

Evidence for the Unexpected Associative Displacement of Adenosyl by Cyanide in Coenzyme B₁₂

Nicola E. Brasch, Mohamed S. A. Hamza,¹ and Rudi van Eldik*

Institute for Inorganic Chemistry, University of Erlangen–Nürnberg, Egerlandstrasse 1, 91058 Erlangen, Germany

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The reaction of coenzyme B₁₂ (adenosylcobalamin) with cyanide has been reinvestigated in detail using spectroscopic and kinetic techniques. It has been shown that this reaction proceeds in one kinetically observable step, contradicting previous findings, with rate-determining attack of the first cyanide ($k = (7.4 \pm 0.1) \times 10^{-3} \text{ M}^{-1} \text{ s}^{-1}$, 25.0 °C, $I = 1.0 \text{ M}$ (NaClO₄)). The activation parameters were found to be $\Delta H^\ddagger = 53.0 \pm 0.6 \text{ kJ mol}^{-1}$, $\Delta S^\ddagger = -127 \pm 3 \text{ J mol}^{-1} \text{ K}^{-1}$ and $\Delta V^\ddagger = -10.0 \pm 0.4 \text{ cm}^3 \text{ mol}^{-1}$, suggesting an associative displacement mechanism. It is postulated that attack of the first cyanide occurs at the β -(5'-deoxy-5'-adenosyl) site rather than at the α -dimethylbenzimidazole site.

Introduction

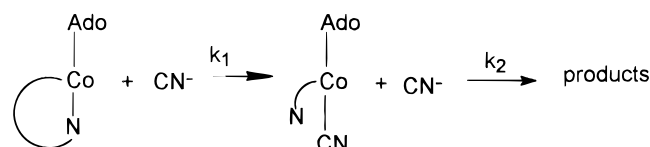
Since the discovery of the Co–C bond in adenosylcobalamin by Hodgkin in 1961,² there has been considerable interest in the chemistry of the cleavage of this bond, which is extremely rare in nature. Vitamin B₁₂ has three biologically active forms, but only methylcobalamin (MeCbl) and adenosylcobalamin (AdoCbl = coenzyme B₁₂), which contain the Co–C bond, are essential for a number of enzyme reactions.³ An important difference between the AdoCbl- and MeCbl-dependent enzyme reactions is that the Co–C bond is homolytically cleaved for the AdoCbl-dependent isomerases and heterolytically cleaved for the MeCbl-dependent transferases.

A considerable amount of effort has been put into trying to understand what causes the 10¹² enhancement of the Co–C cleavage rate of AdoCbl in the presence of the enzyme,⁴ and it is now generally agreed that steric distortion and crowding at the β site play a major role. AdoCbl is stable in neutral solution but is decomposed in strong acid and base in addition to being extremely light sensitive under all conditions.⁵ The Co–C bond can also be cleaved at elevated temperatures.⁶

Unlike MeCbl,^{7a} the Co–C bond of AdoCbl can be cleaved in mildly alkaline solution by cyanide at ambient temperature. The reaction between coenzymes and cyanide has been recognized for several decades, and it was even used as a method of characterization of cobalamins before the advent of X-ray

crystallography and NMR spectroscopy.⁸ The reaction proceeds via heterolytic cleavage of the Co–C bond and the reaction products were isolated and characterized in the 1960s as dicyanocobalamin ((CN)₂Cbl[−]), adenine, and the cyanohydrin of D-erythro-2,3-dihydroxy-4-pentenal.⁹ The decomposition of AdoCbl by cyanide is unexpected, as apart from thiols,⁵ as far as we are aware there are no other nucleophiles known that are capable of cleaving the Co–C bond of AdoCbl or MeCbl. This reaction has therefore been investigated by a number of researchers,^{5,7–12} especially since the reaction between cyanide and cobalamins provides a convenient means for studying factors that control the rate of heterolytic cleavage of the Co–C bond, such as the degree of polarization of the Co–C bond electron density toward the C atom.¹⁰

There are discrepancies in the literature as to the mechanism of cyanation. In the most recent study, the authors claim that two reactions are kinetically observable ($k_{\text{obs}(1)} = 2.9 \times 10^{-2} \text{ s}^{-1}$, $k_{\text{obs}(2)} = 8.4 \times 10^{-4} \text{ s}^{-1}$, 0.1 M KCN, pH 10.5, 0.1 M buffer (NaHCO₃), 25 °C) and propose the following reaction mechanism.⁷



Others have found that only one reaction is observable.¹¹ There is also disagreement as to whether the cyanide first attacks the β -(5'-deoxy-5'-adenosyl) (=adenosyl, β -Ado)^{9,10} or α -5,6-dimethylbenzimidazole (α -DMBI) site,^{5,7} although the latter is preferred by the majority of authors. No attempt has been made

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to characterize the reaction intermediate. Only qualitative studies on the effect of cyanide concentration on the reaction have been performed, and no activation parameters have been measured. This has led us to re-examine this reaction in more detail, and our findings are presented below.

Experimental Section

General Information. Adenosylcobalamin (>96%) was obtained from Fluka. All other chemicals were of AR grade and supplied by either Merck (NaClO₄, NaCN) or Aldrich (TES, CHES, CAPS, TAPS buffers).

Solution pH was measured at 25.0 °C using a pH 537 WTW microprocessor pH meter equipped with an Ingold 402.M657 electrode. A saturated NaCl solution was used for the salt bridge solution, and the electrode was standardized using standard pH 6.86 and 9.18 buffer solutions. Measurement in alkaline solution was carried out under a N₂ atmosphere. Solutions of pH ≥ 11 were prepared from stock NaOH solutions.

The acidity constant of HCN was determined by potentiometric titration. NaCN solution (70 mL; 1.01 × 10⁻² M, I = 1.0 M (NaClO₄)) was titrated with 0.20–0.30 mL aliquots of a HClO₄ solution (0.205 M, I = 1.0 M (NaClO₄)) at 25.0 °C.

UV–visible spectra were measured using a Cary 1 spectrophotometer equipped with a thermostated cell compartment (±0.1 °C). Experiments at elevated pressure were performed using a self-built high-pressure unit¹³ in conjunction with a Shimadzu UV-2101 PC spectrophotometer. The cell compartment was thermostated at 25.0 ± 0.1 °C. All solutions were prepared in the presence of air.

Experimental errors are given as one standard deviation.

Kinetic Measurements (UV–visible Spectroscopy). Unless stated otherwise, an AdoCbl solution of the desired pH ([AdoCbl] ~ 8 × 10⁻⁵ M, (0.10 M buffer), I = 1.0 M (NaClO₄)) was mixed with a NaCN solution of the same pH ([NaCN] = 1.00 × 10⁻²–1.00 M; (0.10 M buffer), I = 1.0 M NaClO₄) in a tandem cuvette and the absorbance of the mixture monitored spectrophotometrically at λ = 367 nm. Buffers were only used for pH ≤ 11.0 solution, and control reactions in the absence of buffer showed that buffer catalysis was negligible. Complete spectra (300–600 nm) were also frequently measured to check that only one reaction was occurring; i.e., clean isosbestic points were observed. AdoCbl solutions were prepared in a dark room using red light and were always protected from light during transportation to the spectrophotometer due to their known light sensitivity.^{3b,5} Preliminary experiments demonstrated that decomposition of AdoCbl by the light beam of the spectrophotometer was negligible. Data were fitted using the Olis data fitting routine OLIS-KINFIT available from On-Line Instrument Systems Inc.

NMR Measurements. ¹H NMR spectra were obtained on a Bruker Avance DRX 400 MHz WB spectrometer equipped with a 5 mm thermostated (25.0 °C) probe. Solutions were prepared in D₂O and TSP was used as a reference. Microliter volumes of concentrated NaOH or HClO₄ solutions were used to obtain the desired solution pH. Exact experimental conditions are detailed in the figure captions.

Results

Figure 1a shows typical UV–visible spectra observed (25.0 °C, λ = 300–650 nm) when an AdoCbl solution is mixed with a cyanide solution ([CN⁻]_f = 0.100 M, pH 11.0, I = 1.0 M (NaClO₄); *f* is the final concentration in reaction mixture) using a tandem cuvette. A comparison of the spectrum before and directly after mixing (~10 s) gave identical spectra and thus demonstrated that negligible reaction had occurred within this time. The reaction was followed for six half-lives and gave clean isosbestic points at 347, 390, and 535 nm. Figure 1b gives a plot of absorbance at λ = 367 nm versus time for these spectra. The data were fitted to a single exponential using the Olis kinetic data fitting routine, giving *k*_{obs} = (7.59 ± 0.08) × 10⁻⁴ s⁻¹.

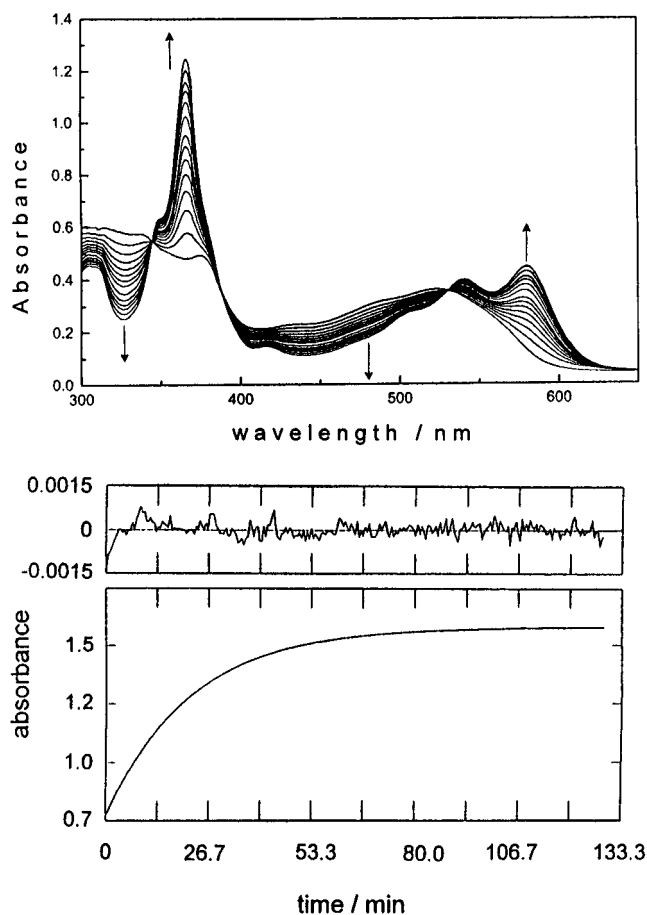


Figure 1. (a) UV–visible spectra measured after mixing (tandem cuvette) for the reaction between AdoCbl (~8 × 10⁻⁵ M, pH 11.0, I = 1.0 M (NaClO₄)) and NaCN (0.200 M, pH 11.0, 25.0 °C, I = 1.0 M (NaClO₄)). The first nine spectra are recorded every 2.50 min. Subsequent spectra taken at times 25, 30, 40, 60, and 100 min. (b) Absorbance at λ = 367 nm versus time for the same experiment as in (a). The data are fitted to a single exponential, giving *k*_{obs} = (7.59 ± 0.08) × 10⁻⁴ s⁻¹. The residuals of the fit are shown above.

Figure 2 shows ¹H NMR spectra of the axial ligands (i.e., the β-adenosyl and α-DMBI) for the same reaction conditions ([CN⁻] = 0.100 M, pD 11.0, 25.0 °C, I = 1.0 M (NaClO₄)). Figure 2a gives the spectrum obtained ~1.5 min after mixing. The ¹H signals of the β-adenosyl and α-DMBI ligands of AdoCbl are clearly visible at 8.22 and 8.01 ppm and 7.17, 6.96, and 6.26 ppm, respectively, and are in excellent agreement with literature values.^{3b} (The ¹H of the ribose group is at 6.28 ppm.) No new peaks are visible, thus once again demonstrating that no reaction has occurred within this time.

Figure 2b gives typical spectra obtained at various times during the subsequent reaction. The signals of AdoCbl decrease during the reaction as expected. New peaks, which are in excellent agreement with the known ¹H NMR chemical shifts of the DMBI ligand of (CN)₂Cbl⁻,¹⁴ can be clearly seen at 8.34, 7.47, and 7.38 ppm and increase in intensity as the reaction progresses. Measurement of the ¹H NMR spectrum of adenine at pH 11.0 confirmed that the two peaks at 8.12 and 7.97 ppm that increase in intensity can be attributed to adenine. During the entire course of the reaction there was no evidence for the formation of an intermediate. The reaction products were also confirmed by ¹³C NMR (spectra not given), and the percentage

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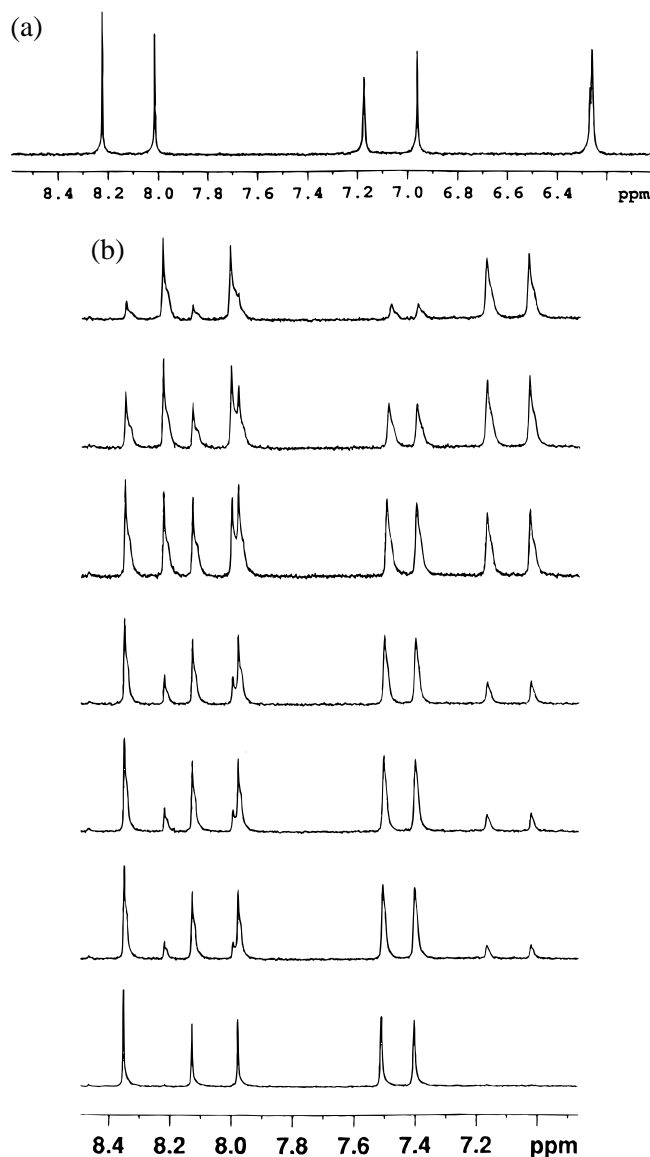


Figure 2. ^1H NMR spectra for the reaction of AdoCbl with CN^- ($[\text{CN}^-]_T = 0.100$ M, $[\text{AdoCbl}]_T = 7 \times 10^{-3}$ M, pD 11.0, 25.0°C , $I = 1.0$ M (NaClO_4), AQ = 2.04 s, D1 = 1.00 s, NS = 1: (a) ~ 1.5 min after adding CN^- to AdoCbl; (b) at longer times. The time delay between the first six spectra is 6.2 min. The last spectrum was measured at the completion of the reaction (ca. 90 min after mixing).

of the adenine reaction product was checked by HPLC ($= 100 \pm 3\%$), to check that only heterolysis of the Co–C bond occurs.¹⁵

Figure 3 gives a plot of the relative peak area of one of the α -DMBI signals of AdoCbl (8.22 ppm) versus time for the same experiment. The reaction was fitted to a single first-order exponential expression (fit shown by the solid line) and gives $k_{\text{obs}} = (6.2 \pm 0.2) \times 10^{-4} \text{ s}^{-1}$. This result is in reasonable agreement with the more accurate value obtained by UV–visible spectroscopy $(7.59 \pm 0.08) \times 10^{-4} \text{ s}^{-1}$; see Figure 1b). Similar analyses of the rate of decrease of the peak areas of other α -DMBI signals and the adenine signals of the β -Ado group of AdoCbl and the rate of increase of the areas of the DMBI signals of the $(\text{CN})_2\text{Cbl}^-$ product gave results that agreed within $\pm 20\%$.

Figure 4 gives a plot of k_{obs} versus $[\text{CN}^-]$ for the reaction between AdoCbl and excess CN^- at pH 11.0 ($[\text{CN}^-]_T = 5.00 \times 10^{-3}$ – 0.500 M). The reaction is pseudo first order in $[\text{CN}^-]$ and gives a second-order rate constant $k = (7.4 \pm 0.1) \times 10^{-3} \text{ M}^{-1} \text{ s}^{-1}$. A negligible intercept shows that the reaction is irreversible and that no other parallel reactions occur. A single

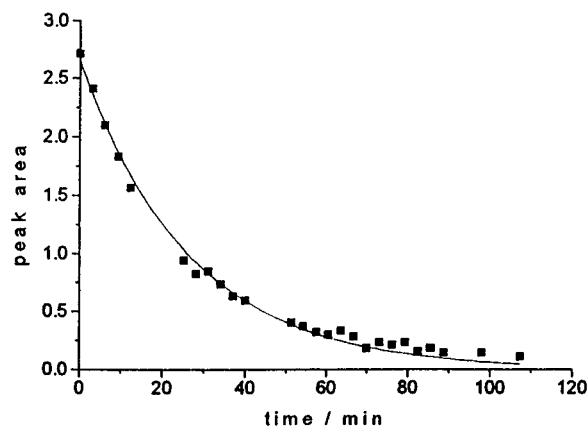


Figure 3. Relative peak area of the ^1H NMR signal at 8.22 ppm versus time for the reaction between CN^- and AdoCbl. Data are fitted to a single first-order exponential expression, giving $k_{\text{obs}} = (6.2 \pm 0.2) \times 10^{-4} \text{ s}^{-1}$. Experimental details are given in the caption to Figure 2.

experiment was also performed with AdoCbl in excess ($[\text{AdoCbl}] = 2.0 \times 10^{-3}$ M, $[\text{CN}^-] = 2.0 \times 10^{-4}$ M, pH 11.0, 25.0°C , $I = 1.0$ M (NaClO_4)) and gave a second-order rate constant $k = (8.9 \pm 0.5) \times 10^{-3} \text{ M}^{-1} \text{ s}^{-1}$, which is in reasonable agreement with the more accurate value obtained with CN^- in excess (since only a small fraction of AdoCbl is converted to products in the former experiment).

Figure 5 gives a plot of k_{obs} versus pH for $[\text{CN}]_T = 0.100$ M, where $[\text{CN}]_T$ refers to the total cyanide concentration. If it is assumed that only CN^- (and not HCN) is the active reactant, it can easily be shown that

$$k_{\text{obs}} = K_{\text{a}(\text{HCN})}k'/(K_{\text{a}(\text{HCN})} + a_{\text{H}^+}) \quad (1)$$

Measurement of the $\text{p}K_{\text{a}(\text{HCN})}$ by potentiometric titration gave $\text{p}K_{\text{a}(\text{HCN})} = 8.74 \pm 0.02$ (25.0°C , $I = 1.0$ M (NaClO_4)). The solid line given in Figure 5 represents the best fit of the data to eq 1 fixing $\text{p}K_{\text{a}(\text{HCN})} = 8.74$ and thus demonstrates that the assumption is correct. CN^- , not HCN, was also found to be the active species for the reaction between cyanide and the adenylyl coenzyme.¹⁶

The dependence of k_{obs} on temperature was investigated for 0.100 M CN^- at pH 11.0. ($I = 1.0$ M (NaClO_4)). Table 1 summarizes this data. Values for ΔH^\ddagger and ΔS^\ddagger were calculated to be $53.0 \pm 0.6 \text{ kJ mol}^{-1}$ and $-127 \pm 3 \text{ J mol}^{-1} \text{ K}^{-1}$, respectively, in the usual manner. The pressure dependence was also measured ($[\text{CN}^-] = 7.50 \times 10^{-2}$ M, pH 11.0, 25.0°C , $I = 1.0$ M (NaClO_4)), and ΔV^\ddagger was found to be $-10.0 \pm 0.4 \text{ cm}^3 \text{ mol}^{-1}$ from the slope of the plot of $\ln k_{\text{obs}}$ versus pressure (see Figure 6).

- (15) This experiment was suggested by a reviewer. HPLC has been demonstrated to be a useful technique for quantification of the nucleoside products arising from Co–C heterolysis (adenine) and homolysis ($5'$ -deoxyadenosine and $8,5'$ -anhydroadenosine) of AdoCbl.^{6,27} AdoCbl (17.3 mg) was dissolved in 25.00 mL of cyanide solution (0.100 M NaCN, pH 11, $I = 1.0$ M (NaClO_4)) and allowed to react to completion ($[\text{AdoCbl}] = 4.38 \times 10^{-4}$ M, 90 min, in dark). A sample for HPLC measurement was prepared by bringing the solution to pH ~ 7 (concentrated NaOH) and 1 mL of solution diluted to 10 mL with 0.05 M phosphate buffer (pH 7; $[\text{nucleoside products}]_T = 4.38 \times 10^{-5}$ M). An authentic adenine solution (Fluka, $>99\%$, 5.00×10^{-5} M) was prepared in 0.045 M phosphate buffer, and the two samples were analyzed by HPLC immediately following one another. Comparison of the peak areas of the adenine (2.7 min) in the two samples gave the percent conversion of AdoCbl to adenine of $100 \pm 3\%$. HPLC operating conditions: 10% $\text{CH}_3\text{CN}/90\%$ phosphate buffer at 1.5 mL/min flow, $\lambda = 260$ nm. A Spectra Physics HPLC (with focus detector) was used equipped with a Machery-Nagel ET 250/4 Nucleosil 100-10 C_{18} column.
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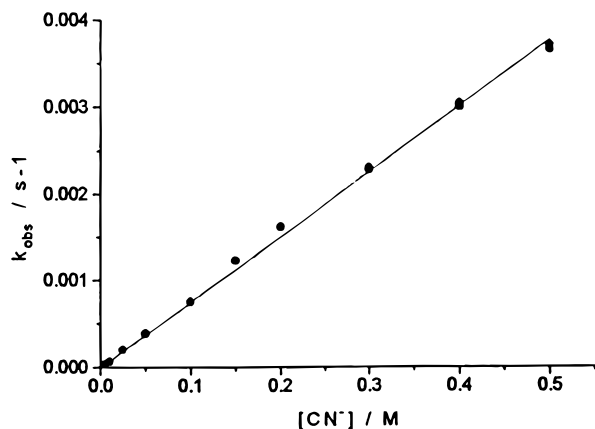


Figure 4. k_{obs} versus $[\text{CN}^-]$ for the reaction between AdoCbl and excess CN^- (pH 11.0, 25.0 °C, $I = 1.0 \text{ M}$ (NaClO_4)). The best fit of the data (solid line) gives $k = (7.4 \pm 0.1) \times 10^{-3} \text{ M}^{-1} \text{ s}^{-1}$.

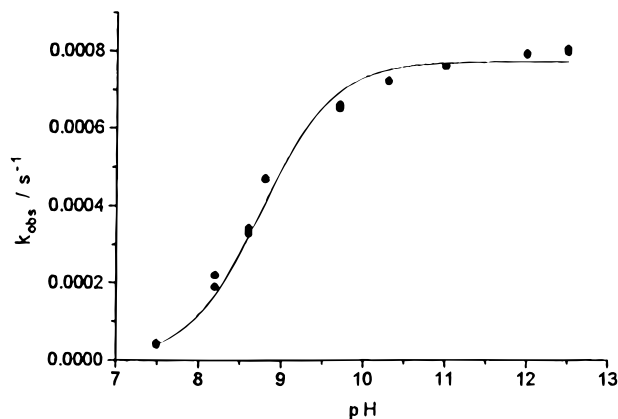


Figure 5. k_{obs} versus pH for the reaction between AdoCbl and excess CN^- ($[\text{CN}]_{\text{T}} = 0.100 \text{ M}$, 25.0 °C, $I = 1.0 \text{ M}$ (NaClO_4)). The best fit to the data to eq 1 (solid line) with $\text{p}K_{\text{a}(\text{HCN})} = 8.6$ gives $k' = (7.7 \pm 0.1) \times 10^{-4} \text{ s}^{-1}$.

Table 1. Effect of Temperature on the Reaction of AdoCbl with CN^- (pH 11.0, $[\text{CN}^-] = 0.100 \text{ M}$, $I = 1.0 \text{ M}$ (NaClO_4))

temp/K	$k_{\text{obs}} \times 10^4/\text{s}^{-1}$
283.0	2.33, 2.44
290.0	4.25, 4.16
298.0	7.59, 7.57
303.0	11.3, 11.0
308.0	16.5, 16.3
$\Delta H^\ddagger/\text{kJ mol}^{-1}$	53.0 ± 0.6
$\Delta S^\ddagger/\text{J mol}^{-1} \text{ K}^{-1}$	-127 ± 3

Figure 7 gives the ^1H NMR spectrum for an equilibrated solution of 1:5 mole equivalent of CN^- :AdoCbl. All signals in the aromatic region could be assigned to unreacted AdoCbl (the peak at 5.94 ppm is from a AdoCbl corrin ring $^1\text{H}^{3\text{b}}$), adenine, and CNCbl (7.28, 7.09, 6.51, 6.37, 6.36, and 6.08 ppm,¹⁷ checked by measuring ^1H NMR of CNCbl at pH 11.0).

Discussion

The following experimental observations in our measurements have led us to question the findings of Rudakova and co-workers.⁷ Rudakova et al. claimed to see two reactions when AdoCbl was reacted with CN^- ($k_{\text{obs}(1)} = 2.9 \times 10^{-2} \text{ s}^{-1}$ ($\lambda = 348 \text{ nm}$), $k_{\text{obs}(2)} = 8.4 \times 10^{-4} \text{ s}^{-1}$ ($\lambda = 370 \text{ nm}$), 0.1 M KCN, 0.1 M NaHCO_3 , pH 10.5, 25 °C^{7a}). We first began our investigations using alkaline experimental conditions where only CN^- (not HCN) is important, which were essentially the same

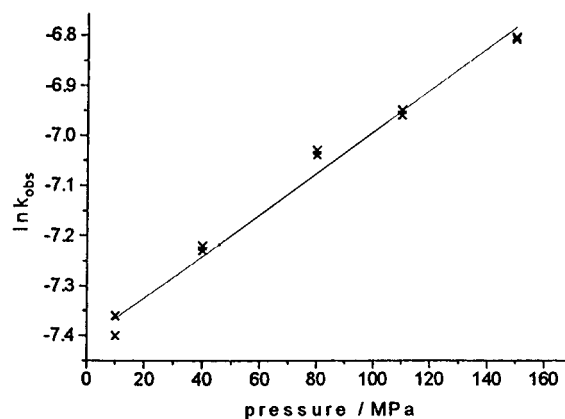


Figure 6. $\ln k_{\text{obs}}$ versus pressure for the reaction between AdoCbl and excess CN^- ($[\text{CN}^-] = 7.50 \times 10^{-2} \text{ M}$, pH 11.0, 25.0 °C, $I = 1.0 \text{ M}$ (NaClO_4)). The best fit of the data (solid line) gives $\Delta V^\ddagger = -10.0 \pm 0.4 \text{ cm}^3 \text{ mol}^{-1}$.

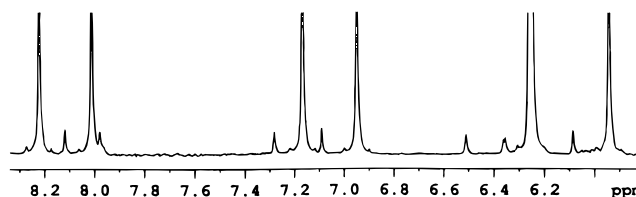


Figure 7. ^1H NMR spectrum for an equilibrated solution of 1:5 mol equiv of CN^- : AdoCbl ($[\text{CN}^-] = 1.4 \times 10^{-3} \text{ M}$, $[\text{AdoCbl}] = 7.0 \times 10^{-3} \text{ M}$, 25.0 °C), AQ = 3.41 s, D1 = 1.00 s, and NS = 128.

(0.100 M NaCN, pH 11.0, 25.0 °C, $I = 1.0 \text{ M}$ (NaClO_4)) as those used by the Russian workers. A comparison of the UV-visible spectrum before and $\sim 10 \text{ s}$ after mixing AdoCbl with 0.100 M CN^- at pH 11.0 showed that negligible reaction had occurred within this time. We also checked for a possible reaction at 347–348 nm (the same λ conditions as the Russian workers used for observation of their fast reaction, i.e., one of the isosbestic points of the slower reaction) and observed no change in absorbance with time. The reaction that was subsequently followed exhibited clean isosbestic points expected for a single reaction. ^1H NMR measurements also gave a spectrum $\sim 1.5 \text{ min}$ after mixing identical to that of the reactants (Figure 2a), thus confirming our suspicion that the “fast” reaction observed by Rudakova and co-workers must have been an artifact. We have no explanation as to why Rudakova and co-workers observed two reactions, other than to suggest that they may have contaminated their reactant by exposing it to light (AdoCbl may have been converted to H_2OCbl^+ by the spectrophotometer light source; for $\text{H}_2\text{OCbl}^+ + \text{CN}^- \rightarrow \text{CNCbl} + \text{CN}^- \leftrightarrow (\text{CN})_2\text{Cbl}^-$, $k_{\text{obs}(1)} = 6.3 \times 10^{-3} \text{ s}^{-1}$ and $k_{\text{obs}(2)} = 3.5 \times 10^{-2} \text{ s}^{-1}$ for $[\text{KCN}] = 0.10 \text{ M}$, pH 11.7, 25 °C¹⁸) or a reactant may have contained an impurity. The Russian workers’ rate constant for the slower reaction ($8.4 \times 10^{-4} \text{ s}^{-1}$, 0.1 M KCN) is in good agreement with our value ($7.59 \times 10^{-4} \text{ s}^{-1}$, 0.100 M NaCN), taking into account the slightly different experimental conditions. Our second-order rate constant (see below) differs significantly from an earlier reported value of Schrauzer and co-workers ($0.83 \text{ M}^{-1} \text{ s}^{-1}$ ^{11b}). Thus, both our UV-visible and ^1H NMR measurements showed that the reactant is converted to products without the buildup of a detectable intermediate.

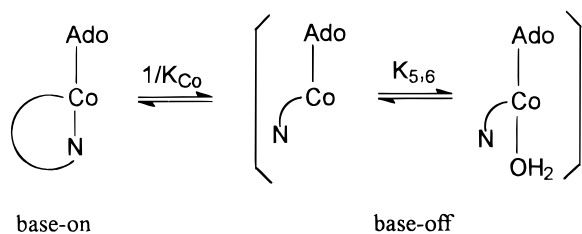
Measurement of the concentration dependence of the cyanation reaction gave a rate constant of $(7.4 \pm 0.1) \times 10^{-3} \text{ M}^{-1} \text{ s}^{-1}$, while the pH dependence of the observed rate constant (and the observation that the absorbance change decreased as the pH decreased) clearly indicated that CN^- , not HCN, is the active

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species and a H^+ is not involved in the rate-determining step (unlike for the acid-catalyzed hydrolysis of AdoCbl; see later). The rate-determining step (i.e., attack of the first CN^-) was further confirmed by measuring the rate of a reaction in which AdoCbl rather than CN^- was in excess, and conversion of k_{obs} to a second-order rate constant gave a value which was in reasonable agreement with that determined under excess CN^- conditions. Measurement of activation parameters resulted in large negative values for both ΔS^\ddagger and ΔV^\ddagger (Table 1), which strongly suggest an associative rate-determining step. To summarize, UV-visible, 1H NMR, and activation parameter data show that the rate-controlling attack of the first cyanide is followed by a fast, kinetically unobservable attack of the second cyanide to give the products and that the first reaction occurs associatively.

AdoCbl is known to exist in aqueous solution in a base-off and base-on form, and the base-off form can be either five-coordinate or six-coordinate (in which a solvent H_2O occupies the α axial position); i.e.,



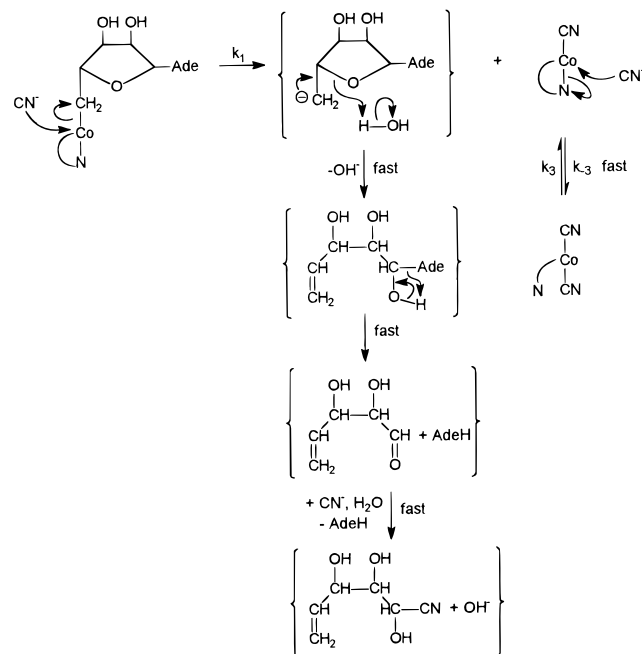
The temperature dependence of K_{Co} for AdoCbl has been reported by Brown et al.¹⁹ ($K_{Co} = 76.6$ at $25.0^\circ C$, i.e. ca. 1.3% base-off), while Pratt et al. have reported that at room temperature base-off AdoCbl exists essentially 100% in its five-coordinate form.²⁰

Johnson and Shaw⁹ were the first to propose that CN^- attacks the β -Ado site in the rate-determining step. This mechanism is outlined in Scheme 1. Hogenkamp et al.¹⁰ further suggested that the electron-withdrawing ribose substituent of the CH_2 of Ado causes polarization of the $Co-C$ electron density toward the C, giving rise to an electrophilic Co, which is attacked by the strong nucleophile CN^- , resulting in bond cleavage. It was also pointed out that $Co-C$ cleavage is particularly favorable for AdoCbl as the cleavage can occur in a concerted manner and does not require a carboanion leaving group. For MeCbl, however, $Co-C$ cleavage does not occur,^{7a} as the $Co-C$ electron density is now polarized toward the Co and the leaving group would be a carboanion.

A further possible mechanism involves rate-determining attack of CN^- at the α -site of base-off AdoCbl, as given in Scheme 2. Since $k_1 \ll k_2$, it was expected that the monocyano (β -Ado)(α - CN)Cbl⁻ intermediate should be undetectable even at low $[CN^-]$ if this mechanism is operating. A single experiment was performed to check this. A solution containing 0.2 mol equiv of CN^- to 1.0 mol equiv AdoCbl was allowed to react ($[CN^-] = 1.4 \times 10^{-3} M$, $[AdoCbl] = 7.0 \times 10^{-3} M$) and the 1H NMR spectrum of the product measured. Only signals attributable to unreacted AdoCbl, CNCbl, and adenine were observed (Figure 7). Thus, the observed products at both low and high $[CN^-]$ do not contradict either of the two schemes.

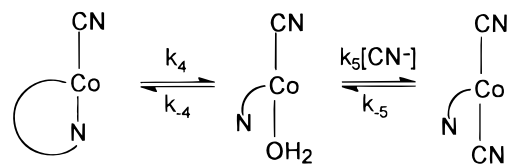
If Scheme 2 is operative, the kinetic treatment must take the base-on/base-off pre-equilibrium, which occurs before the rate-

Scheme 1



determining step, into consideration. It can easily be shown that $k_{obs(corr)} = (K_{Co} + 1)k_{obs} \sim K_{Co}k_{obs}$ (since $K_{Co} \gg 1$), where $k_{obs(corr)}$ represents the value of k_{obs} when the pre-equilibrium in Scheme 2 is taken into account. Using temperature-corrected values of K_{Co} ¹⁹ to calculate $k_{obs(corr)}$ gives $\Delta H^\ddagger_{(corr)} = 42.5 \pm 0.5 \text{ kJ mol}^{-1}$ and $\Delta S^\ddagger_{(corr)} = -126 \pm 3 \text{ J mol}^{-1} \text{ K}^{-1}$; thus inclusion of the temperature-dependent pre-equilibrium has only a small effect on ΔH^\ddagger , and ΔS^\ddagger remains unchanged within experimental error. The pressure dependence of K_{Co} has, however, not been reported in the literature. The nature of the base-on/base-off pre-equilibrium is such that the overall volume change is expected to be positive in the case of a vacant coordination site in the base-off species, or close to zero when water is coordinated in the base-off species. It follows that the convincingly negative volume of activation, irrespective of the possible contribution resulting from the pre-equilibrium in Scheme 2, supports our earlier conclusion that the rate-determining step must be associative in nature.

Kinetics can also be used in general to distinguish between such mechanisms. In this particular case, this would only be possible if there was some experimental condition where the first step was not rate-determining. For Scheme 2, it is expected that the second step (k_2) will always be much faster than the first, since substitution of the Ado by CN^- should be much faster than substitution of the DMBI by CN^- due to the small percentage of base-off species and kinetic translabilization from the α - CN^- in the former substitution process. Reenstra and Jencks¹⁸ measured rate constants for

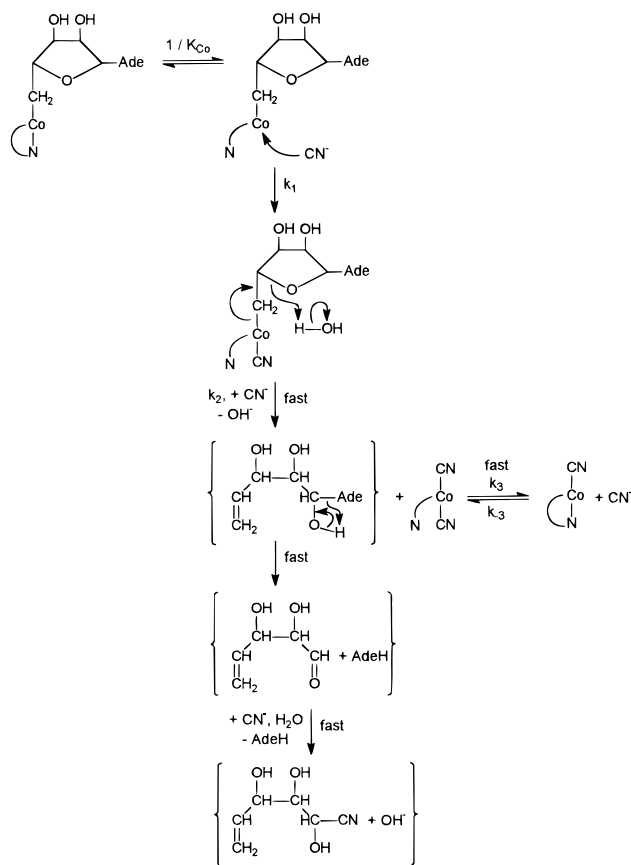


with the corresponding rate law $k_{obs(4,5)} = (k_4k_5[CN^-] + k_{-4}k_{-5}) / (k_{-4} + k_5[CN^-])$, where $k_4 = 4.2 \times 10^{-2} \text{ s}^{-1}$, $k_{-4} = 1.8 \times 10^3 \text{ s}^{-1}$, $k_5 = 8.4 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, and $k_{-5} = 4.0 \times 10^{-4} \text{ s}^{-1}$ (pH 11.7, $25^\circ C$). Since the observed rate constant $k_{obs(4,5)} \gg k_1[CN^-]$ (e.g., at the lowest $[CN^-]$ used ($5 \times 10^{-3} M$), $k_{obs(4,5)} = 8.3 \times 10^{-3} \text{ s}^{-1} \gg k_1[CN^-]$ and at the highest $[CN^-]$ (0.5 M)

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(20) (a) Firth, R. A.; Hill, H. A. O.; Mann, B. E.; Pratt, J. M.; Thorp, R. G.; Williams, R. J. P. *J. Chem. Soc. A* **1968**, 2419. (b) Chemaly, S. M.; Pratt, J. M. *J. Chem. Soc., Dalton Trans.* **1980**, 2267.

Scheme 2

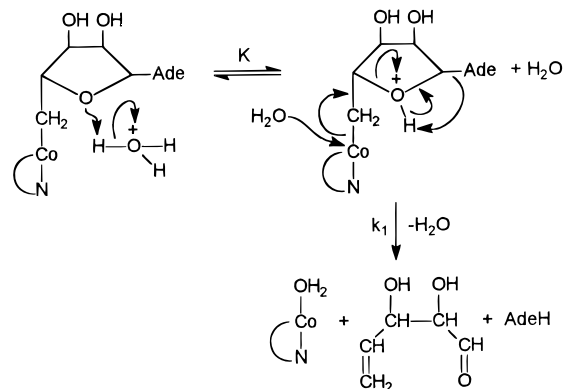


$k_{\text{obs}(4,5)} = 4.0 \times 10^{-2} \text{ s}^{-1} \gg k_1[\text{CN}^-]$ for all experimental conditions, this means that the second step in Scheme 1 and the third step of Scheme 2 will also always be much faster than the first reaction; thus the first reaction will always be the rate-determining step for both Schemes 1 and 2 and examination of the kinetics cannot be used to clarify which scheme is correct.

Pratt proposed that cyanation of AdoCbl proceeds via Scheme 2 from a comparison of the rate constants for cyanation of the adenylyl coenzyme and AdoCbl.⁵ UV-visible spectroscopy measurements claim to show that AdoCbl exists 90% as base-on AdoCbl and 10% in the base-off form (Pratt et al., 20 °C^{20a}), while the adenylyl coenzyme is 100% in the base-off form.⁵ Pratt suggested that the rate of cyanation for AdoCbl is one-tenth that observed for the adenylyl coenzyme as only 10% of the reactant is present in the active (base-off) form. There are, however, two problems which throw doubt on this interpretation. First, the $\text{p}K_{\text{a}}$ of the coordinated H_2O needs to be taken into consideration as it would be expected to occur in the pH 8–10 region, and thus would drastically reduce the fraction of the reactive aqua base-off species, since the hydroxo base-off species is inert to substitution.²¹ The $\text{p}K_{\text{a}}$ would be expected to be similar for the two coenzymes, however, so that the argument still remains valid. Second, the percent base-off AdoCbl present at 25.0 °C has been recently reported to be ~1.3%,¹⁹ which is considerably different from the value estimated by Pratt et al.

Furthermore, the rate-determining step in Scheme 2 involves substitution of the $\alpha\text{-OH}_2$ of base-off AdoCbl by CN^- . There is much evidence in the literature which demonstrates that ligand substitution reactions of a H_2O coordinated to a Co(III) center

Scheme 3



in vitamin B₁₂ chemistry occur dissociatively,²² and we clearly observed activation parameters that strongly suggest that the rate-determining step is associative, suggesting that Scheme 2 is not in operation.

One important consequence of Scheme 1 is that the CN^- must play an important role in the rate-determining Co–C cleavage. This means that the rate-determining step for Scheme 1 is almost certainly associative in nature. It should be kept in mind that the measured activation volume ΔV^\ddagger , which is the favorite parameter to use to investigate the intimate nature of a substitution mechanism, consists in essence of an intrinsic ($\Delta V^\ddagger_{\text{intr}}$) and a solvational component ($\Delta V^\ddagger_{\text{solv}}$).²³ $\Delta V^\ddagger_{\text{intr}}$ is a measure of the associative or dissociative nature of a reaction and $\Delta V^\ddagger_{\text{solv}}$ is concerned with the changes in electrostriction with respect to solvent molecules. $\Delta V^\ddagger_{\text{solv}}$ can be important when dealing with a charged nucleophile (i.e., CN^-) in a polar solvent; however for our system $\Delta V^\ddagger_{\text{solv}}$ must be positive, since the solvent in the transition will be less constricted than in the reactant state due to charge neutralization, so that $\Delta V^\ddagger_{\text{intr}}$ must be even more negative than the observed experimental value; that is the reaction is even more strongly associative in nature than ΔV^\ddagger suggests.

Gerards and Balt²⁴ investigated the acid hydrolysis of AdoCbl. There are remarkable parallels between acid hydrolysis and cyanation with respect to the products of the decomposition of the Ado ligand (adenine and D-erythro-2,3-dihydroxy-4-pentenal) and the activation parameters (for acid hydrolysis $\Delta S^\ddagger = -96 \text{ J K}^{-1} \text{ mol}^{-1}$ and $\Delta V^\ddagger = -11.4 \text{ cm}^3 \text{ mol}^{-1}$). Note, however, that, unlike the cyanation reaction of AdoCbl, acid hydrolysis of AdoCbl requires protonation of (probably) the ribose β -oxygen of the Ado ligand in the rate-determining step. (This was also found to be the case for heterolysis in β -oxoalkyl and oxocarbonyl complexes.²⁵) The authors concluded that the reaction proceeds associatively (see Scheme 3). The route to

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Co–C cleavage is in principle the same for both systems if cyanation of AdoCbl is occurring via Scheme 1. For acid hydrolysis, the authors suggested that protonation of the ribose β -oxygen removes electron density from the ribose, which is compensated by the movement of electron density from the Co–C bond toward the ribose, resulting in Co–C cleavage, while in cyanation the attack of CN^- at the Co induces the electron density of the Co–C bond toward the ribose, resulting once again in Co–C cleavage.

Since the first submission of this paper, a paper by Finke and co-workers²⁶ appeared which demonstrates that α substitution of OH_2 by pyridine and *p*-(dimethylamino)pyridine results in significant heterolysis in addition to homolysis of the Ado Co–C bond (85–110 °C, anaerobic ethylene glycol solvent) for adenosylcobinamide (AdoCbi, (β -Ado)(α - OH_2)Cbi); that is, this is a proven case of where α -ligand substitution results in Co–C heterolysis. On the basis of their results, the authors suggested that heterolysis of the Co–C bond in aqueous solution also occurs via substitution of the α - OH_2 of base-off AdoCbl for the AdoCbl + CN^- reaction (i.e., via Scheme 2). It is questionable, however, whether a mechanism deduced in anaerobic ethylene glycol at temperatures ≥ 85 °C can be extrapolated to water at 25 °C. (It is interesting, however, that while there is no detectable heterolysis of AdoCbl in anaerobic ethylene glycol at 110 °C,^{6a} in aqueous solution at pH 7, 10% heterolysis is observed at 85 °C, and the percentage of heterolysis versus homolysis increases as the temperature decreases^{6b} (85–110 °C).) Importantly, one of the most significant observations we have made is that *there is no apparent reaction between AdoCbl and other strong nucleophiles* (e.g., SO_3^{2-} , $\text{S}_2\text{O}_3^{2-}$, I^- , thiourea, Im), which suggests to us that the reaction between AdoCbl and CN^- is rather unique and does not simply involve trans-labilization by CN^- , which

results in Co–C cleavage. Further experiments are of course necessary to resolve this aspect.

A reviewer pointed out that a study of the reaction between adenosylcobinamide and cyanide could be a further test to prove which mechanism is operating. On the basis of the results of the above-mentioned paper, cyanation of AdoCbi would almost certainly occur via α attack of the first CN^- for a cobinamide. Whether or not an intermediate is observed would indicate if the addition of the second or first CN^- is rate-determining, respectively. If the first step was rate-determining, comparison of the rate constant (k) with k_1 for the reaction between AdoCbl and CN^- could give a better indication of whether Scheme 1 or 2 is operating in the latter case (since $k \cong k_1$ if Scheme 2 is operating).

In summary, the reaction between AdoCbl and CN^- has been shown to occur in one kinetically observable step with rate-determining attack of the first CN^- . Activation parameters typical for an associative reaction strongly suggest that the first CN^- attacks the β -Ado site followed by a subsequent fast attack of a second CN^- at the α -DMBI. Measurements of the activation parameters for cyanation of MeCbl could further confirm the reliability of associative activation parameters as an indication of rate-controlling attack of CN^- at the β -Ado site of AdoCbl, since cyanation of MeCbl to form (β -Me)(α -CN)Cbl⁻ would be expected to be dissociative in nature. Experiments with MeCbl are currently in progress.

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