Interaction of Vitamin B_{12a} with 8-Azaguanine and 6-Mercaptopurine: Kinetic and Thermodynamic Characterizations

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Rate constants for the formation, $k_1^{app.}$, and decomposition, $k_{-1}^{app.}$, of 6-mercaptopurine and 8-azaguanine adducts of vitamin B_{12a}, B_{12}–mpur and B_{12}–agua, and hence their stability constants $K^{app.} = k_1^{app.}/k_{-1}^{app.}$, have been determined in aqueous buffered solutions as a function of pH at 25.0 °C. The pH–rate profiles for the formation of B_{12}–mpur and B_{12}–agua are bell shaped with maxima at pH 7.5 and 7.0, respectively. Rate constants for the decomposition of B_{12}–mpur decrease curvilinearly with increasing pH, having a short plateau in the pH 7—9 region. $k_{-1}^{app.}$ Values for the decomposition of B_{12}–agua do not change between pH 6 and 8, but they increase exponentially with increasing hydrogen-ion concentration at pH <6. Kinetic treatment of the data in terms of dissociation constants for vitamin B_{12a}, the ligand, and the vitamin B_{12} complexes, and in terms of the reactivities of these species, affords pH-independent rate constants for the formation, k_1, and for the decomposition, k_{-1}, of these vitamin B_{12} complexes. k_1 and k_{-1} values for B_{12}–mpur are 800 $dm^3\ mol^{-1}\ s^{-1}$, and those for B_{12}–agua are 220 $dm^3\ mol^{-1}\ s^{-1}$ and $2.0 \times 10^{-2}\ s^{-1}$, respectively. The mechanism of these reactions and their pharmaceutical potential are discussed.

LIGAND-EXCHANGE reactions of vitamin B_{12a} (aquocobalamin, bzm–Co–OH_2) [equation (1)] have been

$$Bzm\text{–}Co\text{–}OH_2 + L \underset{k_{-1}^{app.}}{\overset{k_1^{app.}}{\rightleftharpoons}} Bzm\text{–}Co\text{–}L + H_2O; \quad K^{app.} = k_1^{app.}/k_{-1}^{app.} \quad (1)$$

extensively investigated.[1-3] Rate constants for the formation, $k_1^{app.}$, and dissociation, $k_{-1}^{app.}$, of vitamin B_{12} complexes have been determined for the ligands (L) $[N_3]^-$, $[OCN]^-$, $[SCN]^-$, $[SO_3]^{2-}$, $[NCO]^-$, I^-, Br^-, imidazole, glycine, and L-cysteine.[4-11] The interaction of some purines with vitamin B_{12a} has also been qualitatively established.[6,8,12]

The importance of 6-mercaptopurine (mpur) and 8-azaguanine (agua) in cancer treatment[13,14] and our interest in encapsulating these drugs and their complexes in liposomes[15-17] have prompted the present investigation. Rate and equilibrium constants for reaction (1) using mpur and agua as ligands are reported. Additionally, we have isolated the mpur and agua complexes of vitamin B_{12}.

The present study has also important bearings on the recognized role of vitamin B_{12}-dependent methionine synthetase in cancer chemotherapy.[18] Methionine synthetase catalyzes the reaction between 5-methyltetrahydrofolate and homocysteine to form tetrahydrofolate and methionine. In the absence of this enzyme, 5-methyltetrahydrofolate accumulates with the resulting inhibition of cell replication. The *in vivo* inhibition of methionine synthetase can be affected by the use of substrate or methylcobalamin analogues.[19,20] Transport of vitamin B_{12} and its derivatives into the cells is mediated through binding to serum protein, transcobalamin-II.[19,20] Transcobalamin-II binds vitamin B_{12} tightly but non-selectively; many vitamin B_{12} analogues are readily transported into the cell.[21] Apparently, binding of vitamin B_{12} derivatives to the protein occurs through the 5,6-dimethylbenzimidazole occupying the fifth coordination position, while ligands at the sixth position do not appreciably influence the interaction. A similar situation has been encountered in the interaction of vitamin B_{12} derivatives with aqueous micelles.[22-24] These corrinoids bind strongly to anionic micellar sodium dodecyl sulphate but do not interact with cationic micellar hexadecyltrimethylammonium bromide. By this analogy, the binding site at transcobalamin-II is likely to be negatively charged. 6-Mercaptopurine and 8-azaguanine complexes of vitamin B_{12} may have, therefore, dual roles. They act as inhibitors for

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methionine synthetase and purine antimetabolites. The experimental verification of these ideas must, surely, rest on an understanding of the basic chemistry involved. The primary purpose of the present work has been to obtain such an understanding.

EXPERIMENTAL

Best available grades of vitamin B_{12a} (E. Merck), 8-azaguanine (agua) (Calbiochem), and 6-mercaptopurine (mpur) (Nutritional Biochemical Co.) were used. The purity of these compounds was found to be satisfactory and they were used, therefore, as received. Stock solutions of mpur were deaerated by nitrogen bubbling and kept in the dark to prevent oxidation of the thiol. Using these precautions only a minimum of oxidation took place as established by the criteria of reproducible rate measurements and of spectrophotometric parameters.

The vitamin B_{12} complex of 8-azaguanine, B_{12}–agua, was isolated by treating vitamin B_{12a} (0.20 mmol) with agua (0.20 mmol) in water (200 cm^3) at room temperature for 24 h under vigorous stirring. The solvent was rotary-evaporated to dryness and the solid material (*ca.* 280 mg) separated from starting material by column chromatography (30 × 1.5 cm column) on silica gel using methanol as the eluant. Unchanged vitamin B_{12a} remained on the top of the column. The obtained material showed only one spot on thin-layer chromatography (pre-coated t.l.c. silica gel sheets, 60-F-254, E. Merck, using methanol as developer) with $R_f = 0.21$. The vitamin B_{12} complex of 6-mercaptopurine, B_{12}–mpur, was prepared by treating vitamin B_{12a} (0.20 mmol) with mpur (0.20 mmol) in water (50 cm^3), whose pH was adjusted to 8.5 by trace amounts of $K_2[CO_3]$, for 30 min at room temperature under vigorous stirring. The solvent was evaporated to dryness and the obtained solid was dried over P_4O_{10} *in vacuo*. Thin-layer chromatography (see above) showed that *ca.* 5% of unchanged aquocobalamin remained, but the low stability of B_{12}–mpur in water and in methanol precluded further purification.

All the other chemicals were the best available reagent grade. Water was deionized and distilled from all-glass equipment. . The pH of buffered solutions was measured by using a radiometer pHM-26 pH-meter. Sodium acetate (0.10 mol dm^{-3}, $Na[O_2CMe]$–$MeCO_2H$), sodium phosphate (0.67×10^{-2} mol dm^{-3}, $Na[H_2PO_4]$–$Na_2[HPO_4]$) and sodium tetraborate (0.40 mol dm^{-3}, $Na_2[B_4O_7]$) were used as buffers in the pH 4.00—5.49, 6.00—8.00, and 8.00—10.00 regions.

Spectrophotometric determinations were made on a Cary 118-C spectrophotometer whose cell compartment was thermostatted at 25.0 ± 0.1 °C. In general, the complete spectral range was recorded on the 0—1.0 A scale at a speed of 10 nm in^{-1} and 0.2 nm s^{-1}. The pK_a of agua was determined by spectrophotometric titration, recording the complete u.v. spectra of samples of identical concentration whose pH had previously been adjusted. Kinetic data were obtained on the Cary 118-C spectrophotometer and on a Durrum model 110 stopped-flow spectrophotometric system at 25.0 ± 0.1 °C. Some kinetic solutions were deaerated by purging with ultrapure nitrogen for 15 min. All the reactions were followed under pseudo-first-order conditions, concentrations of ligands being at least 25-fold greater than that of vitamin B_{12a}. Concentrations of vitamin B_{12a} were determined by measuring the optical density at 350 nm and by using the reported values of molar absorption coefficients.[10] Use of this technique allowed the determination of the true vitamin B_{12a} concentration, independent of the water of crystallization of our sample. The compounds mpur and agua were dried overnight *in vacuo* prior to making up stock solutions. Observed pseudo-first-order rate constants, k_ψ values, were calculated from plots of $\log(A_\infty - A_t)$ against time. Good linearity was observed in all cases up to 3—4 half-lives. k_ψ Values are considered to be accurate to ±3%. Second-order rate constants for complex formation, $k_1^{app.}$, and first-order rate constants for its decomposition, $k_{-1}^{app.}$, were calculated from k_ψ values at different ligand concentrations at each pH value. Calculations were carried out on a PDP-11 minicomputer (Digital Equipment Corp.) using a linear regression-analysis program. Correlation coefficients were better than 0.98.

RESULTS

Interaction of 6-Mercaptopurine with Vitamin B_{12a}.—The absorption spectra of vitamin B_{12a} in water (ε 2.6×10^4 at 350, 7.9×10^3 at 497, and 8.3×10^3 dm^3 mol^{-1} cm^{-1} at 523 nm)[10] undergoes pronounced changes on addition of dilute aqueous solutions of mpur. Absorbances at 350, 497, and 523 nm decrease with the concurrent appearance of absorbances at 370, 424, 534, and 560 nm. These spectral changes are indicative of the formation of a complex between vitamin B_{12a} and mpur. The shift of the absorption maximum of the γ band of vitamin B_{12a} (350 nm) to a higher wavelength (370 nm), as well as the concomitant appearance of several bands of comparable intensities in the 300—350 nm region, are characteristic for the formation of Co–S bonds.[25] Vitamin B_{12} complexes of L-cysteine, 2-mercaptoacetic acid, and 3-mercaptopropionic acid follow this pattern.[11] Figure 1 illustrates the differential spectra of B_{12}–mpur. It is seen that increasing ligand concentrations in the 4×10^{-4}—13×10^{-4} mol dm^{-3} range result in increasing absorbance at 376 and 434 nm. A saturation of absorbance is reached at 1.4×10^{-3} mol dm^{-3} mpur after which further addition of the ligand does not alter the absorption spectra. This behaviour corresponds to the equilibrium build-up of the B_{12}–mpur complex. Quantitation of the absorption data according to the Benesi–Hildebrand treatment[26] allows the calculation of the stability constant for complex formation [equation (2)], where ΔD and $\Delta\varepsilon$ are the

$$\frac{1}{\Delta D} = \frac{1}{K^{app.}\Delta\varepsilon[\text{bzm–Co–OH}_2][\text{mpur}]} + \frac{1}{\Delta\varepsilon[\text{bzm–Co–OH}_2]} \quad (2)$$

differences in absorbance and molar absorption coefficients between the uncomplexed vitamin B_{12a} and the B_{12}–mpur complex at a given concentration of mpur. Good linearity was obtained on treating the data according to equation (2) (Figure 1), substantiating the formation of 1 : 1 complexes. Similarities of the absorption spectrum of B_{12}–mpur to a number of vitamin B_{12}–thiol complexes and the observed 1.0 : 1.0 stoicheiometry of complex formation render unlikely the displacement of benzimidazole by mpur and agua. Substitution does occur by replacing the water molecule in the sixth co-ordination position. At pH 10, values for $K^{app.}$ and $\Delta\varepsilon$(376 nm) were calculated to be 2.3×10^3 dm^3 mol^{-1} cm^{-1}.

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Rate constants for the equilibrium attainments of B_{12}–mpur complex formation, k_ψ values, are given in Table 1 as functions of mpur concentrations and pH. Plots of k_ψ with the concurrent appearance of absorbances at 323, 356, 411, and 504 nm. These spectral changes are indicative of the formation of a vitamin B_{12}–8-azaguanine complex,

TABLE 1

Interaction of 6-mercaptopurine with vitamin B_{12a} in water at 25.0 °C

	pH [a]								
10^4[mpur] / mol dm^{-3}	6.03 [b]	6.50 [b]	6.99	7.45	8.00	8.51	9.01	9.49	10.0
	10^2k_ψ/s^{-1}								
1.99									0.362
2.50	6.03	10.7							
2.66									0.410
3.00	19.60	11.3	8.88	7.18	5.85	4.20	1.73	0.997	
3.50	21.40	11.7							
3.65									0.406
4.00	23.00	12.3	10.20	9.12	7.62	4.65	2.11	1.21	0.485
4.40	22.40	12.7							
4.65									0.488
5.00	20.40	13.2	12.30						0.488
5.98			13.90	12.30	10.70	6.03	2.71	1.43	0.570
6.65									0.657
6.98			16.10	14.90	12.80				
7.97			16.00	15.90	13.10	8.12	3.38	1.82	
8.97									
9.97				19.00	16.30	9.90	3.65	2.06	0.815
11.96						11.70	4.44	2.36	0.976
13.95									1.057

[a] Stoicheiometric [vitamin B_{12a}] = 8.25×10^{-6} mol dm^{-3}. Buffer for pH 6.03—7.45 was 6.67×10^{-2} mol dm^{-3} Na[H_2PO_4]; for pH 8.0—10.0 buffer was 0.40 mol dm^{-3} $Na_2[B_4O_7]$. All the experiments were carried out under nitrogen, unless stated otherwise. [b] In air-saturated solutions.

against mpur concentration were linear at each pH. Rate constants for the formation, $k_1^{app.}$, and decomposition, $k_{-1}^{app.}$, of the B_{12}–mpur complex were calculated from the gradients and intercepts of these linear relations using equation (3). Values for $k_1^{app.}$, $k_{-1}^{app.}$, and $K^{app.}$ ($K^{app.} = k_1^{app.}/k_{-1}^{app.}$) are collected in Table 2. Rate constants for

$$k_\psi = k_1^{app.}[\text{mpur}] + k_{-1}^{app.} \quad (3)$$

the decomposition of B_{12}–mpur were also determined directly by following the decomposition of the isolated complex in the appropriate buffer solution ($k_{-1}^{app.\prime}$ values in Table 2). The agreement between corresponding pairs of $k_{-1}^{app.}$ and $k_{-1}^{app.\prime}$ values is considered to be reasonable.

Interaction of 8-Azaguanine with Vitamin B_{12a}.—Addition of an aqueous solution of agua to vitamin B_{12a} also results in a marked alteration of the absorption spectrum of the corrinoid. Absorbances at 350, 497, and 523 nm decrease

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B_{12}–agua. Since the differential absorption spectra for the interaction 8-aza-adenine with vitamin B_{12a} [12,27] are similar to that shown in Figure 2, co-ordination of agua to

TABLE 2

Apparent rate and equilibrium constants for the interaction of 6-mercaptopurine with vitamin B_{12a} in water at 25.0 °C

pH	$k_1^{app.}$ / dm^3 mol^{-1} s^{-1}	$10^3k_{-1}^{app.}$ [a] / s^{-1}	$10^3k_{-1}^{app.}$ [b] / s^{-1}	$10^3K^{app.}$ [c] / dm^3 mol^{-1}	$10^{-3}K^{app.}$ [c] / dm^3 mol^{-1}
4.00			9 340.0		
5.00			1 580.0		
6.03		205.0	106.0		
6.50	100.0	82.0	46.8	1.22	2.07
6.99	157.0	42.8	25.7	3.66	7.08
7.45	171.0	22.7	16.3	7.53	10.4
8.00	148.0	16.8	14.7	8.82	10.1
8.51	85.9	12.9	10.4	6.64	8.78
9.01	28.9	9.37	7.32	3.09	4.10
9.49	15.1	5.70	3.74	2.64	3.93
10.00	6.06	2.22	1.73	2.73	3.44

[a] Obtained from the intercepts of plots of k_ψ against [mpur]. [b] Obtained from following the decomposition of the isolated bzm–Co–mpur complex in 0.33% (v/v) MeOH under anaerobic conditions. [c] Calculated from $K^{app.} = k_1^{app.}/k_{-1}^{app.}$ or $K' = k_1^{app.}/k_{-1}^{app.\prime}$.

vitamin B_{12a} is likely to occur through one of the heterocyclic nitrogens without removal of the benzimidazole in the fifth co-ordination position. The pK_a for protonation of agua was determined to be 6.20 by spectrophotometric titration at 247 nm. The kinetic and thermodynamic behaviour for the formation of the B_{12}–agua complex follow a similar pattern to those observed for B_{12}–mpur. Treatment of the spectrophotometric data for B_{12}–agua formation as a function of agua concentration at pH 4 by an equation analogous to (2) yielded a good straight line (Figure 2) from which $K^{app.} = 5.77 \times 10^3$ dm^3 mol^{-1} and $\Delta\varepsilon = 8.27 \times 10^3$ dm^3 mol^{-1} cm^{-1} at 363 nm were calculated.

Rate constants for the equilibrium attainment for the formation of B_{12}–agua, k_ψ values, as functions of agua concentration and pH are in Table 3. Treating the data according to an equation analogous to (3) resulted in good

straight lines, from the gradients of which $k_1^{app.}$ were calculated (Table 4). The intercepts of these lines were very

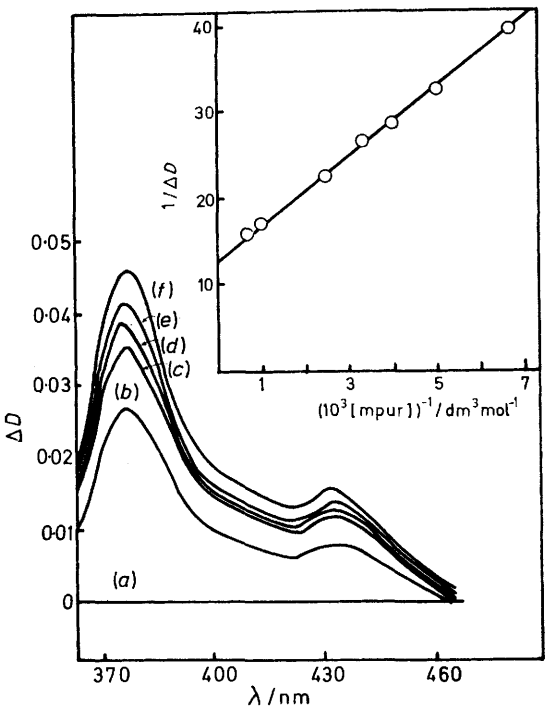


FIGURE 1 Differential absorption spectra of 8.25×10^{-6} mol dm^{-3} vitamin B_{12a} and 0 (*a*), 1.5×10^{-4} (*b*), 2.0×10^{-4} (*c*), 2.5×10^{-4} (*d*), 3.0×10^{-4} (*e*), and 4.0×10^{-4} mol dm^{-3} (*f*) 6-mercaptopurine at pH 10.0 (0.40 mol dm^{-3} borate buffer) and 25.0 °C. Four optically matched 1.00-cm cells were used; two were placed in the reference beam and two in the sample beam. One of the cells in the reference beam contained vitamin B_{12a}, while appropriate concentrations of mpur were placed in the other. The first cell in the sample beam contained mixtures of vitamin B_{12a} and mpur in concentrations identical to that in the second cell placed in the reference beam. The second cell in the sample compartment contained only aqueous buffer solution. Inset is a plot of the data according to equation (2)

small, thus $k_{-1}^{app.}$ could not be meaningfully calculated from k_ψ values. Rate constants for the decomposition of the

TABLE 3

Interaction of 8-azaguanine with vitamin B_{12a} in water at 25.0 °C

	pH *						
10^4[agua]/mol dm^{-3}	4.00	4.97	5.49	6.00	7.00	7.50	8.00
	$10^2 k_\psi$/s^{-1}						
2.50	2.83	2.09	2.65	3.46	4.23	3.28	1.73
3.00		2.48	2.81	4.04	4.92	4.13	2.59
3.50	3.40	2.87	3.79	4.56	5.71	4.23	2.55
4.00	3.70	3.03	3.85	5.54	6.42	6.03	2.85
4.50	4.06	3.27	4.44	6.13	6.88	6.73	3.94
5.00	4.13	3.96	4.33	6.93	7.83	6.28	4.15

* Stoicheiometric [vitamin B_{12a}] = 8.25×10^{-6} mol dm^{-3}. Buffer for pH 4.0—5.49 was Na[O_2CMe]–MeCO$_2$H (0.10 mol dm^{-3}); for pH 6.00—8.00 the buffer was Na[H_2PO_4]. All the experiments were carried out on air-saturated solutions.

isolated B_{12}–agua, $k_{-1}^{app.\prime}$ values, were determined, however, and are given in Table 4. Stability constants, calculated from $K^{app.} = k_1^{app.}/k_{-1}^{app.\prime}$, are also in Table 4.

DISCUSSION

pH-Rate profiles for the formation of B_{12}–mpur and B_{12}–agua complexes are similar (Figures 3 and 4).

TABLE 4

Apparent rate and equilibrium constants for the interaction of 8-azaguanine with vitamin B_{12a} in water at 25.0 °C

pH	$10^{-2}k_1^{app.}$/dm^3 mol^{-1} s^{-1}	$10^3 k_{-1}^{app.}$ [a]/s^{-1}	$10^{-5}K^{app.}$ [b]/dm^3 mol^{-1}
4.00	0.558	11.6	0.048 1
4.97	0.746	2.77	0.269
5.49	0.930	1.43	0.650
6.00	1.36	1.11	1.26
7.00	1.44	0.915	1.57
7.50	1.30	0.840	1.55
8.00	0.993	0.880	1.13

[a] Obtained by following the decomposition of the isolated bzm–Co–agua complex. [b] Calculated from $K^{app.} = k_1^{app.}/k_{-1}^{app.}$.

Decreasing the hydrogen-ion concentration of the medium, up to pH 7.5, results in an increase of the apparent rate constant for the formation of B_{12}–mpur.

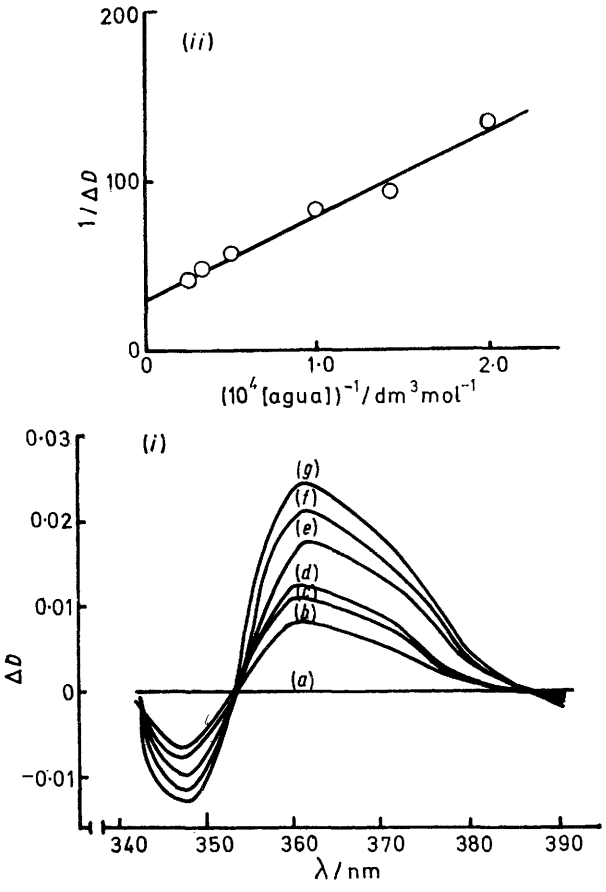


FIGURE 2 (*i*) Differential absorption spectra of 8.25×10^{-6} mol dm^{-3} vitamin B_{12a} and 0 (*a*), 5.0×10^{-5} (*b*), 7.0×10^{-5} (*c*), 1.0×10^{-4} (*d*), 2.0×10^{-4} (*e*), 3.0×10^{-4} (*f*), and 4.0×10^{-4} mol dm^{-3} (*g*) 8-azaguanine at pH 4.0 (0.10 mol dm^{-3} acetate buffer) and 25.0 °C. Procedure as in Figure 1. (*ii*) A plot of the data according to equation (2)

At pH 7.5 a rate maximum is attained after which the rate decreases (left-hand side of Figure 4). A similar pH

dependency has been observed for the formation of B_{12}–agua with a rate maximum centred around pH 7 (insert in Figure 3).

Conversely, pH–rate profiles for the decomposition of B_{12}–mpur and B_{12}–agua are dissimilar (Figures 3 and 4). In the pH 6—8 region, $k_{-1}^{app.}$ for the decomposition of B_{12}–agua is independent of the hydrogen-ion concentration. Increasing the latter causes the decomposition rate to increase logarithmically (Figure 3). $k_{-1}^{app.}$ Values for the decomposition of B_{12}–mpur change little between pH 7 and 9 but they increase with increasing $[H^+]$ on both the acid and basic side of this short plateau (right-hand side of Figure 4).

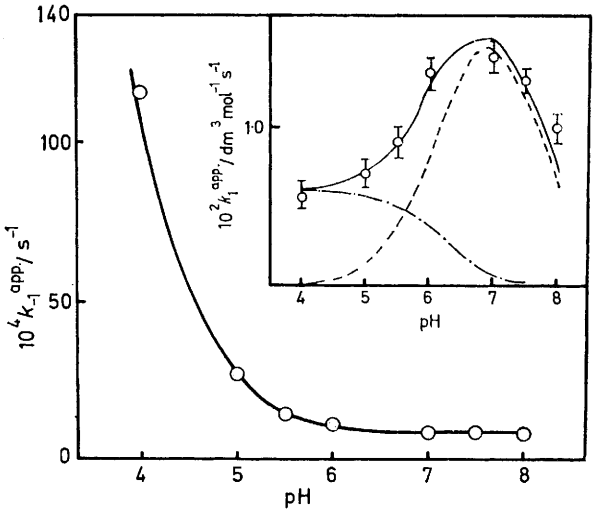
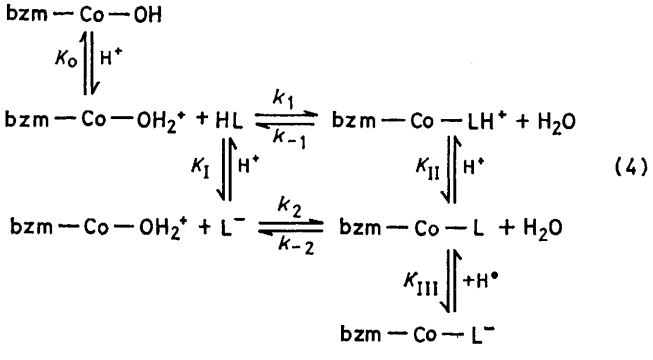


FIGURE 3 Experimental rate constants for the aquation of the isolated vitamin B_{12a} complex (○) as a function of pH at 25.0 °C. The full line was calculated by means of equation (6). Inset: experimental rate constants for the anation of vitamin B_{12a} by 8-azaguanine as a function of pH at 25.0 °C. The lines were calculated by means of equation (5). The full line represents $k^{app.}$ calculated, while the separate contributions due to k_1 and k_2 are indicated by — — — and — · — · —, respectively

Equilibrium constants for the formation of B_{12}–mpur and B_{12}–agua are described by bell-shaped pH dependences (not shown) having maxima at pH 7.5 and 7.0, respectively.



(4)

The dependences of the rate and equilibrium constants on pH can be rationalized by considering the dissociation constants of vitamin B_{12}, mpur, agua, B_{12}–mpur, and B_{12}–agua, as well as the reactivities of the different species involved. The complete reaction scheme is described by equation (4) assuming that vitamin B_{12b} (bzm–Co–OH) is not reactive. This assumption is justified since the reaction of bzm–Co–OH only becomes important at high pH where the observed rate constants decrease with increasing hydroxide-ion concentration.

Equations (5) and (6) describe the observed rate constants for the formation and decomposition of the vitamin B_{12} complexes at any pH value (see Appendix).

$$k_1^{app.} = \frac{k_1}{(1 + K_0[H^+]^{-1})(1 + K_I[H^+]^{-1})} + \frac{k_2}{(1 + K_0[H^+]^{-1})(1 + [H^+]K_I^{-1})} \quad (5)$$

$$k_{-1}^{app.} = \frac{k_{-1}}{1 + K_{II}[H^+]^{-1}} + \frac{k_{-2}}{1 + [H^+]K_{II}^{-1} + K_{III}[H^+]^{-1}} \quad (6)$$

K_0, K_I, K_{II}, and K_{III} are dissociation constants for aquocobalamin, for the attacking ligand, and for the different protonated forms of the vitamin B_{12} complex as defined in equation (4). The value for pK_0 has been reported to be 7.5—7.7.[1,22] pK_I Values of 7.6 (ref. 28) and 6.2 have been used for mpur and agua, respectively. Values for K_{II} and K_{III} were used as adjustable parameters to fit our experimental data. Best fits were obtained taking pK_{II} 4.0 and pK_{III} 9.0 for B_{12}–mpur, and pK_{II} 4.0 for B_{12}–agua. The constancy of $k_{-1}^{app.}$ in the range pH 6—8 implies that $K_{III}/[H^+] \ll 1$; therefore, in the case of the decomposition of B_{12}–agua, pK_{III} need not be considered and equation (6) simplifies to (7). Using these parameters, pH dependences of

$$k_{-1}^{app.} = \frac{k_{-1}([H^+]/K_{II}) + k_{-2}}{(1 + [H^+]K_{II}^{-1}} \quad (7)$$

$k_1^{app.}$ and $k_{-1}^{app.}$ have been calculated and are indicated as full lines in Figures 3 and 4. Satisfactory agreements between the experimentally obtained and calculated pH–rate profiles are evident and substantiate the validity of the assumptions involved in using equations (5)—(7). The contributions of HL and L^- to the overall reactivities of mpur and agua are also indicated in Figures 3 and 4.

Table 5 contains the calculated pH-independent rate and equilibrium constants [k_1, k_2, k_{-1}, k_{-2}, K_1, and K_2 as defined in (4)] for the interaction of mpur and agua with vitamin B_{12a}. Reactivities of unprotonated mpur and agua toward vitamin B_{12a} are seen to be greater than those of their protonated species. Decomposition of the protonated vitamin B_{12} complexes (bzm–Co–LH^+), on the other hand, is more ready than that of their unprotonated analogues. These rate effects are also manifested in the equilibrium constants. For both ligands $K_2 \gg K_1$. Analogies may be drawn between the greater stability of bzm–Co–L compared to bzm–Co–

[28] J. J. Fox, I. Wempen, A. Hampton, and I. L. Doerr, *J. Org. Chem.*, 1958, **80**, 1669.

LH^+ and the release of benzimidazole from the fifth co-ordination position of Co on protonation.

Available data for the interaction of ligands with either by an S_N1 limiting type mechanism, or in terms of an outer-sphere–inner-sphere complex formation in which a fast exchange occurs.[29,30]

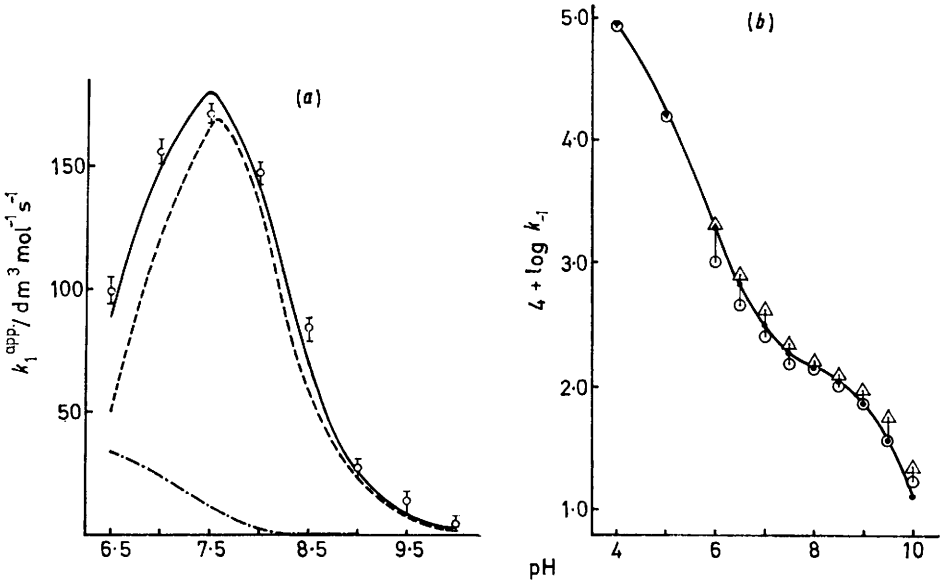


FIGURE 4 (*a*) Experimental rate constants for the anation of vitamin B_{12a} by 6-mercaptopurine as a function of pH at 25.0 °C. The lines were calculated by the use of equation (5). The full line represents $k_1^{app.}$ calculated, while the separate contributions due to k_1 and to k_2 are indicated by — — — and — · — · —, respectively. (*b*) Experimental rate constants for the equation of the *in situ* formed (△) and the isolated (○) B_{12}–mpur complex as a function of pH at 25.0 °C. The full line was calculated by means of equation (6)

vitamin B_{12a} are collected in Table 5. 6-Mercaptopurine and agua behave as any other ligand. Indeed, rate constants for anation (k_2 values in Table 5) are relatively independent of the nature of the incoming nucleophile.

Formation of stable, readily isolable, vitamin B_{12} complexes of purine antimetabolites has been demonstrated in the present study. The effective binding of these complexes to transcobalamin-II ensures their

TABLE 5

Ligand-exchange reactions of vitamin B_{12a} [a]

Ligand, L	k_1	k_2	k_{-1}	k_{-2}	K_1	K_2	Ref.
	dm^3 mol^{-1} s^{-1}		s^{-1}		dm^3 mol^{-1}		
mpur	40.0	800	18.4	1.4×10^{-2}	2.17	5.71×10^4	*b*
agua	62.0	220	2.0×10^{-2}	8.8×10^{-4}	3.1×10^3	2.5×10^6	*b*
L-Cysteine	11.5	50	5.2×10^{-5}	3.6×10^{-5}	2.2×10^5	1.4×10^6	23
$[SCN]^-$		7.1×10^3		1.8 ± 0.6		3.9×10^3	4
		2.3×10^3		1.8		1.2×10^3	9
$[N_3]^-$	100.0	1.7×10^3	0.7	0.03	1.4×10^2	5.66×10^4	5
		1.2×10^3		2.9×10^{-2}		5.6×10^4	9
$[NCO]^-$		7.3×10^2		0.95		7.7×10^2	5
		4.7×10^2		1.1		5.3×10^2	9
$[CN]^-$		1.5×10^3		10^{-9}		1.5×10^{12}	5
I^-		1.4×10^3		3.5×10^1		3.2×10^1	9
Br^-		1.0×10^3		5.9×10^2		1.9	9
Imidazole		27 ± 6		6×10^{-4}		4.5×10^4	5
$[S_2O_3]^{2-}$		2.0×10^2		3.5×10^{-2}		7.3×10^3	9
$[SO_3]^{2-}$		$\simeq 2 \times 10^2$		$\simeq 1 \times 10^{-5}$		2.2×10^7	9

[a] See (4) for definitions of k_1, k_2, k_{-1}, k_{-2}, K, and K_2. [b] This work.

These results, coupled with the observed spectral similarities between B_{12}–mpur and a variety of vitamin B_{12}–thiol complexes (see above),[11] and those between the 8-azaguanine and 8-aza-adenine complexes of vitamin B_{12a}, rule out the proposed charge-transfer complex formation between vitamin B_{12a} and purine derivatives.[12,27] Even though our data can be explained transportation into damaged cells.[18] Rate and equilibrium constants, determined in the present study, have provided vital information on the stabilities of these complexes through their passage into cells. Ideally, the maximum stability should correspond to the physiological pH. B_{12}–mpur and B_{12}–agua have maximum stabilities at the physiological pH since $(pK_0 + pK_1)/2 \simeq$

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

[30] F. Basolo and R. G. Pearson, 'Mechanisms of Inorganic Reactions,' Wiley, New York, 1967.

7 for these ligands for the equilibria given in (4). Pharmacological ramifications of the present work are being actively pursued in our laboratories.

APPENDIX

The rate of product formation, for the reaction described by (4) is given by (A1)—(A6) where $[B_{12}]_T$ = [bzm–Co–OH_2^+] + [bzm–Co–OH], $[L]_T$ = [HL] + $[L^-]$, and $[B_{12}–L]_T$ = [bzm–Co–LH^+] + [bzm–Co–L] + [bzm–Co–L^-].

$$dx/dt = k_1[\text{bzm–Co–OH}_2^+][\text{HL}] + k_2[\text{bzm–Co–OH}_2^+][L^-] - k_{-1}[\text{bzm–Co–LH}^+] - k_{-2}[\text{bzm–Co–L}] \quad (A1)$$

$$[\text{bzm–Co–OH}_2^+] = [B_{12}]_T/(1 + K_0[H^+]^{-1}) \quad (A2)$$

$$[\text{HL}] = [L]_T/(1 + K_I[H^+]^{-1}) \quad (A3)$$

$$[L^-] = [L]_T/(1 + [H^+]K_I^{-1}) \quad (A4)$$

$$[\text{bzm–Co–LH}^+] = \frac{[B_{12}–L]_T}{1 + [H^+]K_{II}^{-1} + K_{III}[H^+]^{-1}} \quad (A5)$$

$$[\text{bzm–Co–L}] = \frac{[B_{12}–L]_T}{1 + [H^+]K_{II}^{-1} + K_{III}[H^+]^{-1}} \quad (A6)$$

$$\frac{dx}{dt} = \frac{k_1[B_{12}]_T[L]_T}{(1 + K_0[H^+]^{-1})(1 + K_I[H^+]^{-1})} + \frac{k_2[B_{12}]_T[L]_T}{(1 + K_0[H^+]^{-1})(1 + [H^+]K_I^{-1})} - \frac{k_{-1}[B_{12}–L]_T}{1 + K_{II}[H^+]^{-1}} - \frac{k_{-2}[B_{12}–L]_T}{1 + [H^+]K_{II}^{-1} + K_{III}[H^+]^{-1}} \quad (A7)$$

Substituting equations (A2)—(A6) into (A1) yields (A7) which can be rearranged to (A8), which corresponds to equations (5) and (6) in the text.

Further, equation (1) can be rewritten as (A9) and hence

$$\frac{dx}{dt} = \left\{\frac{k_1}{(1 + K_0[H^+]^{-1})(1 + K_I[H^+]^{-1})} + \frac{k_2}{(1 + K_0[H^+]^{-1})(1 + [H^+]K_I^{-1})}\right\}[B_{12}]_T[L]_T - \left(\frac{k_{-1}}{1 + K_{II}[H^+]^{-1}} + \frac{k_{-2}}{1 + [H^+]K_{II}^{-1} + K_{III}[H^+]^{-1}}\right)[B_{12}–L]_T \quad (A8)$$

$$\underset{(a-x)}{\text{Total } B_{12}} + \text{total ligand} \underset{k_{-1}^{app.}}{\overset{k_1^{app.}}{\rightleftharpoons}} \underset{x}{\text{Total } (B_{12}–L)} \quad (A9)$$

we obtain (A10) and (A11). Since at equilibrium we have (A12), expression (A13) follows. Substituting (A13) into

$$dx/dt = k_1^{app.}[L]_T(a - x) = k_{-1}^{app.}x \quad (A10)$$

$$dx/dt = k_1^{app.}[L]_T a - (k_1^{app.}[L]_T - k_{-1}^{app.})x \quad (A11)$$

$$\frac{x_e}{a - x_e} = \frac{k_1^{app.}[L]_T}{k_{-1}^{app.}} \quad (A12)$$

$$k_1^{app.}[L]_T a = x_e(k_1^{app.}[L]_T + k_{-1}^{app.}) \quad (A13)$$

equation (A11) leads to (A14), which can be integrated to (A15), which corresponds to equation (3) in the text.

$$dx/dt = (k_1^{app.}[L]_T + k_{-1}^{app.})(x_e - x) \quad (A14)$$

$$k_1^{app.}[L]_T + k_{-1}^{app.} = (1/t)\ln[x_e/(x_e - x)] \quad (A15)$$

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