Continuous-Wave Quantum Yields of Various Cobalamins Are Influenced by Competition between Geminate Recombination and Cage Escape[†]

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ABSTRACT: Quantum yields of photolysis of the cobalt–carbon bond for three cobalamin compounds were measured with a continuous-wave laser at 442 nm under both aerobic and anaerobic conditions. Aerobically, the initial homolysis product, Co(II) cobalamin, is trapped by oxygen to form aquocobalamin. Use of an excess of the radical trapping reagent 2,2,6,6-tetramethyl-1-piperidinyloxyl, under anaerobic conditions, scavenges the carbon radical and allows detection of the cobalt(II) photoproduct. Quantum yields measured under anaerobic conditions for 5′-deoxyadenosylcobalamin ($\phi_{(\text{Co–C}\alpha),442} = 0.20 \pm 0.03$) and methylcobalamin ($\phi_{(\text{Co–C}\alpha),442} = 0.35 \pm 0.03$) are in agreement with the values obtained under aerobic conditions ($\phi_{(\text{Co–C}\alpha),442} = 0.19 \pm 0.04$ and $\phi_{(\text{Co–C}\alpha),442} = 0.36 \pm 0.04$, respectively). Additionally, the quantum yield values for 5′-deoxyadenosylcobalamin and its base-off derivative ($\phi_{(\text{Co–C}\alpha),442} = 0.045 \pm 0.015$) match those obtained on a nanosecond time scale [Chen, E., & Chance, M. R. (1990) *J. Biol. Chem. 256*, 12987–12994]. A comparison of quantum yields obtained anaerobically for 5′-deoxyadenosylcobalamin and methylcobalamin in H_2O versus ethylene glycol shows a 4-fold decrease for the former cobalamin and no change for the latter. These quantum yields are evaluated in terms of time-independent radical separation distances.

Homolytic cleavage of the cobalt–carbon (Co–Cα)[1] bond in 5′-deoxyadenosylcobalamin (AdoCbl), which generates Co(II) cobalamin [Co(II)] and a radical, is recognized to be critical for initiation of several enzymatic reactions (Lee & Abeles, 1963; Wagner et al., 1966; Babior, 1970; Cockle et al., 1972; Finlay et al., 1972; Orme-Johnson et al., 1974; Valinsky et al., 1974; Finlay et al. 1973; Babior et al., 1972; Toraya et al., 1982). Therefore, characterization of homolytic reaction mechanisms can give insight into possible enzyme–cobalamin interactions that lead to bond cleavage. Many model compound studies suggest that the predominant influence on bond lability is steric rather than electronic (Chemaly & Pratt, 1980b,c; Mealli et al., 1987; Christianson & Lipscomb, 1985; Marzilli et al., 1979; Zhu & Kostic, 1987; Ng et al., 1982; Schrauzer & Grate, 1981; Schrauzer et al., 1968, 1970). Although there are series of ligands that exhibit trends explained solely by steric or inductive effects, there are as many or more that demonstrate the complexities of bond lability (Guschl et al., 1974; Chemaly & Pratt, 1976; Toscano & Marzilli, 1979; Weiss et al., 1979; Rossi et al., 1985; Sagi et al., 1990; Taraszka et al., 1991; Sagi & Chance, 1992). For example, ^{13}C NMR studies by Toscano and Marzilli (1979) show that CH_3- and i-C_3H_7-substituted cobaloximes have very similar ligand substitution rates and trans influences, although the bond distances vary significantly (1.998 and 2.085 Å, respectively; Marzilli et al., 1979). An explanation for this may be secondary steric effects, preventing the i-C_3H_7 group from being a superior electron donor. Co–Cα bond lability would appear to be governed by a combination of steric and electronic effects; however, it is not implicit that under photolytic (or enzyme-induced) conditions the bond will respond in a manner that reflects its strength.

While many studies of alkylcobalamin photolysis have focused on the rates and products of light-induced bond cleavage (Hogenkamp et al., 1962; Hogenkamp, 1963, 1966; Johnson et al., 1962, 1963; Dolphin et al., 1964; Pratt, 1964; Yamada et al., 1966a,b; Schrauzer et al., 1970; Endicott & Ferraudi, 1977; Pailes & Hogenkamp, 1968; Hartshorn et al., 1978; Rudakova et al., 1978; Chemaly & Pratt, 1980a), only a few have measured the quantum yields of photolysis of the Co–Cα bond. Quantum yields have been reported for several cobalamins on continuous-wave (CW) (Pratt & Whitear, 1971; Taylor, 1973) and ultrafast time scales (Endicott & Netzel, 1979; Chen & Chance, 1990). Spectral dynamics investigations show that quantum yields measured at CW and nanosecond (ns) times are equivalent.[2] The CW and ns quantum yields are not primary yields of original Co(II) and radical partners (Noyes, 1955), but "secondary" quantum yields subsequent to the geminate events of the radicals. Unfortunately, the separately reported CW quantum yields for AdoCbl and methylcobalamin (MeCbl) do not agree with each other or with those measured at ns times. These previous studies were performed with single-wavelength detection methods, which have a disadvantage to full-spectrum techniques in that the actual products can be misidentified. It is the primary goal of this paper to describe a CW laser photolysis system that was developed with full-spectrum detection to measure accurate quantum yield ($\phi_{(\text{Co–C}\alpha),442}$ or ϕ) values. Relative to the ns configuration we described

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[1] Abbreviations: Co–Cα, cobalt–α carbon bond; Co(II), Co(II) cobalamin; AdoCbl, 5′-deoxyadenosylcobalamin; MeCbl, methylcobalamin; $\phi_{(\text{Co–C}\alpha),442}$ or ϕ, quantum yield of photolysis of the Co–Cα bond; CW, continuous wave; AqCbl, aquocobalamin; Co–N*ax* cobalt–axial nitrogen bond; TEMPO, 2,2,6,6-tetramethyl-1-piperidinyloxyl; HyCbl, hydroxocobalamin; EthCbl, ethylcobalamin.

[2] Quantum yields obtained at nanosecond and continuous-wave time scales are often referred to as ns quantum yields and CW quantum yields. Photolysis experiments performed under anaerobic or aerobic conditions are sometimes referred to as anaerobic photolysis or aerobic photolysis.

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previously (Chen & Chance, 1990), the CW system has advantages in that quantum yield measurements are less complex. With the apparent success of the technique, we also propose a mechanism of photolysis that depends primarily upon the kinetics of geminate recombination and solvent cage behavior of the nascent radicals. An observed parallel between our FTIR data (Taraszka et al., 1991) and the quantum yields of various cobalamins suggests structural barriers to geminate recombination based on the ability of the nascent Co(II) species to regain its original Co(III) conformation.

In an aerobic environment, the initial photolysis product, Co(II), is rapidly oxidized by O_2, generating aquocobalamin (AqCbl). Anaerobically, diffusional recombination due to rapid recombination of Co(II) with radical makes it difficult to measure quantum yields on a CW time scale. Thus, in order to quantitate the anaerobic photoproducts, we have taken advantage of the nitroxide radical trapping agent 2,2,6,6-tetramethyl-1-piperidinyloxyl (TEMPO), which (1) has been used previously in investigations of Co–Cα bond thermolysis; (2) is the method of choice for trapping a wide range of alkylcorrin radical products; and (3) is well characterized in its reactions (Smith, 1982; Finke et al., 1983; Geno & Halpern, 1987a,b; Martin & Finke, 1992). Using TEMPO as a radical trap for anaerobic experiments and O_2 as a Co(II) trap for aerobic photolysis, quantum yields were obtained for MeCbl, AdoCbl, and base-off AdoCbl. Acidification of AdoCbl forms base-off AdoCbl where the 5,6-dimethylbenzimidazole group is protonated and the cobalt–nitrogen axial (Co–N*ax*) bond is cleaved. The quantum yield for MeCbl is measured at two pH values, under aerobic conditions, and the quantum yields obtained in H_2O versus 80% ethylene glycol are compared for MeCbl and AdoCbl. The effect on the quantum yields of different solvents is evaluated quantitatively by comparing the relative initial separations of the methyl and 5′-deoxyadenosyl radicals.

MATERIALS AND METHODS

Sample Preparation. MeCbl and TEMPO were purchased from Aldrich. Monobasic and dibasic potassium phosphate, AdoCbl, and a separate stock of MeCbl were obtained from Sigma. Reagent grade ethylene glycol came from J. T. Baker Chemical Co. All solids were used as purchased. Cobalamin samples were prepared in two different solvents, distilled H_2O and 80% ethylene glycol/H_2O, and buffered with potassium phosphate. Final phosphate buffer concentrations were 16 mM in H_2O and 70 mM in 80% ethylene glycol. For anaerobic experiments, AdoCbl was prepared with pH 7.45 phosphate buffer and 80% ethylene glycol. Under aerobic conditions of MeCbl photolysis, pH 6.12 and 9.00 buffers were chosen to avoid a product mixture of AqCbl and hydroxocobalamin (HyCbl), which appears between pH 6.50 and 8.50. For AdoCbl, experiments were performed only at pH 6.12. Base-off AdoCbl for aerobic experiments was obtained by adding the parent cobalamin to pH 2.05 phosphate buffer. The samples for aerobic experiments were prepared without further treatment of the buffer solvents. For anaerobic experiments, the buffers were carefully deoxygenated with nitrogen or argon gas in a glove box to prevent generation of AqCbl side products. All cobalamin samples for these experiments were prepared in the glove box. These treatment methods are sufficient since no AqCbl byproduct was detected in the optical spectra. AdoCbl and MeCbl (3–4 mM) were used as stock solutions, with the final sample concentrations ranging between 30 and 150 μM.

The 1.0–1.2-mL samples used in anaerobic photolysis were prepared in a similar way. However, in addition to cobalamin, the solution also contained TEMPO (8 mM stock solution) in degassed and pH 7.45 buffered solvent. The final cobalamin concentrations ranged between 30 and 150 μM, whereas the TEMPO concentration was at least 10 times (1.5 mM) that of the cobalamin solution. The solution was prepared this way to ensure that enough TEMPO is present to capture each radical formed (Hay & Finke, 1988) and to prevent underestimation of the quantum yield due to diffusion-controlled recombination of Co(II) and radical.

All samples were prepared at 25 °C in the absence of light and loaded into a 1-cm × 1-cm quartz cuvette with a 0.5-cm × 0.15-cm cylindrical micro spinbar. The cuvette for anaerobic samples was sealed with a Teflon cap under positive nitrogen pressure in the glove box, and the top of the cuvette was wrapped several times with Teflon tape. The experimental results were determined to be unaffected by stirring. Most experiments were performed with stirring only between photolysis intervals so that the probe beam intercepted optically homogeneous sample. Although previous quantum yield experiments had O_2 bubbling through the sample during photolysis, a series of tests were performed to determine if this was necessary. The quantum yields obtained under various treatments of the sample with O_2 did not vary from that measured with the ambient content of O_2. Therefore, all aerobic experiments were performed without flowing O_2 to minimize sample disturbance. All photolysis experiments were carried out at 26 °C.

Experimental Apparatus. The light source for the CW photolysis experiments was a Liconix 4240NB HeCd laser operated at 442-nm, TEM_{00} mode, with a maximum power of 42 mW. The laser beam, which was initially 0.12 cm in diameter, was expanded and collimated with Newport Corp. lenses to a diameter of 2.0 cm. The beam diameter was then reduced using a variable Newport optical iris with an aperture setting of 1.5 cm. Therefore, the sample, which was usually 1.0–1.2 mL in volume and 1.0 cm in width, sat comfortably within the beam. Neutral density filters from Newport were used to attenuate the power, which was measured with a Liconix 45PM laser power meter. The measured power is less than the actual power incident to the sample since the sensor head is only 7 mm in diameter. Mathematical extrapolation of beam intensity with a Gaussian profile was used to determine the correction factor. The power was measured at approximately 20 points every 0.5 mm to the left and right of the beam hotspot. A close approximation to a Gaussian curve was obtained by plotting power (mW) versus distance (across the beam diameter, mm). The relative intensity of a 10-mm diameter beam was calculated and compared to that measured by the 7-mm diameter sensor head. The actual power incident to the sample was determined to be 1.16–1.25 times that measured by the power meter.

Subsequent to irradiation, the changes in optical spectra as a function of photolysis were probed using optical spectroscopy. Spectra were recorded on an HP8452A UV/vis spectrophotometer interfaced with an AT&T XT computer. The wavelength range of the optical spectra was 190–820 nm, and the resolution of the instrument was approximately 2 nm. Since the beam diameter of the UV/vis was 8 mm, it is certain that the area of sample region probed was within the region photolyzed.

Photolysis Experiments. A typical photolysis experiment consisted of six to eight intervals of photolysis by the laser beam, followed by probing of the changes in optical spectra after each interval with a UV/vis spectrophotometer (probe source). The spectra recorded after each photolysis interval are referred to as "partially photolyzed". Optical spectra of

Table I: Difference Extinction Coefficients (OD·mM^{-1}·cm^{-1})

	Cobalamin to Co(II), Anaerobic (pH 7.45)			
AdoCbl	378 nm	408 nm	470 nm	528 nm
	−3.82 ± 0.09	2.91 ± 0.09	3.16 ± 0.10	−4.47 ± 0.17
AdoCbl (ethylene glycol)	378 nm	408 nm	470 nm	528 nm
	−3.76 ± 0.12	2.84 ± 0.06	2.91 ± 0.09	−4.28 ± 0.10
MeCbl	378 nm	408 nm	470 nm	528 nm
	−4.19 ± 0.07	3.20 ± 0.09	3.14 ± 0.10	−4.89 ± 0.13
MeCbl (ethylene glycol)	378 nm	408 nm	470 nm	528 nm
	−4.65 ± 0.09	3.10 ± 0.08	2.89 ± 0.18	−5.53 ± 0.09
	Cobalamin to Aquocobalamin, Aerobic			
AdoCbl (pH 6.12)	350 nm	380 nm	434 nm	562 nm
	12.42 ± 0.21	−6.43 ± 0.17	−1.88 ± 0.12	−2.42 ± 0.14
base-off AdoCbl[a] (pH 2.05)	352 nm	384 nm	450 nm	530 nm
	15.83 ± 0.21	−4.97 ± 0.15	−5.50 ± 0.11	6.67 ± 0.14
MeCbl (pH 6.12)	352 nm	378 nm	434 nm	560 nm
	11.98 ± 0.25	−6.36 ± 0.14	−1.22 ± 0.06	−2.16 ± 0.07
MeCbl (pH 9.00)	356 nm	380 nm	438 nm	544 nm
	9.56 ± 0.31	−5.96 ± 0.10	−1.17 ± 0.12	−0.96 ± 0.06

[a] Concentrations of base-off AdoCbl were calculated using the following measured extinction coefficients: $-\epsilon_{380}$ = 9.14 ± 0.26 and ϵ_{460} = 9.73 ± 0.16. Calculated values of ϵ_{380} and ϵ_{460} for base-off AdoCbl at pH 2 were different from those previously published (Chemaly & Pratt, 1980a; Ladd et al., 1961).

the pure cobalamin before photolysis and pure cobalamin photoproduct were also recorded. The time interval of irradiation varied depending upon the sample concentration and the laser power. Under optimal conditions a maximum of 30% photoproduct was obtained after accumulation of 8–10 data points. These conditions required sample photolysis for a total of 1–2 min at 5–15 s intervals. The laser powers suitable to obtain these optimal conditions were determined through investigation of how the quantum yield is affected by varying powers. An appropriate laser beam power range was determined to be the region of a plot of power versus quantum yield where the slope is zero (ϕ is constant). For all the experiments reported here, the power range was from 1.03 to 0.26 mW.

The formation of Co(II) due to photolysis by the probe source (probe photolysis) and thermolysis by the laser light was studied and considered insignificant. MeCbl was used to investigate possible probe photolysis since our results show that its CW quantum yield is greater than that for AdoCbl and base-off AdoCbl. Therefore, if probe photolysis is not observed with MeCbl, it is reasonable to assume it is negligible for the other cobalamins. The integrated scan time for each optical spectrum recorded by the spectrophotometer was only 1 s, and the total scan time per experiment was at most 7 s. After 85 s of irradiation by the probe source, conversion of an MeCbl sample to AqCbl was calculated to be less than 5%. The contribution of Co(II) product through thermolysis was examined using a temperature probe. After 20 min of exposure to the laser beam, the temperature of the solution increased by 0.3 °C under an 11-mW beam and remained constant with a 0.65-mW beam. According to Hay and Finke (1988), significant thermolytic products were observed at 100.0 °C after 48 min and complete thermolysis after 25 h. Therefore, even with a maximum power of 11 mW and time of 90 s the thermal energy generated does not cause sample thermolysis.

The photolyzability and photon absorbing capacity at 442 nm of TEMPO, potassium phosphate, and ethylene glycol were also studied. Each species underwent a typical photolysis experiment in the absence of cobalamin. TEMPO exhibited an absorption feature at 442 nm; however, the extinction coefficient at this wavelength is a factor of 100–200 times less than that for AdoCbl. Additionally, the optical spectrum of TEMPO does not change during laser photolysis.

Calculation of the Quantum Yield of Photolysis. Optical data were analyzed using difference spectra, obtained by subtracting the spectrum of the parent cobalamin from that of each partially photolyzed species. The appearance of product was monitored by wavelength maxima of the difference spectra and the disappearance of cobalamin by wavelength minima. Table I gives the difference extinction coefficients at these wavelengths (along with their standard deviation) that were required to calculate the concentration of product formed per time interval. These values were obtained from the difference spectrum of the photoproduct spectrum and its parent spectrum.

In order to calculate the concentration of photons absorbed by the sample, several equations were used. The incident concentration of photons per second, [photons]$_0$/s (μM/s), is calculated from $P_b/(E_{442}N_AV_s)$, where P_b is the power of the laser beam (mW), E_{photon} is the energy (mJ) of the photon at 442 nm, N_A is Avogadro's number and V_s is the volume (L) of the sample. The product of the fraction of photons absorbed, χ_a, the time length of photolysis, t_p, and [photons]$_0$/s gives the concentration of photons absorbed by the sample, [photons]$_{abs}$ (=[photons]$_0$/s)$\chi_a t_p$]). Over periods of irradiation, the optical spectrum of the sample reflects a changing mixture of photolyzed and unphotolyzed species. Although there are no secondary effects of photolysis, the photoproduct is not transparent to light at 442 nm. Therefore, a correction factor, χ_i, is applied to the calculated value of [photons]$_{abs}$ in order to account for the fraction of remaining unphotolyzed species that are available for photolysis. χ_i was calculated for the beginning of each interval from the extinction coefficients at 442 nm for the alkylcobalamins (ACbl) and their photolysis products [PP, either Co(II) cobalamin or aquocobalamin as appropriate] and the concentrations of these species (also at the beginning of each interval). Thus $\chi_i = \epsilon_{ACbl,442}[ACbl]_i/(\epsilon_{ACbl,442}[ACbl]_i + \epsilon_{PP,442}[PP]_i)$. A more elaborate correction, which interpolates the average remaining photoproduct *within* an interval does not increase the precision of the measurements, since the intervals were very short. The final correction, P_c, is for measurement of the power at the face of the sample (vide infra). P_c was determined to be 1.25 for the measured power of 0.65 mW. Thus, eq 1 was the final expression used to calculate total [photons]$_{abs}$ at the end of irradiation interval i.

$$\text{total [Photons]}_{abs} = \sum_{i=1}^{i} \{([\text{photons}]_0/\text{s})\chi_a\chi_i t_p P_c\}_i \quad (1)$$

Quantum yields of CW photolysis were obtained by measuring the slope of a plot of [photolysis product] versus

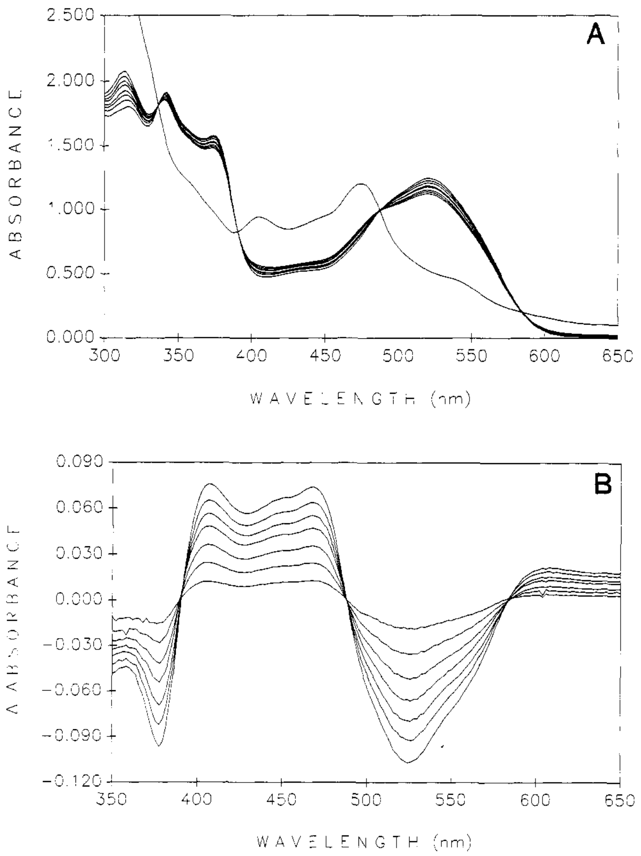


FIGURE 1: Raw optical and difference absorption spectra of photolytic conversion of MeCbl to Co(II) under anaerobic conditions. The raw optical spectra of MeCbl (A) show changes in characteristic bands (520, 373, 340, and 315 nm) as the sample is exposed to successive irradiation intervals of 5 s, for a total of 35 s. Under anaerobic conditions complete photolysis of MeCbl yields Co(II), which is identified by wavelength maxima at 474 and 402 nm. The difference spectra (B) are obtained by subtracting the optical spectra (A) of the partially photolyzed MeCbl from the parent MeCbl. The increasing absorption maxima indicate the appearance of photolysis product, while the increasing absorption minima show the disappearance of MeCbl (Table I). In this experiment a 1-mL sample of 119 μM MeCbl at pH 7.45, sealed under positive nitrogen pressure, was exposed to a 1.00-mW laser beam.

$[\text{photons}]_{\text{abs}}$. The slopes of these plots are linear at low photon concentrations and at low percentages of photolysis. As the percentage of photolysis increases beyond 50%, the product concentration reaches saturation. This results from a gradual depletion over time of the cobalamin concentration relative to the increasing absorbed photon concentration. Although the corrections in eq 1 are applied to counteract the saturation effect, linearity of the plot is ensured by maintaining a level of $\leq 30\%$ photolysis. All spectra collected on the UV/vis spectrophotometer were imported into Spectra Calc (Galactic Industries Corp.) for data analysis. Data used in the calculations and presented in all figures are unsmoothed.

RESULTS

Quantum yields, under anaerobic conditions, were measured for MeCbl and AdoCbl, as well as under aerobic conditions for AdoCbl, MeCbl, and base-off AdoCbl. Figure 1A shows the changes in optical spectra for the conversion of MeCbl to Co(II) at pH 7.45, under anaerobic conditions, as it is photolyzed consecutively at 5-s irradiation intervals. The conversion of MeCbl to Co(II) passes cleanly through isosbestic points (388, 368, and 490 nm), with disappearance of less than 30% of the parent cobalamin over the total irradiation period of 40 s. Figure 2A shows the progression of AdoCbl at pH 6.12 to its photolysis product AqCbl, under aerobic conditions. Photolysis of MeCbl at pH 6.12 and base-off AdoCbl at pH 2.05, in the presence of O_2, also generates photoproducts with optical spectra that resemble AqCbl. At pH 9.00 MeCbl photolysis yields HyCbl, as evidenced by a band at 356 nm. The difference in photolysis product formation due to variations in pH does not affect the quantum yield values.

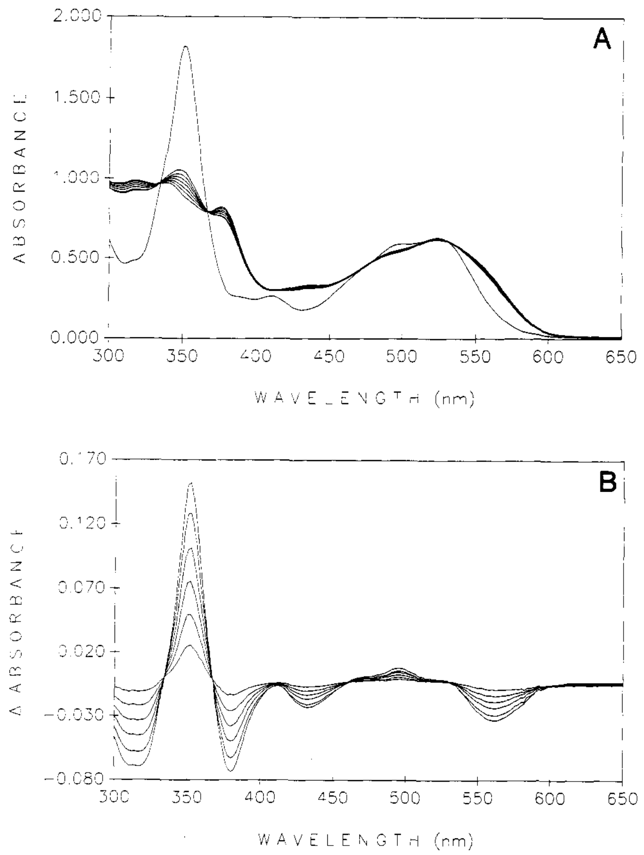


FIGURE 2: Raw optical and difference absorption spectra of the progression of AdoCbl to AqCbl under aerobic photolytic conditions. The optical spectra of AdoCbl (A) exhibit wavelength maxima at 522, 376, 341, and 318 nm. As a 1-mL sample of 77 μM AdoCbl at pH 6.12 is irradiated for 10-s intervals with a 0.65-mW laser beam, the optical spectra show the gradual appearance of a distinct band at 350 nm. Complete photolysis of AdoCbl in the presence of O_2 gives an optical spectrum that resembles AqCbl. The difference spectra (B) are obtained directly from the spectra in panel A. The gradual appearance of AqCbl is shown in the increasing OD at 350 nm. The absorption values of the wavelength extrema (Table I) are used to calculate the concentration of AqCbl formed and the concentration of AdoCbl that disappears.

The changes due to irradiation are observable in the raw optical spectra (Figures 1A and 2A); however, they are significantly enhanced in the difference spectra. Figures 1B and 2B are difference spectra of MeCbl to Co(II) and AdoCbl to AqCbl conversions, obtained directly from the optical spectra in Figures 1A and 2A. The optical spectrum of AdoCbl is very similar to that of MeCbl, in both H_2O and 80% ethylene glycol. Therefore, their difference maxima and minima obtained from anaerobic photolysis are identical (Figure 1B and Table I). Under aerobic conditions, difference extrema for AdoCbl (Figure 2B) and MeCbl at pH 6.12 are very similar. MeCbl at pH 6.12 and pH 9.00 have different wavelength extrema since the photolysis products are AqCbl and HyCbl, respectively. Figure 3 shows the base-off AdoCbl to AqCbl difference spectra obtained under aerobic conditions.

Figure 4 shows 0–15% photolysis for MeCbl (A), AdoCbl (B), and base-off AdoCbl (C) and their relative quantum yields under anaerobic (TEMPO trap, O) and aerobic

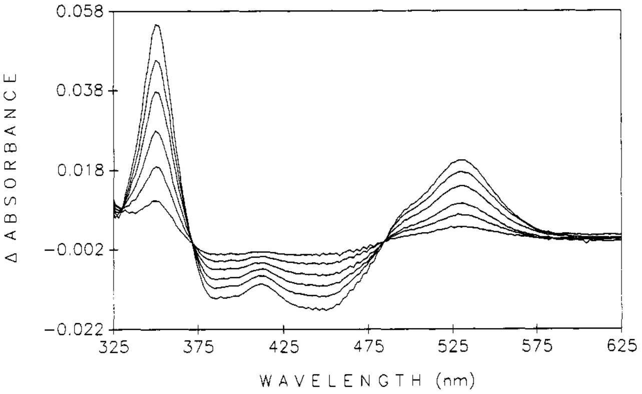


FIGURE 3: Base-off AdoCbl difference spectra. The photolysis of base-off AdoCbl in the presence of O_2 gives an optical spectrum which resembles AqCbl. Difference spectra of partially photolyzed base-off AdoCbl and pure base-off AdoCbl show characteristic wavelength extrema at 352, 384, 450, and 530 nm. The raw optical data used to obtain these difference spectra were collected after each 5-s interval of irradiation with a 1.00-mW beam (total photolysis time was 30 s). A lower initial concentration of base-off AdoCbl, 41 μM, was used due to a lower concentration of photolysis product after 5 s.

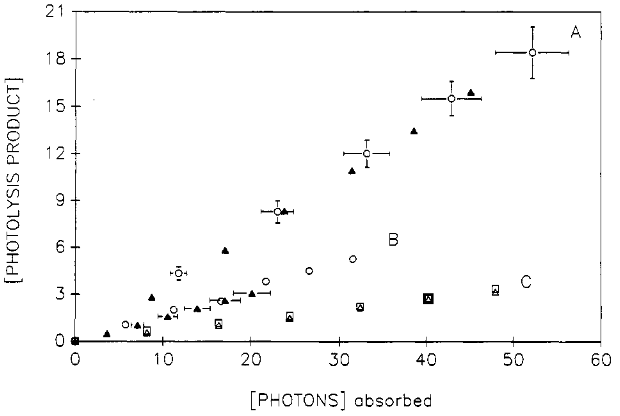


FIGURE 4: Comparison of CW quantum yields for MeCbl, AdoCbl, and base-off AdoCbl. The relative quantum yields for MeCbl (A), AdoCbl (B), and base-off AdoCbl (C) are obtained from the slopes of these plots. There is agreement between the anaerobic and aerobic quantum yields for AdoCbl and MeCbl. This is clearly shown for AdoCbl (B, $\phi_{(Co\text{–}C\alpha),442}$ = 0.20 ± 0.03 and $\phi_{(Co\text{–}C\alpha),442}$ = 0.19 ± 0.04) and MeCbl (A, $\phi_{(Co\text{–}C\alpha),442}$ = 0.36 ± 0.04 and $\phi_{(Co\text{–}C\alpha),442}$ = 0.35 ± 0.03), where two plots, anaerobic (○) and aerobic (▲), are overlaid. Additionally, the quantum yields calculated at each wavelength extremum shown in Table I for each cobalamin also match. This is demonstrated in part C where two plots of base-off AdoCbl ($\phi_{(Co\text{–}C\alpha),442}$ = 0.045 ± 0.015), one from 530 nm (△) and one from 384 nm (□), are overlaid. The linear plots show ca. 17% photolysis of 147 μM (anaerobic) and 99 μM (aerobic) MeCbl, a maximum of ca. 16% photolysis of 37 and 45 μM AdoCbl, and ca. 5% photolysis of 76 μM base-off AdoCbl. Data for these plots were obtained from photolysis experiments using a laser beam of 0.65–1.00 mW at 5-s intervals. The error bars for MeCbl and AdoCbl represent less than 10% error for both axes, while those for base-off AdoCbl are less than 5%.

conditions (O_2 trap, ▲). The slopes of these lines give quantum yield values within the error of the results shown in Table II. A comparison of quantum yields measured in pH 7.45 H_2O and 80% ethylene glycol for AdoCbl and MeCbl shows no change for MeCbl in ethylene glycol. A 4-fold decrease of the quantum yield for AdoCbl is observed when the solvent is changed from H_2O ($\phi_{(Co\text{–}C\alpha),442}$ = 0.20 ± 0.03) to 80% ethylene glycol ($\phi_{(Co\text{–}C\alpha),442}$ = 0.053 ± 0.009).

Table II: Quantum Yields of Photolysis of the Co–Cα Bond at 442 nm

cobalamin	CW aerobic $\phi_{(Co\text{–}C\alpha),442}$	CW anaerobic $\phi_{(Co\text{–}C\alpha),442}$	ns anaerobic $\phi_{(Co\text{–}C\alpha)}$[a]
AdoCbl			
pH 7.45		0.20 ± 0.03	0.23 ± 0.04
ethylene glycol		0.053 ± 0.009	
pH 6.12	0.19 ± 0.04		
base-off AdoCbl	0.045 ± 0.009		0.045 ± 0.015
MeCbl			
pH 7.45		0.35 ± 0.03	
ethylene glycol		0.38 ± 0.06	
pH 6.12	0.36 ± 0.04		
pH 9.00	0.37 ± 0.05		

[a] See Chen and Chance (1990).

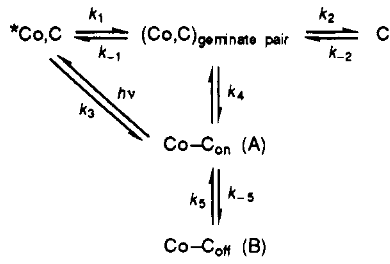
There is agreement between the quantum yields obtained under anaerobic conditions and those obtained under aerobic conditions, supporting the use of either method. Additionally, the quantum yields calculated at each extremum shown in Table I for each cobalamin also match. This is demonstrated in Figure 4C, where two data sets for base-off AdoCbl, one obtained from a maximum (△) and one from a minimum (□), are overlaid. Thus, the decreasing concentration of parent cobalamin photolyzed within each time interval is exactly accounted for by the increasing concentration of Co(II) or AqCbl formed. This, along with the clean isosbestic points shown in Figures 1B, 2B and 3, indicates that there are no other corrin products or intermediate states identifiable within the experimental conditions.

DISCUSSION

Investigations of light-induced anaerobic conversion of AdoCbl and MeCbl to Co(II) have indicated a slow photolysis rate that results from the rapid back-reaction. Co(II) photoproduct from photolysis of AdoCbl under anaerobic and CW experimental conditions is only observable after a considerable time of irradiation. During this time MeCbl appears unphotolyzable, since Co(II) is not detected (Pratt, 1964). Factors which remove an essential component of the back-reaction facilitate spectroscopic observation of the photolysis products (Pratt, 1964, 1972a,b; Schrauzer et al., 1968; Hogenkamp, 1963, 1966; Hogenkamp et al., 1962; Johnson et al., 1963). For MeCbl, hydrogen abstraction and dimerization of the methyl radical lead to Co(II) detection, although only after ca. 20 h of irradiation. Anaerobically, cyclization of the 5′-deoxyadenosyl radical species acts as a natural radical trap, allowing for detection of Co(II) (Hogenkamp, 1963, 1966; Pratt, 1964; Johnson et al., 1963; Yamada et al., 1966a,b; Hay & Finke, 1986). Thus, acceleration of photolysis under anaerobic conditions is dependent upon the irreversible consumption of radical or Co(II), which removes the recombination potential.

The measured CW quantum yields of this paper agree with our previous ns quantum yield measurements for AdoCbl ($\phi_{(Co\text{–}C\alpha)}$ = 0.23 ± 0.04 at 532 and 355 nm) and base-off AdoCbl ($\phi_{(Co\text{–}C\alpha)}$ = 0.045 ± 0.015 at 532 nm) (Chen & Chance, 1990). These results are consistent with our previous suggestion that the quantum yield values measured aerobically by Endicott and Netzel (1979) and Taylor et al. (1973) are low (Chen & Chance, 1990). Using CW photolysis with O_2 scavenging, Taylor's group reported that at pH 7.0 the quantum yield for AdoCbl falls in a range between 0.088 and 0.135 for irradiation wavelengths from 570 to 250 nm. For MeCbl there was an increase in quantum yield at pH 7.0 from 0.12 at 570 nm to 0.35 at 250 nm. These results contradict investigations of MeCbl by Pratt and Whitear (1971) that gave a quantum yield value of ca. 0.30 with no wavelength dependence. The quantum yields for MeCbl obtained aerobically by Pratt and Whitear agree with our numbers within the experimental error. The low quantum yields for MeCbl and AdoCbl at pH 7.0 and ca. 570 nm may be explained by

Scheme I



the apparent use of deionized H_2O buffered at pH 7.0 and single-wavelength detection at 250 nm. In our experiments, we detected the formation of a mixture of AqCbl and HyCbl photolysis product at pH 7.0. Since AqCbl was the expected product, Taylor's OD value at 350 nm underestimates the concentration of photolysis product formed since the absorption maximum for a mixture of products is actually ca. 354 nm to pH 7.0. However, the difference between the OD values at 350 nm vs 354 nm is only able to account for 43% of the factor (2.90) by which the quantum yield is low. The use of a focused photolyzing beam as described by Taylor et al. may contribute to the remaining 57%, since only a small fraction of the 3-mL cobalamin solution can be photolyzed per irradiation interval.

Several investigations have suggested that six-coordinate alkylcobalamins exhibit a temperature-dependent axial–base equilibrium (Chemaly & Pratt, 1980a,b; Firth et al., 1968; Brown, 1987; Brown et al., 1988). In the presence of an axial–base equilibrium, a complete mechanism (Scheme I) involves an equilibrium between the six-coordinate ($Co–C_{on}$, A) form and the unprotonated base-off ($Co–C_{off}$, B) species, where B is inclusive of several unprotonated base-off forms. As the concentration of B increases, ϕ_{on} is masked by ϕ_{off}, and $\phi \approx f\phi_{on} + (1 - f)\phi_{off}$ (where f is the fraction of base-on).

The quantum yield values obtained in this study were based on the assumption that at 25–26 °C MeCbl and AdoCbl are substantially (ca. 95%) A in H_2O and ethylene glycol. This assumption is supported by comparison of AdoCbl and MeCbl optical spectra at 25 °C in water vs 80% ethylene glycol and by temperature-dependent axial–base equilibrium studies that indicate that A is predominant at 25 °C for AdoCbl and MeCbl (Chemaly & Pratt, 1980a,b; Hogenkamp et al., 1975; Finke & Haye, 1984; Firth et al., 1968; Martin & Finke, 1992). Thus, when $[A] \gg [B]$, the previously described ϕ expression (eq 2) (Chen & Chance, 1990) is generated. $\phi \approx \phi_{on}$ can be justified under conditions of 97–95% base-on cobalamin, where an upper limit of $\phi_{off} = 1$ changes ϕ_{on} by only 0.03–0.05.

$$\phi \approx \phi_{on} = \frac{k_1 k_2}{(k_1 + k_3)(k_2 + k_4)} \qquad (2)$$

The quantum yield results measured in H_2O vs ethylene glycol can be explained considering the possible role of the solvent "cage". The "cage" describes a solvent pocket which surrounds the geminate radical pair, such as Co(II) and the methyl radical (Franck & Rabinowitsch, 1934; Lamp & Noyes, 1954; Noyes, 1955; Hammond & Turro, 1963; Rice, 1985). Radicals that are trapped in a "cage" experience an increased probability of geminate recombination. Geminate recombination rates of AdoCbl in H_2O ($k_4 \approx 1 \times 10^9$ s^{-1}) are competitive with cage escape ($k_2 \approx 1.8 \times 10^9$ s^{-1}; Endicott & Netzel, 1979; Tsou et al., 1982). Since the rate of geminate rebinding is assumed to be independent of solvent (Nodelman & Martin, 1976), a solvent cage that decreases the rate of cage escape will reduce ϕ.

The important role of the "cage" in Co–Cα bond homolysis has been previously suggested in cobalamins and related systems (Ng et al., 1982; Endicott & Netzel, 1979). A relationship between the solvent cage and the quantum yield is established by using the expression for cage efficiency, F_c (Koenig & Fischer, 1973; Owens & Koenig, 1974; Koenig et al., 1988; Koenig & Finke, 1988), in eq 2. F_c, defined by Scheme I as $k_4/(k_4 + k_2)$, is directly correlated to ϕ, eq 3.

$$\phi \approx \frac{k_1 k_2 F_c}{k_4(k_1 + k_3)} \qquad (3)$$

Although little is known about the values of k_1 and k_3 in the photodissociation of cobalamins, they may be of the same magnitude as those found in iron hemes. If we assume that k_1 ($\approx 3 \times 10^{12}$ s^{-1}) and k_3 ($\approx 3 \times 10^{11}$ s^{-1}) (Anfinrud et al., 1989; Martin et al., 1983) are independent of the solvent system, then $k_1/(k_1 + k_3)$ may be redefined as a constant K ($K \approx 0.9$). The effects of large or negligible cage efficiencies on ϕ are clearly identified in the simplified form of ϕ ($\approx Kk_2F_c/k_4$). With a large cage efficiency, where the geminate recombination rate is faster than the rate of cage escape ($k_4 \gg k_2$ and $F_c \approx 1$), ϕ approaches zero. Alternatively, ϕ approaches 1 when F_c becomes small ($k_2 \gg k_4$).

Analysis of the quantum yield results requires consideration of radical side reactions. Some competitive side reactions may lead to misinterpretation of the level of geminate recombination and the quantum yield. In the cobalamin system, possible reactions include electron transfer, hydrogen abstraction by the carbon radical from the solvent and/or the corrin ring, dimerization, cyclization, and diffusional recombination (Pratt, 1972b; Ng et al., 1982; Hay & Finke, 1986; Turro, 1978; Jacobsen & Bergman, 1985). An additional "induced" trapping process is introduced by the presence of TEMPO. Co(I) and Co(III) are not detected by direct spectroscopic observation, confirming the absence of electron-transfer reactions. The rates of cyclization (5′-deoxyadenosyl radical, 10^4–10^5 s^{-1}), TEMPO radical trapping [$(3–5) \times 10^8$ M^{-1} s^{-1}], and hydrogen abstraction (10^2–10^4 M^{-1} s^{-1}) have been estimated in both ethylene glycol and H_2O (Finke & Hay, 1984; Hay & Finke, 1986, 1987, 1988; Chen & Chance, 1990; Nigam et al., 1976; Thomas, 1967). Methyl dimerization rates (10^{10} M^{-1} s^{-1}) have also been measured in H_2O (Pailes & Hogenkamp, 1968; Pryor, 1966). Recombination of the methyl (10^9 M^{-1} s^{-1}) and 5′-deoxyadenosyl (10^8–10^9 M^{-1} s^{-1}) radicals with Co(II) is found to be near the diffusion-controlled limit in H_2O (Chen & Chance, 1990; Finke & Hay, 1984; Endicott & Ferraudi, 1977; Endicott & Netzel, 1979; Roche & Endicott, 1974). In the limit of 30% photolysis of the cobalamin solution [or 30 μM Co(II)], the above-mentioned processes are orders of magnitude smaller than the rate of TEMPO trapping. Thus, the quantum yield values reflect the differences in primary photophysics and the competition between cage escape and geminate recombination and are not contaminated by artifactual side reactions.

Evaluation of the quantum yield values measured in H_2O and ethylene glycol suggests that factors such as size and mass affect the probability of radical escape from the geminate pair state in more viscous media (Endicott & Netzel, 1979; Rice, 1985; Roche & Endicott, 1974; Herk et al., 1961; Booth & Noyes, 1960). The effect of the two solvents on the carbon radicals is examined by comparing the time-independent initial separation distances of the radicals. According to Noyes (1955), the initial separation distance, $\sim [(6mk_bT)^{1/2}]/6\pi\eta a$ (a and m, radical radius and mass; η, viscosity of the solvent; k_b, Boltzmann constant), is reached when the energy ($3k_bT/2$) with which the geminate pair separates is lost to viscous drag. The estimated radii for the methyl and 5′-deoxyadenosyl radicals are 1.1 and 6 Å, respectively (Weast, 1981). The radius for the 5′-deoxyadenosyl radical was calculated according to the distance between Cα and the NH_2 of the adenine. The calculated separation distances decrease in ethylene glycol (vs H_2O) by 40% for the 5′-deoxyadenosyl radical and 15% for the methyl radical, suggesting that the stronger solvent cage decreases the cage escape probability. Although the calculation for MeCbl in ethylene glycol predicts a small drop in quantum yield, no change is observed. This result for MeCbl has at least two explanations. First, the initial separation distance traveled by the methyl radical in ethylene glycol (0.11 Å) is still larger than the distance traveled by the 5′-deoxyadenosyl radical in H_2O (0.10 Å). Thus, although the predicted distance decreases in ethylene glycol, the distance may still be long enough so that the recombination potential is unaffected. Second, structural barriers unrelated to the initial separation distances may influence the geminate recombination. This is consistent with FTIR data of corrin ring equatorial modes (Taraszka et al., 1991).

It is established that upon cleavage of the Co–Cα bond the Co atom moves out of the corrin plane toward the DMB group (Glusker, 1982, Krautler et al., 1989). EXAFS studies have provided evidence that the Co–N*ax* distance decreases at the same time (Sagi et al., 1990; Wirt et al., 1992). One requirement for successful recombination of Co(II) with the radical is the reversal of this process. Although structural data by Krautler et al. (1989) show that the "butterfly bending" of the corrin ring is similar for Co(II) cobalamin and alkyl–Co(III) cobalamins, some variations in the corrin ring structure are likely. FTIR studies have identified a corrin ring breathing mode that is highly sensitive to the inductive effects of the axial ligands. It is interesting to note that, as the position of this mode decreases and moves closer to the value for Co(II) cobalamin [AdoCbl, 1570 cm^{-1}; MeCbl, 1568 cm^{-1}; ethylcobalamin (EthCbl), 1566 cm^{-1}; Co(II), 1563 cm^{-1}], the quantum yield of photolysis increases (AdoCbl, 0.23; MeCbl, 0.35; EthCbl, 0.50, Chance et al., unpublished observations for EthCbl). If ethylcobalamin more closely resembles Co(II) cobalamin in important features of the corrin ring, this could lower the barrier to photolysis. As such, it would tend to have its effect on the primary yield of photolysis, which could be investigated with ultra-fast optical methods (subnanosecond).

Our data strongly support the existence of a "geminate" solvent cage surrounding AdoCbl that influences the fate of radicals generated by homolytic cleavage of the cobalt–carbon bond. The 4–5-fold decrease in quantum yield due to variation in solvent is unlikely to be due to changes in the photophysics of the reaction and is related to an increase in the cage efficiency (F_c). By contrast, MeCbl is much less sensitive to the influence of solvent, and we find it reasonable that the methyl radical is less inhibited by a solvent cage. Also, the quantum yield value for base-off AdoCbl suggests a high cage efficiency for this species, assuming the primary quantum yields of base-on and base-off AdoCbl are similar. A recent paper by Garr and Finke (1992) demonstrates that high concentrations of TEMPO can function as an in-cage trap. Thermolysis experiments with 5′-deoxyadenosylcobinamide indicate a cage efficiency of 0.94–1.00. Thus, if base-off AdoCbl and its cobinamide are structurally similar [and there is evidence that this is the case, see Wirt and Chance (1992)] and if the primary quantum yield of base-off AdoCbl is near unity, an F_c value of 0.95 would exactly account for the quantum yields observed here.

In conclusion, these results must be examined in light of the relevant enzymology of the MeCbl and AdoCbl cofactors. In all cases demonstrated so far, AdoCbl cofactors are associated with homolytic cleavage reactions, while MeCbl cofactors are associated with methylation and heterolytic cleavage of the cobalt–carbon bond [for a discussion see Wirt et al. (1992) and references therein]. As has been previously suggested (Chen & Chance, 1990; Pett et al., 1987; Krouwer et al., 1980), the function of the protein is to orient and manipulate the nascent radical species, transferring them to substrate directly or through a protein radical intermediary. The solvation state in the interior of proteins is poorly understood in most cases. In cobalamin enzymes it is likely that the protein provides the "cage" environment for the generated radicals. It is possible that the AdoCbl cofactor is a useful radical generator for the following reasons: (1) since it is bulky, it cannot escape easily from the cobalamin "pocket"; (2) it has numerous polar and nonpolar groups to which the enzyme can attach so as to orient and control the radical. By contrast, the methyl radical is particularly unsuited to ultimate transfer to a substrate. Since it is small and highly reactive, it is much more difficult to control. Thus, the dynamic interactions of the enzyme and the AdoCbl radical, with the enzyme functioning as a sophisticated cage, are critical areas for future research.

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