitive equilibrium was detected at pH 14. We conclude that the only major equilibria involved are those represented by equilibrium (1), together with the possible change in relative orientation of the two ligands in ahc. The kinetic and t.l.c. results, however, provide evidence for the existence of additional minor equilibria.

$$HO{-}Co{-}OH \rightleftharpoons O{=}Co{-}OH_2 \text{ (or } O{=}Co + H_2O) \quad (3)$$

$$2\ HO{-}Co{-}OH \rightleftharpoons HO{-}Co{-}O{-}Co{-}OH + H_2O \quad (4)$$

The kinetics of substitution of co-ordinated H_2O in dac and ahc by I^-, CN^-, and $[Co(CN)_6]^{3-}$ (see Table and Figure 5) show two features which require an explanation, namely the occurrence of bi- and even tri-phasic kinetics and the variation of these rate constants with pH. The latter can be qualitatively explained as follows. Starting from the pH-independent reactions of dac at pH 1—2, the increase in rate above pH 5 corresponds to an increasing conversion of dac into the more labile ahc (and of HCN into CN^-) and the fall in rate at higher pH reflects conversion into the kinetically inert dhc. The pH profile for cyanide can be quantitatively interpreted according to a scheme analogous to that used by Reenstra and Jencks [11] in their study of the reaction of aquocobalamin with cyanide; full details will be published later. The pH profile for the hexacyanide, on the other hand, cannot be quantitatively explained in terms of just the two p*K* values of dac and no p*K* for the hexacyanide. We suspect that, because of the high charge on the hexacyanide, ion-pairing may play some role and that the apparent effects of varying the pH may include effects due to changes in ion-pairing; but this has not been proved.

It is mathematically impossible [20] to differentiate between *parallel* rate constants, where the incoming ligand reacts with different forms of the cobinamide which are not in ' instantaneous ' equilibrium with each other, and *consecutive* rate constants, where the initial adduct undergoes successive steps before yielding the final product; the latter possibility can, however, be excluded because similar anomalous kinetics are observed with all three ligands. The ratio of these rate constants is independent of cobalt concentration (see Table), *i.e.* they all represent reactions of corrinoids having the same degree of aggregation, hence presumably monomers. The only reasonable conclusion is that dac and ahc exist in solution as a pH-independent mixture of two or three ' isomers ' and that the rate of interconversion between the isomers is slow compared to the rate of reaction with the incoming ligand and also to the rate of loss or gain of a proton. The fact that the same biphasic kinetics are observed at pH > 10 (*i.e.* where the complex is present as dhc) strongly suggests that, although dhc is kinetically inert, it must also be present as two ' isomers ' which can each be rapidly converted into the analogous isomer of ahc but only slowly into the other isomer of dhc.

We have pointed out [13] that one of the most important factors which determines the R_f values of corrinoids in t.l.c. is the hydrogen-bonding capacity of the axial ligand(s), which implies a significant effect on the conformation of the side-chains; this is supported both by the *X*-ray diffraction data [21] for cobyric acid and by the ^{13}C n.m.r. spectra of its two isomers in solution (see later). We also noted the marked streaking of aquocobalamin and dac; [13] we have now confirmed these observations, identified the main complexes present as hydroxocobalamin and ahc, and shown that streaking does not involve any irreversible decomposition. The existence of ahc as a mixture of slowly interconverting isomers provides an explanation for the unusual streaking, as well as the unusual kinetics, of ahc. The observation of two waves in the reduction of dac by cyclic voltammetry [3] may be a related phenomenon. We therefore have to find some structural feature which can provide the basis for a new type of pH-independent ' isomerism ' common to dac, ahc, probably dhc, and possibly even vitamin B_{12a}. The structure of cobyric acid (Factor V_{1a}) provides a clue.

In Factor V_{1a} the upper (or b) co-ordination site is occupied by H_2O (though the possibility that the ligand is OH^- cannot be excluded) and the lower (or a) site by CN^-. The terminal N atom of the cyanide is hydrogen-bonded to one water molecule, while the co-ordinated H_2O is hydrogen-bonded to the carbonyl O atom of the acetamide side-chain of ring B and also to a water molecule, *i.e.* the co-ordinated H_2O provides the

protons for both hydrogen bonds, while the co-ordinated CN^- provides the lone pair of electrons for its single hydrogen bond. The bonding between the co-ordinated H_2O and the amide is apparently strong enough both to cause a detectable displacement of the co-ordinated O atom and to affect the pucker of the corrin ring.[21] This same acetamide side-chain also forms a hydrogen bond to the co-ordinated cyanide in dry vitamin B_{12},[22] but in wet B_{12}[23] and in the co-enzyme [24] it turns outward to form intermolecular hydrogen bonds. The *X*-ray evidence therefore shows that the axial ligands can form hydrogen bonds to the side-chains; that the ability to form such bonds is probably greater with H_2O than with CN^- and will obviously be non-existent with alkyl ligands; and that the conformation of the side-chains can be affected by the hydrogen-bonding capacity of the ligands.

It appears that all cyanoaquo-/cyanohydroxo-corrinoids (including Factor V_{1a}) exist in solution as a mixture of two isomers which differ in the relative orientation of the CN^- and H_2O/OH^- ligands. The ratio of the two isomers varies from 1.5 : 1 to 0.25 : 1 at pH $\geq$ 5 (*i.e.* where the ligands are CN^- and H_2O); the dependence of this ratio on the number of ionised carboxylate side-chains provides fairly direct evidence for hydrogen-bonding between the axial ligands and the side-chains in solution.[7b] A comparison of the ^{13}C n.m.r. spectra of the two isomers of Factor V_{1a} (apparently in neutral solution where the ligands would be CN^- and H_2O) revealed surprising differences, which extended even to some of the carbon atoms in the side-chains; it was concluded than these differences must reflect ' some conformational changes of the corrin ring.' [25] All the isomeric pairs of cyanocorrinoids show similar large differences in R_{B12} values, [7b] which can again be ascribed to significant effects of the axial ligands on the conformation of the side-chains. In the case of Factor B the main species present in the t.l.c. solvent has been identified as the cyanohydroxo-complex (see Results section); this suggests that co-ordinated OH^- has a similar effect to co-ordinated H_2O. Furthermore, the fact that Factor B exists as an approximately equal mixture of two isomers and that the two t.l.c. spots have a similar diffuseness (see Results section), in spite of very different R_{B12} values, strongly suggests that there is little difference between the side-chains above and below the corrin ring in their ability to form hydrogen bonds to co-ordinated H_2O and OH^-. It therefore appears that in cyanoaquo-/cyanohydroxo-corrinoids the co-ordinated H_2O or OH^- is hydrogen-bonded to some side-chain in both stereoisomers; the slight diffuseness observed in the t.l.c. of Factor B might indicate that the H_2O or OH^- can be hydrogen-bonded to more than one side-chain, but the evidence is clearly not very definite.

We therefore propose (*a*) that the different isomers observed with dac, *etc.* represent hydrogen-bonding between the axial ligand(s) and different side-chains or combinations of side-chains; (*b*) that co-ordinated H_2O and HO^- show fairly similar behaviour, such that the isomers of dac, ahc, and dhc have analogous structures; and (*c*) that the relatively slow rate of interconversion of the isomers is due to the slow conformational change of one or more side-chains. Isomers of this type would be expected to show only slight differences in properties such as spectra, p*K* values, and rate constants; the resolution of only two or three rate constants does not therefore necessarily mean that this is the maximum number of such isomers present in solution. There is no evidence to suggest whether the two or more isomers observed for ahc have OH^- in the same co-ordination position or not. The existence of such isomers with slight differences in spectra and p*K* probably explains why the changes in the spectrum corresponding to pK_2 were not as regular as expected in the 300—600 nm region.

The streaking of B_{12a} in t.l.c. could indicate the existence of isomers, but neither we nor others [11,26,27] have obtained any evidence for such isomers from the study of ligand substitution reactions. However, Kenyhercz *et al.*[28] have reported that high-pressure liquid chromatography of B_{12a} gave two closely spaced yet distinct peaks, while B_{12} apparently gave only a single peak; this interesting observation clearly requires confirmation. They also found that, under the conditions of their spectroelectrochemical experiments, the redox behaviour of the Co^{III}/Co^{II} couple of B_{12a} showed pronounced hysteresis with two different waves for reduction. They were forced to conclude that B_{12a} (in contrast to B_{12}) existed in aqueous solution pH 7.0 as a 65 : 35 mixture of two species and suggested that these were the ' base-on ' and ' base-off ' forms, but admitted that this explanation was contradicted by their own results on the Co^{II}/Co^{I} redox reactions. These observations might conceivably be related to the existence of isomers of the type discussed above.

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