# Interaction of Cysteine with Vitamin $B_{12a}$: Kinetic and Thermodynamic Investigations

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Rate and equilibrium constants for the interaction of L-cysteine with vitamin $B_{12a}$ have been determined in aqueous solutions in the absence and in the presence of air as functions of pH, buffer concentration, and temperature. Kinetic treatment of the data has afforded the pH-independent rate constants for the anation of aquo- and hydroxocobalamins by L-cysteine, for the aquation of the vitamin $B_{12}$–L-cysteine complexes, and for the formation of vitamin $B_{12r}$ from the L-cysteine complex of vitamin $B_{12a}$. The mechanism of these reactions is discussed.

In spite of their obvious importance, relatively few detailed kinetic studies have been carried out on ligand-exchange reactions with vitamin $B_{12a}$.[1-4] Rate constants for the anation, $k_1$, and those for the aquation, $k_{-1}$, of vitamin $B_{12a}$, and aquocobalamin (bzm–Co–$OH_2$) have been determined for the ligands (L) $N_3^-$, $OCN^-$, $SCN^-$,

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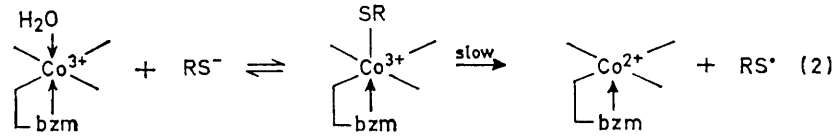
[2] D. G. Brown, *Progr. Inorg. Chem.*, 1973, **18**, 177.
[3] R. H. Prince and D. A. Stotter, *J. Inorg. Nuclear Chem.*, 1973, **35**, 321.
[4] G. N. Schrauzer, *Pure Appl. Chem.*, 1973, **33**, 545.

$SO_3^{2-}$, $NCO^-$, $I^-$, $Br^-$, imidazole, and glycine.[5-11] Although the structure of cobalamin is far from simple,

$$bzm\text{-}Co\text{-}OH_2 + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} bzm\text{-}Co\text{-}L + H_2O \quad (1)$$

chelation of the benzimidazole (bzm) side chain precludes substitution in a stoicheiometry other than 1 : 1, at least in the range pH 4—10.[1] Kinetic treatment of the data for simple ligand substitutions of aquocobalamin is, therefore, unexpectedly simple.

The recognized role of sulphydryl groups in reactions catalyzed by vitamin $B_{12}$ enzymes prompted the numerous exploratory investigations of the interactions of thiols with methylcobalamin,[12] aquocobalamin,[13-16] and $B_{12}$ coenzymes.[17] Surprisingly, however, there is only meagre information on the detailed kinetics of these processes. Reaction of thiols with methylcobalamin was suggested to result in the formation of vitamin $B_{12r}$ and the methylated thiol with a subsequent one-electron reduction to yield vitamin $B_{12s}$.[3,12] Alternatively, vitamin $B_{12s}$ was postulated to form directly in the interaction of thiols with methylcobalamin.[4,18-20] No such dispute exists, however, on the steps involved in the interaction of thiols with vitamin $B_{12a}$. Investigations of the kinetics of reaction (2) are, therefore, not




Vitamin $B_{12a}$ Vitamin $B_{12}$

only inherently important but they may provide additional insight into the analogous methyl transfer. The present work reports the results of our kinetic and thermodynamic studies of L-cysteine with vitamin $B_{12a}$ in aqueous solutions.

## EXPERIMENTAL

The best available grades of vitamin $B_{12a}$, aquocobalamin, bzm-Co-$OH_2$ (Merck), L-methionine (Sigma), L-cysteine (Sigma), and 2-mercaptoacetic acid and 3-mercaptopropionic acid (Aldrich) were used as received. Stock solutions of the thiols were prepared under a stream of nitrogen immediately prior to their use. Using these precautions, only a minimum of oxidation took place as established by the criteria of reproducibility of rate measurements and of ultraviolet absorbance of the thiols for the duration of the experiments. All stock solutions were prepared in double glass-distilled water; the pH of the buffered solutions was adjusted with Na[OH] and HCl and was determined by means of a Radiometer pHM-26 instrument. The pH of the solutions for kinetic runs at temperatures other than 25.0 °C were corrected by the temperature compensator of the pH meter.

Spectrophotometric determinations were made using a Cary 118-C spectrophotometer whose cell compartment was thermostatted at 25.0 ± 0.1 °C. Initially, the complete spectral range was recorded, generally on the scale 0—2.0 A at a speed of 10 nm $in^{-1}$ and 0.2 nm $s^{-1}$. Kinetic data were obtained on a Cary 118-C spectrometer. Temperatures for the kinetic runs were maintained by water circulation. Some kinetic runs were carried out in degassed solutions. For the slower runs, using the Cary 118-C spectrophotometer, solutions were placed in the two limbs of a specially constructed vessel which additionally had a quartz cell and a high-vacuum stopcock attached to it. The solutions in the separate compartments were degassed on a high-vacuum line by repeated freeze–pump–thaw cycles. Subsequent to the removal of air (*ca.* $10^{-6}$ Torr), the high-vacuum stopcock was closed and the vessel was removed from the vacuum line. The solutions were brought to the desired temperature, rapidly mixed, and transferred to the limb containing the quartz cell.

Rate constants for the reactions of L-cysteine with the vitamin $B_{12}$–cysteine adduct were determined in some systems by pH jump. An unbuffered solution of vitamin $B_{12a}$ and L-cysteine at *ca.* pH 4.5 was allowed to react to completion in one of the arms of the special degassing vessel, while the other arm contained a buffered solution adjusted to an appropriate, but at least three units higher, pH value. Subsequent to degassing, the two solutions were mixed and formation of vitamin $B_{12r}$ was followed at the appropriate wavelength.

Polarographic titration of vitamin $B_{12a}$ with L-cysteine was carried out by means of a Sargent model XXI polarograph. All reactions were followed under pseudo-first-order conditions to at least 98% completion. Plots of $\log(A_\infty - A_t)$ against time were linear and the error in the reported rate constants is ±3%.

## RESULTS

Addition of excess of L-cysteine, 2-mercaptoacetic acid, or 3-mercaptopropionic acid to a buffered (pH 5.5) solution

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[17] P. Y. Law and J. M. Wood, *J. Amer. Chem. Soc.*, 1973, **95**, 914.
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[20] G. N. Schrauzer, *Bioinorg. Chem.*, 1974, **3**, 353.

of vitamin $B_{12a}$ resulted in marked alteration of the absorption spectra (Table 1). Conversely, addition of excess of methionine or L-cysteine did not alter the spectra of aquocobalamin over a period of several days. The spectra of the products formed in the interaction of the three thiols with vitamin $B_{12a}$ were identical (Figure 1), indicating the

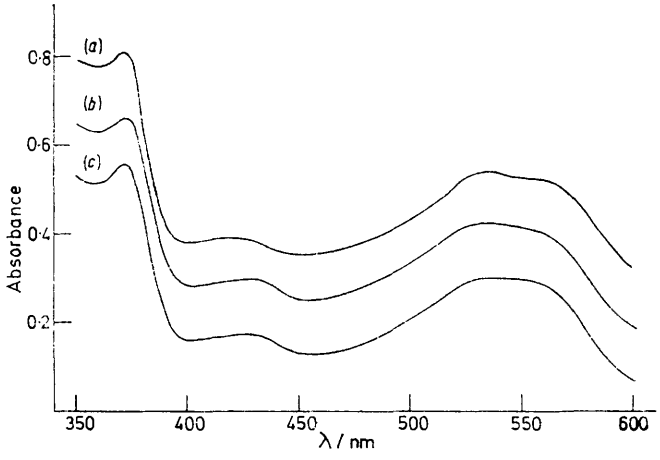



FIGURE 1 Absorption spectra of vitamin $B_{12}$–cysteine (*a*), vitamin $B_{12}$–3-mercaptopropionic acid (*b*), and vitamin $B_{12}$–2-mercaptoacetic acid (*c*) adducts. Substrate concentrations were $4.0 \times 10^{-5}$ mol dm$^{-3}$. Spectra were recorded in a 1.00 cm cell; those due to (*b*) and (*a*) are displaced by 0.1 and 0.2 absorbance units

formation of a product which is independent of the structure of the thiols. An identical spectra was also reported for the interaction of glutathione with aquocobalamin.[17] The cysteine did not change the spectra. This fact and the observed isosbestic points at 337, 362, 445, and 532 nm indicate the equilibrium formation of a product between vitamin $B_{12a}$ and L-cysteine. Addition of L-cysteine to vitamin $B_{12a}$ at pH > 7.0 resulted in a somewhat more complex behaviour. Subsequent to a time lag of several minutes, there was a time-dependent change in the 'limiting' spectra of the vitamin $B_{12a}$–L-cysteine adduct. The time lag was somewhat irreproducible and represents the consumption of oxygen in the stoppered cell. In degassed buffered solutions of L-cysteine and vitamin $B_{12a}$ at pH >7.30, these changes were observed without the time lag. A typical time-dependent spectral change for this process is illustrated in Figure 2(*b*). Compared to the limiting spectra of the vitamin $B_{12a}$–L-cysteine adduct [Figure 2(*a*)], absorbances increased at 400 and 470 nm at the expense of those at 370, 530, and 552 nm with isosbestic points at 388 and 491 nm. The limiting spectrum is stable for several days and corresponds to that attributed to vitamin $B_{12r}$.[1] The observed spectral changes are compatible with the reaction sequence:

$$\text{bzm–Co–OH}_2 + \text{RSH} \underset{k_{-1}^{app.}}{\overset{k_1^{app.}}{\rightleftharpoons}} \text{bzm–Co–SHR} + \text{H}_2\text{O} \quad (3)$$

$$\text{bzm–Co–SHR} + \text{RSH} \xrightarrow{k_2^{app.}} \text{Vitamin } B_{12r} + \text{RS}\cdot \quad (4)$$

Table 1 gives absorption maxima and absorption coefficients for aquocobalamin, hydroxocobalamin, the vitamin $B_{12}$–thiol complexes, and vitamin $B_{12r}$. Absorption coefficients were related to those of vitamin $B_{12a}$ and consequently they are independent of the amounts of water of crystallization in our samples. Development of the vitamin $B_{12}$–L-cysteine adduct and vitamin $B_{12r}$ could be followed

TABLE 1

Absorption spectral parameters in water at 25.0 °C

| Species | $\lambda_{max.}$ / nm | $10^{-4}\,\varepsilon$ / dm$^3$ mol$^{-1}$ cm$^{-1}$ | $\lambda_{max}$ / nm | $10^{-4}\,\varepsilon$ / dm$^3$ mol$^{-1}$ cm$^{-1}$ | $\lambda_{max.}$ / nm | $10^{-4}\,\varepsilon$ / dm$^3$ mol$^{-1}$ cm$^{-1}$ |
|---|---|---|---|---|---|---|
| bzm–Co–$OH_2$ | 350 | 2.60 | 497 | 0.79 | 523 | 0.83 |
| | (350) [a] | (2.62) [a] | | | | |
| bzm–Co–OH | 357 | 2.10 | | | | |
| bzm–Co–L-cysteine | 370 | 1.40 | 532 | 0.75 | 552 | 0.74 |
| | (370) [b] | (1.41) [b] | | | | |
| bzm–Co–3-mercaptopropionic acid | 371 | 1.40 | 534 | 0.83 | 554 | 0.79 |
| bzm–Co–2-mercaptoacetic acid | 371 | 1.38 | 534 | 0.76 | 552 | 0.74 |
| Vitamin $B_{12}$ | | | 401 | 0.72 | 471 | 0.93 |
| | | | (402) [a] | (0.75) [a] | (473) [a] | (0.92) [a] |

[a] From ref. 1. [b] From ref. 12.

stoicheiometry of the interaction of cysteine with aquocobalamin was established by polarography. Titration of a $1.0 \times 10^{-4}$ mol dm$^{-3}$ solution of vitamin $B_{12a}$ with a concentrated solution of L-cysteine in the polarography cell, using 0.1 mol dm$^{-3}$ sodium acetate at pH 5.5 or 0.1 mol dm$^{-3}$ sodium tetraborate at pH 9.3 as supporting electrolytes, gave end-points at $8.5 \times 10^{-5}$, $8.0 \times 10^{-5}$, $9.5 \times 10^{-5}$, and $8.7 \times 10^{-5}$ mol dm$^{-3}$ cysteine. Interaction of thiols with vitamin $B_{12a}$ is, therefore, likely to involve formation of a 1 : 1 complex.

Development of the absorption spectra of the vitamin $B_{12a}$–thiol adducts was dependent on the concentration of the thiols. This is illustrated for L-cysteine in Figure 2(*a*). Gradual addition of L-cysteine to vitamin $B_{12a}$ at pH < 7.0 resulted in increases in the absorbances at 552 and 370 nm at the expense of the absorbances at 495 and 525 nm. At sufficiently high cysteine concentration, addition of further spectrophotometrically. Knowledge of the appropriate spectral parameters (Table 1) allows meaningful dissection of $k_1^{app.}$ and $k_2^{app.}$ [equations (3) and (4)]. Rate constants for the equilibrium attainment of the vitamin $B_{12a}$–L-cysteine complex [equation (3)] were best obtained in air-saturated solutions at wavelengths where bzm–Co–SHR and vitamin $B_{12r}$ have isosbestic points (388 and 490 nm).

Similarly, the formation of vitamin $B_{12r}$ from bzm–Co–SHR [equation (4)] could be best followed at wavelengths where bzm–Co–$OH_2$ and bzm–Co–SHR have isosbestic points (337, 362, 445, and 532 nm). The observed pseudo-first-order rate constants, $k_\psi$, for the interaction of L-cysteine with vitamin $B_{12a}$ at different pH values, buffer concentrations, and temperatures are given in Table 2. The error in the second-order rate constants is considered to be ±8%. Using the method described in the Experimental section, reactions (3) and (4) were independently determined

for the interaction of L-cysteine with vitamin $B_{12a}$ at the appropriate isosbestic points (see above). Data obtained for these separate processes are also given in Table 2.

The observed pseudo-first-order rate constants for the equilibrium attainment of vitamin $B_{12a}$–L-cysteine complex of these plots yielded rate constants for the formation of the vitamin $B_{12a}$–L-cysteine complex, *i.e.* values for $k_1^{app.}$. Values of $k_1^{app.}$ at a given pH were independent of the concentration of buffers (Table 3). Furthermore, the reaction proceeded at the same rate in the dark as in the

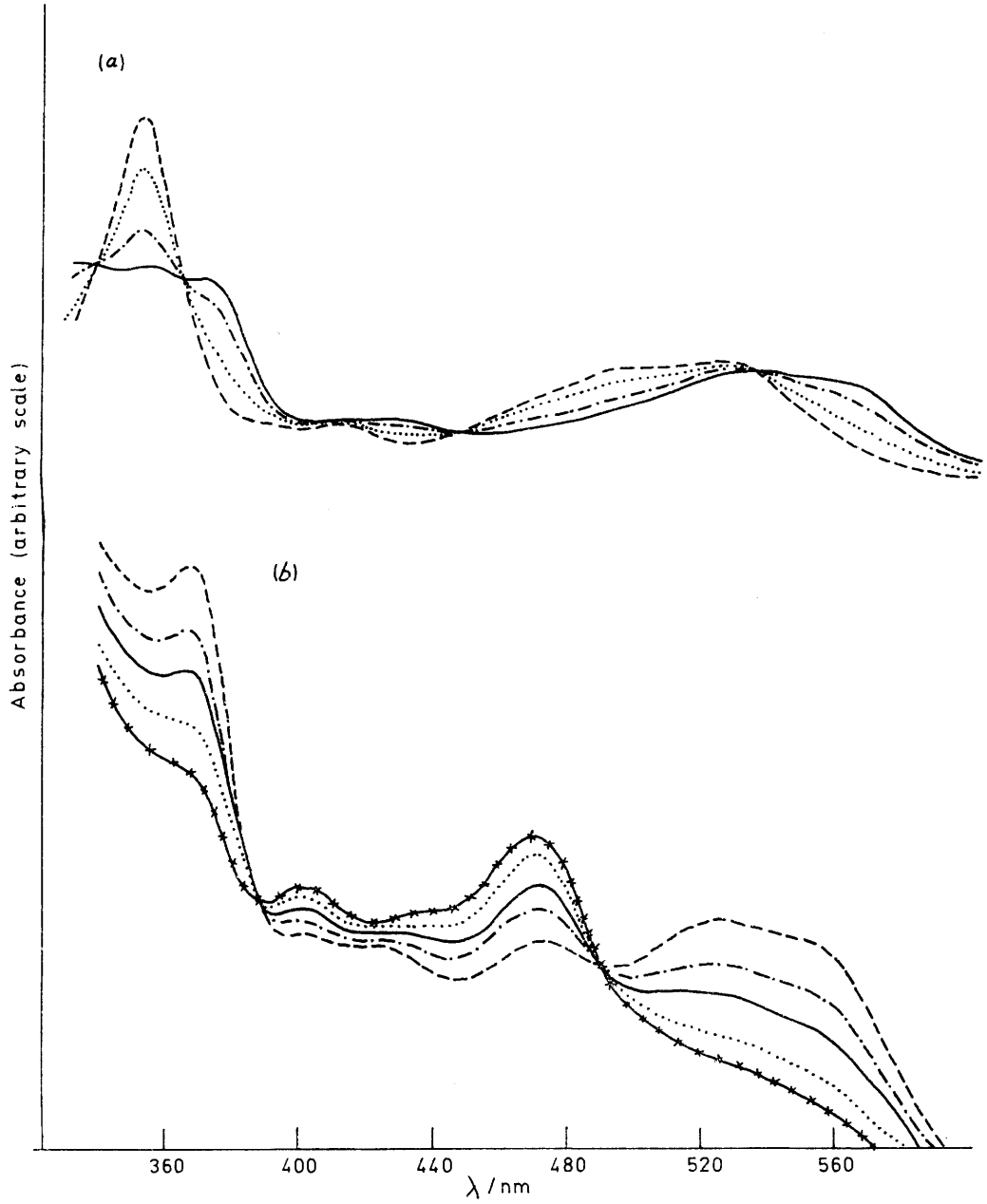



FIGURE 2 Absorption spectra of (*a*) $5.0 \times 10^{-5}$ mol dm$^{-3}$ vitamin $B_{12a}$ at pH 5.5 in water (— — —), in the presence of $3.5 \times 10^{-5}$(· · ·), $6.0 \times 10^{-5}$ (— · — · —), and $1.0 \times 10^{-3}$ mol dm$^{-3}$ mol dm$^{-3}$ L-cysteine (——), (*b*) of the vitamin $B_{12}$–L-cysteine adduct ($5.0 \times 10^{-5}$ mol dm$^{-3}$) in water at pH 7.3 and 2 (– – – –), 12 (— · — · —), 30 (——), 80 (· · · ·), and 150 min ($\overset{\vee\vee\vee\vee}{\wedge\wedge\wedge\wedge}$) after mixing

formation, $k_\psi$, allows, under favourable conditions, the calculation of the apparent (*i.e.* pH-dependent) rate constant for the anation, $k_1^{app.}$, and aquation, $k_{-1}^{app.}$, from equation (5). Good linear relations were obtained for all systems

$$k_\psi = k_1^{app.}[RSH] + k_{-1}^{app.} \quad (5)$$

on plotting the data according to equation (5). Gradients

light. Anation of bzm–Co–$OH_2$, therefore, is not photocatalyzed. Since for most cases the intercepts of these lines did not diverge appreciably from the origin, values of $k_{-1}^{app.}$ must be small and cannot be meaningfully obtained by treatment of the kinetic data according to equation (5).

Attempts were made to obtain the equilibrium constants for the formation of vitamin $B_{12a}$–L-cysteine adducts,

TABLE 2

Interaction of L-cysteine with vitamin $B_{12a}$ [a]

| Reaction | Conditions [b] | λ/nm | pH | $10^3$[L-Cysteine]/mol dm$^{-3}$ | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 0.50 | 0.67 | 0.83 | 1.00 | 1.50 | 2.00 | 2.33 | 2.50 | 2.66 | 3.00 | 3.33 | 3.50 |
| | | | | $10^2k\psi$/s$^{-1}$ | | | | | | | | | | | |
| $B_{12a}$ ⟶ $B_{12}$Cys | A | 350 | 3.85 [c] | | | | | 1.70 | 2.22 | 2.62 | | 3.12 | 3.50 | 4.30 | |
| | D | 350 | 4.32 [c] | | | | | 1.51 | 1.98 | | 2.58 | | 3.21 | | 3.46 |
| | A | 350 | 4.45 [c] | | | | | 1.65 | 2.10 | 2.43 | | 2.72 | 3.28 | 3.48 | |
| | A [d] | 350 | 5.00 [c] | 1.23 | 1.52 | 1.89 | 2.20 | 3.35 | | | | | | | |
| | A [e] | 350 | 5.00 [c] | 2.94 | 3.08 | 4.20 | 5.37 | 8.88 | | | | | | | |
| | A | 350 | 5.07 [c] | | | | | 1.77 | 1.99 | 2.27 | | 2.82 | 3.56 | 3.73 | |
| | A | 350 | 5.20 [c] | | | | | 1.95 | 2.31 | 2.75 | | 2.88 | 3.50 | 3.98 | |
| | D | 350 | 5.50 [c] | | | | | 1.93 | 2.34 | | 2.80 | 3.51 | | | 4.01 |
| | A [d] | 350 | 5.50 [c] | 1.26 | 1.51 | 1.87 | 2.10 | 3.45 | | | | | | | |
| | A [e] | 350 | 5.50 [e] | 2.94 | 3.03 | 4.28 | 5.25 | 8.60 | | | | | | | |
| | A | 350 | 5.78 [c] | | | | | 1.70 | 2.25 | 2.95 | | 3.20 | 3.20 | 4.12 | |
| | A | 350 | 6.40 [f] | | | | | 1.75 | 2.22 | 2.57 | | 2.88 | 3.84 | 4.12 | |
| | D | 350 | 6.50 [f] | | | | | 1.41 | 2.10 | 2.56 | | | 3.10 | | 3.30 |
| | A | 352 | 7.20 [f] | | | | | 1.20 | 1.70 | 2.40 | | 2.31 | 2.56 | 2.95 | |
| | A | 354 | 7.50 [f] | | | | | 1.40 | 1.72 | 2.06 | | 2.40 | 2.66 | 3.23 | |
| | A | 355 | 8.17 [f] | | | | | 0.90 | 1.19 | 1.34 | | 1.61 | 1.65 | 2.13 | |
| | A | 357 | 8.68 [g] | | | | | 0.66 | 0.93 | 1.14 | | 1.35 | 1.60 | 1.69 | |
| | A | 357 | 9.07 [g] | | | | | 0.75 | 1.06 | 1.12 | | 1.38 | 1.53 | 1.78 | |
| | A [d] | 357 | 9.30 [g] | 0.396 | 0.480 | 0.605 | 0.764 | 1.15 | | | | | | | |
| | A [e] | 357 | 9.30 [g] | 1.03 | 1.19 | 1.57 | 1.98 | 3.01 | | | | | | | |
| $B_{12a}$ ⟶ $B_{12r}$ | D | 357 | 9.40 [g] | | | | | 0.77 | 0.82 | 1.03 | | 1.18 | 1.30 | 1.43 | |
| $B_{12a}$ ⟶ $B_{12}$Cys | A | 357 | 9.40 [g] | | | | | 0.61 | 0.86 | 1.05 | | 1.15 | 1.19 | 1.44 | |
| $B_{12}$Cys ⟶ $B_{12r}$ | D | 362, 532 | 7.30 | | | | | 0.025 [h] | 0.030 [h] | | 0.029 [h] | | 0.032 [h] | | 0.035 [h] |
| | D | 362, 532 | 8.85 | | | | | 0.137 [h] | 0.197 [h] | | 0.224 [h] | | 0.289 [h] | | 0.330 [h] |
| | D | 362, 532 | 9.20 | | | | | 1.10 [h] | 1.50 [h] | | 1.75 [h] | | 1.98 [h] | | 2.31 [h] |
| | D | 362, 532 | 9.65 | | | | | 2.24 [h] | 2.90 [h] | | 3.42 [h] | | 4.02 [h] | | 4.65 [h] |
| $B_{12a}$ ⟶ $B_{12}$Cys | A | 350 | 5.07 [i] | | | | | 1.63 | 2.13 | 2.57 | | 2.88 | 3.12 | 3.85 | |
| | A | 350 | 5.07 [j] | | | | | 1.74 | 2.30 | 2.40 | | 2.70 | 3.20 | 3.82 | |
| | A | 352 | 7.20 [k] | | | | | 1.22 | 1.68 | 2.38 | | 2.46 | 2.56 | 2.91 | |
| | A | 352 | 7.20 [l] | | | | | 1.21 | 1.73 | 2.12 | | 2.41 | 2.70 | 2.95 | |
| | A | 357 | 9.07 [m] | | | | | 0.77 | 1.08 | 1.10 | | 1.40 | 1.55 | 1.76 | |
| | A | 357 | 9.07 [n] | | | | | 0.76 | 1.05 | 1.10 | | 1.40 | 1.50 | 1.75 | |

[a] In water at 25.0 °C, unless stated otherwise. [Vitamin $B_{12a}$] = $5.0 \times 10^{-5}$ mol dm$^{-3}$. Followed by use of the Cary 118 instrument. [b] A = Air saturated, D = degassed. [c] 0.10 mol dm$^{-3}$ Sodium acetate buffer. [d] At 35.0 °C. [e] At 45.0 °C. [f] 0.10 mol dm$^{-3}$ Sodium dihydrogenphosphate buffer. [g] 0.10 mol dm$^{-3}$ Sodium tetraborate buffer. [h] Average values from two wavelengths. [i] 0.30 mol dm$^{-3}$ Sodium acetate buffer. [j] 0.50 mol dm$^{-3}$ Sodium acetate buffer. [k] 0.30 mol dm$^{-3}$ Sodium dihydrogenphosphate buffer. [l] 0.50 mol dm$^{-3}$ Sodium dihydrogenphosphate buffer. [m] 0.050 mol dm$^{-3}$ Sodium tetraborate buffer. [n] 0.075 mol dm$^{-3}$ Sodium tetraborate buffer.

TABLE 3

Apparent kinetic and thermodynamic parameters for the reaction of L-cysteine with vitamin $B_{12a}$ in aqueous solutions [a]

| pH | Conditions [b] | $k_1^{app.}$/dm$^3$ mol$^{-1}$ s$^{-1}$ | $10^5k_{-1}^{app.}$/s$^{-1}$ | $10^{-4}K^{app.}$/dm$^3$ mol$^{-1}$ | pH | Conditions [b] | $k_1^{app.}$/dm$^3$ mol$^{-1}$ s$^{-1}$ | $10^5k_{-1}^{app.}$/s$^{-1}$ | $10^{-4}K^{app.}$/dm$^3$ mol$^{-1}$ | $k_5^{app.}$/s$^{-1}$ |
|---|---|---|---|---|---|---|---|---|---|---|
| 3.85 | A | 12.1 | | | 7.20 [c] | A | 9.85 | | | |
| 4.00 | A | | 5.41 [d] | 21.7 | 7.20 [e] | A | 9.60 | | | |
| 4.32 | D | 10.8 | | | 7.30 | D | | | | 0.041 |
| 4.45 | A | 10.5 | | | 7.50 | A | 9.50 | | | |
| 5.00 [f] | A | 22.8 | | | 8.17 | A | 5.80 | | | |
| 5.00 [g] | A | 67.0 | | | 8.40 | A | | 5.96 [d] | 8.38 | |
| 5.07 | A | 12.0 | | | 8.68 | A | 6.30 | | | |
| 5.07 [c] | A | 12.2 | | | 8.85 | D | | | | 0.940 |
| 5.07 [e] | A | 11.9 | | | 9.07 | A | 5.07 | | | |
| 5.20 | A | 11.5 | | | 9.07 [h] | A | 5.15 | | | |
| 5.50 | A | | 5.06 [d] | 23.2 | 9.07 [i] | A | 4.95 | | | |
| 5.50 | D | 11.5 | | | 9.20 | D | | | | 6.10 |
| 5.50 [f] | A | 22.6 | | | 9.30 [f] | A | 10.0 | | | |
| 5.50 [g] | A | 63.0 | | | 9.30 [g] | A | 26.0 | | | |
| 5.78 | A | 11.5 | | | 9.40 | A | 4.25 | | | |
| 6.40 | A | 13.2 | 5.43 [d] | 21.6 | 9.40 | D | 4.35 | | | |
| 7.20 | A | 9.75 | | | 9.65 | D | | | | 12.1 |

[a] In water at 25 °C and 0.1 mol dm$^{-3}$ buffer, unless stated otherwise; [Vitamin $B_{12a}$] = $5 \times 10^{-5}$ mol dm$^{-3}$. [b] A = Air saturated, D = degassed. [c] 0.3 mol dm$^{-3}$ buffer. [d] Calculated from the equilibrium constant and $k_1$. [e] 0.5 mol dm$^{-3}$ buffer. [f] At 35.0 °C. [g] At 45.0 °C. [h] 0.075 mol dm$^{-3}$ buffer. [i] 0.05 mol dm$^{-3}$ buffer.

$K^{app.} = k_1^{app.}/k_{-1}^{app.}$, thermodynamically from absorption spectroscopic data at different reactant concentrations. Because of the apparently high values of $K^{app.}$ and the rapid decomposition of the vitamin $B_{12a}$–L-cysteine complexes at approximately equimolecular reactant concentrations, equilibrium constants could not be obtained by the use of the Benesi–Hildebrand or analogous equations.[21]

Values for $K^{app.}$ were obtained, however, from observing absorbances due to the vitamin $B_{12a}$–L-cysteine complex in the presence of Na[SCN], $Na[N_3]$, and imidazole as competitors. Typically, $5.0 \times 10^{-5}$ mol $dm^{-3}$ vitamin $B_{12a}$ and $3.33 \times 10^{-2}$ mol $dm^{-3}$ sodium azide were mixed in the presence of 0.10 mol $dm^{-3}$ sodium acetate buffer at pH 5.5. This solution gave the limiting spectra of the vitamin $B_{12a}$–sodium azide complex.[1-7] Addition of L-cysteine ($1.6 \times 10^{-3}$—$5.0 \times 10^{-3}$ mol $dm^{-3}$) caused the absorbance at 357 nm to decrease. Plots of the left-hand side of equation (6) against $[NaN_3]$/[L-cysteine] allowed the calculation of the

$$\frac{[bzm\text{–}Co\text{–}N_3]}{[bzm\text{–}Co\text{–}L\text{-cysteine}]} = \frac{K^{app.}(B_{12}N_3)}{K^{app.}(B_{12}Cys)} \times \frac{[NaN_3]}{[L\text{-cysteine}]} \quad (6)$$

quotient of the equilibrium constant $K^{app.}(B_{12}N_3)/K^{app.}(B_{12}Cys)$. Typical plots of data according to equation (6) are illustrated in Figure 3. Taking a value of $3.2 \times 10^4$ $dm^3$ $mol^{-1}$ for $K^{app.}(B_{12}N_3)$ at pH 5.5,[6] a value of $2.4 \times 10^5$ $dm^3$ $mol^{-1}$ was obtained for the apparent equilibrium constant for the formation of the vitamin $B_{12a}$–L-cysteine adduct. This value was substantiated by carrying out analogously the competition between L-cysteine and $SCN^-$ and imidazole for vitamin $B_{12a}$ using values of 2 300 and $5.5 \times 10^2$ $dm^3$ $mol^{-1}$ for $K^{app.}(B_{12}SCN)$ and $K^{app.}(B_{12}im)$, and gave the values in $2.10 \times 10^5$ and $2.55 \times 10^5$ $dm^3$ $mol^{-1}$ for $K^{app.}(B_{12}Cys)$, respectively. Agreement among the $K^{app.}(B_{12}Cys)$ values, obtained by the use of three different

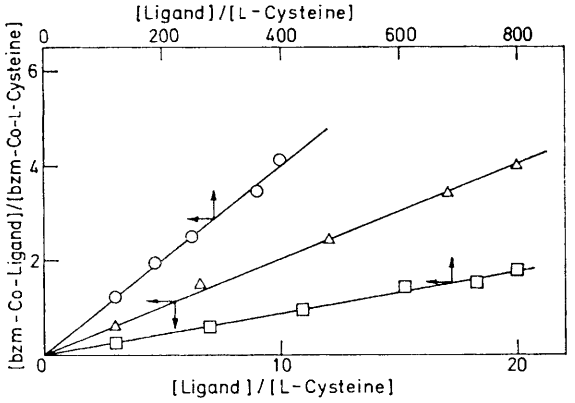



FIGURE 3 Competition plots for the formation of the vitamin $B_{12}$–L-cysteine adduct [equation (6)] at pH 5.5 using $SCN^-$ (○), $N_3^-$ (△), and imidazole (□) as competitors

ligands as competitors, is quite satisfactory and lends credence to this approach. Using values of the kinetically determined $k_1^{app.}$ in conjunction with $K^{app.}$, values for $k_{-1}^{app.}$ have been calculated and are given in Table 3.

[21] R. Foster, ' Organic Charge Transfer Complexes,' Academic Press, New York, 1969.
[22] G. Jung, E. Breitmaier, and W. Voelter, *Eur. J. Biochem.*, 1972, **24**, 438.

DISCUSSION

Absorption maxima and absorption coefficients of the thiol complexes of vitamin $B_{12a}$ (Table 1) follow the established pattern.[1,15] As expected for electronegative ligands, the absorption maxima of the $\gamma$ bands for bzm–Co–SHR are at higher wavelengths than that for bzm–Co–$OH_2$. This ' atypical ' spectrum has been taken to indicate the formation of Co–S bonds.[15] Lack of reaction of methionine and cystine with vitamin $B_{12a}$ also substantiates this mode of binding.

Apparent rate constants for the anation of vitamin $B_{12a}$ by L-cysteine, $k_1^{app.}$, are independent of the hydrogen-ion concentration. At higher pH values, however, $k_1^{app.}$ decreases sigmoidally with increasing hydroxide-ion concentration. The observed pH–rate profile (Figure 4)

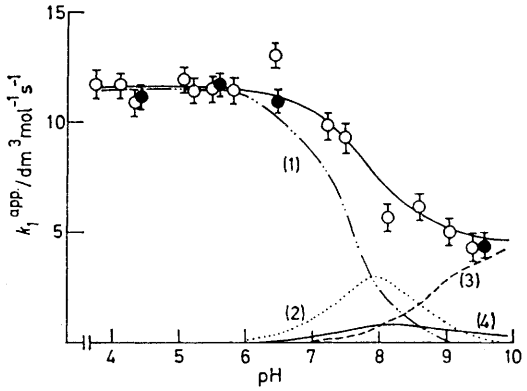
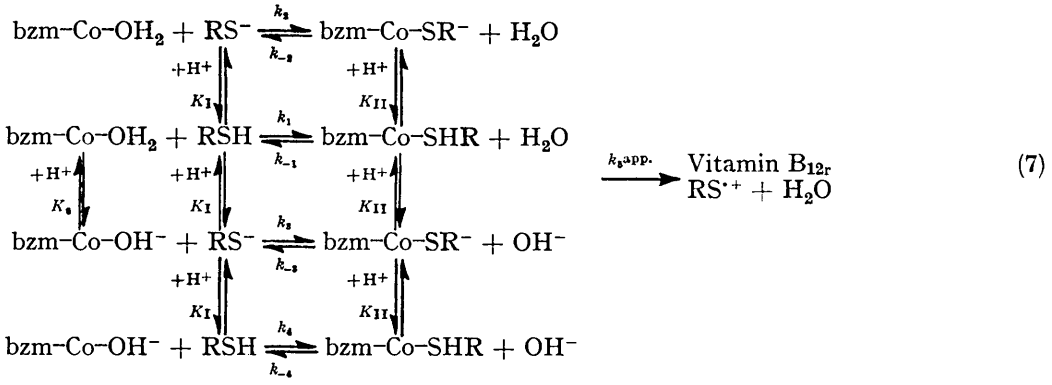



FIGURE 4 pH–rate profile for the interaction of L-cysteine with vitamin $B_{12a}$ in water. Experimental points for $k_1^{app.}$ are: (○), for air saturated; and (●), for degassed solutions. The solid line was calculated from equation (8). Contributions from $k_1$, $k_2$, $k_3$, and $k_4$ are given by lines (1), (2), (3), and (4), respectively

is explicable in terms of the different reactivities of bzm–Co–$OH_2$ and bzm–Co–$OH^-$ and L-cysteine and L-cysteinate ion. Although the sites of protonation of L-cysteine are not known with certainty,[22,23] the seven microscopic dissociation constants for the pH dependence of the concentrations of the eight species in solution have been calculated.[24] This information allows the assessment of the concentrations and reactivities of the thiol and thiolate anion at different pH values. If L-cysteinate ions are the only reacting species, values of $k_1^{app.}$ would approach $10^9$ $dm^3$ $mol^{-1}$ $s^{-1}$ in the pH 3.0—5.0 region. In the light of the available data for analogous anations ($k_1^{app.}$ values for a variety of ligands are $10^2$—$10^3$ $dm^3$ $mol^{-1}$ $s^{-1}$) [5-11] such a large rate constant is highly unlikely. The lack of reactivity of L-methionine, a neutral thiol analogue, can be rationalized in terms of the greater steric hindrance encountered in its reaction with aquocobalamin than that with cobaloxime.[1-4] At higher pH values, however, bzm–Co–$OH_2$ is ionized to bzm–Co–$OH^-$,[1-11] and the concentration of L-cysteinate ion becomes appreciable. The aquation rates, and hence

[23] G. Jung, E. Breitmaier, W. A. Gunzler, M. Ottnad, W. Voelter, and L. Flohe, *Proc. 16th Conf. German Soc. Biol. Chem.*, Tubingen, March 1973, George Thiene Publishers, Stuttgart, 1974.
[24] R. G. Kallen, *J. Amer. Chem. Soc.*, 1971, **93**, 6227.

the equilibrium constants for the formation of the L-cysteine complex of vitamin $B_{12a}$, also depend on the protonation equilibrium bzm-Co-$SR^-$ + $H^+$ $\overset{K_{II}}{\rightleftharpoons}$ bzm-Co-SRH. The scheme describes the complete mechanism for the interaction of L-cysteine with vitamin $B_{12a}$. Here $k_1$, $k_2$, $k_3$, and $k_4$ are the pH-independent rate constants for the formation of the vitamin $B_{12}$–L-cysteine complex and $k_{-1}$, $k_{-2}$, $k_{-3}$, and $k_{-4}$ are the corresponding aquation rate constants; $k_5^{app.}$ is the pH-dependent rate




(7)

constant for the production of vitamin $B_{12r}$. pH-Independent equilibrium constants are defined as $K_1 = k_1/k_{-1}$, $K_2 = k_2/k_{-2}$, $K_3 = k_3/k_{-3}$, and $K_4 = k_4/k_{-4}$. Equation (7) affords the expression of $k_1^{app.}$ at any pH value:

$$k_1^{app.} = \frac{k_1}{1 + (K_0/[H^+])}\left(\frac{[RSH]}{[RSH] + [RS^-]}\right) + \frac{k_2}{1 + (K_0/[H^+])}\left(\frac{[RS^-]}{[RSH] + [RS^-]}\right) + \frac{k_3}{1 + ([H^+]/K_0)}\left(\frac{[RS^-]}{[RSH] + [RS^-]}\right) + \frac{k_4}{1 + ([H^+]/K_0)}\left(\frac{[RSH]}{[RSH] + [RS^-]}\right) \quad (8)$$

Values of $k_1$, $k_2$, $k_3$, and $k_4$ (Table 4) were calculated from equation (8), by using $pK_0$ 7.60 and the appropriate thiol concentration. The concentrations of L-cysteine and L-cysteinate were calculated from the microscopic dissociation constants,[22-24] assuming that the four forms of the neutral thiols and the four forms of the thiolate ions have similar reactivities. Table 4 also contains data on the pH-independent equilibrium constants, $K_1$, $K_2$, $K_3$, and $K_4$. Values of $K_1$, $K_2$, $K_3$, and $K_4$ were obtained from the thermodynamically obtained $K^{app.}$ values (Table 3) by means of an equation analogous to (8) which includes $[OH^-]$ for $K_3$ and $K_4$. Individual contributions of $k_1$, $k_2$, $k_3$, and $k_4$ to $k_1^{app.}$ at different pH values are given in Figure 4. Below pH 6 the only important path for anation is governed by $k_1$. Titration of $5.0 \times 10^{-5}$ mol $dm^{-3}$ vitamin $B_{12}$–L-cysteine complex spectrophotometrically at 370 nm resulted in a $pK_{II}$ value of 10.9 ± 0.1.

Several points emerge from the data in Table 4. L-Cysteinate ion is 4—5 times more reactive than neutral L-cysteine both with bzm-Co-$OH_2$ ($k_2/k_1$ = 4.3) and with bzm-Co-$OH^-$ ($k_3/k_4$ = 5.0). Similarly, aquocobalamin is 8—9 times more reactive than hydroxocobalamin both with L-cysteine ($k_1/k_4$ = 8.8) and with L-cysteinate ion ($k_2/k_3$ = 7.7). Although rate constants for the anation of bzm-Co-$OH_2$ are always greater than those for bzm-Co-$OH^-$, binding of L-cysteine to bzm-Co-$OH^-$ occurs to an appreciable extent. It is recalled that reactions of imidazole, glycine, azide, and thiocyanate ions occur almost exclusively with bzm-Co-$OH_2$.[1-11] The rate constant for the aquation of bzm-Co-$SR^-$ is similar to that of bzm-Co-SHR either by water ($k_{-1} \approx k_{-2}$) or by $OH^-$ ($k_{-3} \approx k_{-4}$). Stability of the vitamin $B_{12}$–L-cysteine complexes is governed, therefore, by the rate of anation rather than by that of the aquation. The good agreement between the $K_2$ values determined in the present work ($1.4 \times 10^6$ $dm^3$ $mol^{-1}$) and that given in the literature ($10^6$ $dm^3$ $mol^{-1}$) [15] lends credence to our method of evaluating rate and equilibrium constants.

Activation parameters for the anation were calculated from the temperature dependencies of $k_1^{app.}$ at pH 5.0 and 5.5. At these hydrogen-ion concentrations the overall reaction is predominantly governed by $k_1$. The obtained values, $\Delta H^{\ddagger} = 14.9 \pm 0.3$ kcal $mol^{-1}$ and $\Delta S^{\ddagger} = -3.6 \pm 2.0$ cal $K^{-1}$ $mol^{-1}$, resemble closely those determined for the interaction of a variety of ligands with vitamin $B_{12a}$.[10] This is consistent with an $S_N1$ limiting type mechanism which involves the dissociation of water in the rate-limiting step or with outer-sphere complex formation in which a fast exchange occurs.[25]

TABLE 4

pH-Independent rate and equilibrium constants for the interaction of L-cysteine with vitamin $B_{12}$

| | | |
|---|---|---|
| $k_1$ = 11.5 $dm^3$ $mol^{-1}$ $s^{-1}$ | $k_{-1} = 5.22 \times 10^{-6}$ $s^{-1}$ | $K_1 = 2.2 \times 10^5$ $dm^3$ $mol^{-1}$ |
| $k_2$ = 50 $dm^3$ $mol^{-1}$ $s^{-1}$ | $k_{-2} = 3.57 \times 10^{-5}$ $s^{-1}$ | $K_2 = 1.4 \times 10^6$ $dm^3$ $mol^{-1}$ |
| $k_3$ = 6.5 $dm^3$ $mol^{-1}$ $s^{-1}$ | $k_{-3}$ = 250 $dm^3$ $mol^{-1}$ $s^{-1}$ | $K_3 = 2.6 \times 10^{-2}$ |
| $k_4$ = 1.3 $dm^3$ $mol^{-1}$ $s^{-1}$ | $k_{-4}$ = 197 $dm^3$ $mol^{-1}$ $s^{-1}$ | $K_4 = 6.6 \times 10^{-3}$ |

[25] C. H. Langford and H. B. Gray, 'Ligand Substitution Processes,' W. A. Benjamin, New York, 1965.

The prompt formation of vitamin $B_{12r}$, governed by $k_5^{app.}$ in equation (7), only proceeds in the absence of air. This reaction is, therefore, likely to involve free radicals and it is more complex than is indicated in equation (7).[16] The observed pseudo-first-order rate constants for the formation of vitamin $B_{12r}$ from bzm–Co–L-cysteine increase linearly with increasing L-cysteine concentration. The second-order rate constants for these reactions, $k_5^{app.}$, were obtained from good straight lines on plotting $k_\psi$ against L-cysteine concentration (Table 3). Values of $k_5^{app.}$ increase exponentially with increasing pH. At pH $< 9$, $k_5^{app.}$ is rate determining. Above pH 9.2, however, $k_5^{app.}$ becomes larger than $k_1^{app.}$ and the overall formation of vitamin $B_{12r}$ is increasingly being governed by the rate-limiting formation of bzm–Co–L-cysteine. Lack of data on $k_5^{app.}$ for other thiols does not allow a comparison of the relative efficiency of this process.

The present work has demonstrated the formation of vitamin $B_{12r}$ in the interaction of L-cysteine with vitamin $B_{12a}$. This finding is significant since it is analogous to the proposed first step in the enzymatic synthesis of methionine.[3] The spectrophotometric observation of vitamin $B_{12r}$ in enzymatic methyl transfer [26] supports this postulate. Interactions of other thiols with vitamin $B_{12a}$ and with alkyl cobalamins are being investigated mechanistically in our laboratories.

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[26] H. Rüdiger, *Eur. J. Biochem.*, 1971, **21**, 264.