Studies on the Coordination of Reduced Glutathione to B_{12} Coenzymes

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Abstract: Reduced glutathione coordinates (S bonded) to cobalt in 5′-deoxyadenosylcobalamin at pH 7.4 and in methylcobalamin at pH 4.0 by displacing the 5,6-dimethylbenzimidazole group from the sixth coordination site. Uv–visible spectral studies, photolability, and pH optima for these two glutathione complexes with B_{12} confirm that 5,6-dimethylbenzimidazole has been displaced by reduced glutathione. The Co–C σ bond in the two coenzymes is much less stable to light when glutathione is coordinated in place of 5,6-dimethylbenzimidazole. Electron spin resonance spectra of the reduced glutathione–5′-deoxyadenosylcobalamin complex and the diaquocobinamide-reduced glutathione complex were compared after photolysis under anaerobic conditions. The results from this experiment show that reduced glutathione does not form a stable complex with Co^{2+}, and after quantitative homolysis of the Co–C σ bond, then 5,6-dimethylbenzimidazole recoordinates to Co^{2+} in place of reduced glutathione.

Sulfhydryl groups are believed to be very important in vitamin B_{12} dependent enzymes. With the exception of bacterial methyl malonyl coenzyme A (CoA) mutase, all B_{12}-requiring enzymes so far discovered have a sulfhydryl group either as a functional group on a subunit of an enzyme, or else a thiol compound of low molecular weight is necessary as a coenzyme. Inhibition of enzyme catalysis by sulfhydryl specific reagents is found in the B_{12} enzymes ethanolamine ammonialyase,[1] glutamate mutase,[2,3] diol dehydrase,[4] and methionine synthetase.[5] A low molecular weight thiol, such as lipoic acid or dithioerythritol, is essential for the enzymes ribonucleotide reductase,[6] L-β-lysine mutase,[7] methionine synthetase,[5] and glutamate mutase.[3] The reaction between thiols and aquocobalamin has been studied extensively.[8–10] For this reaction aquocobalamin is either reduced to cob(II)alamin (B_{12}-r) or else the thiol coordinates in place of water in the fifth coordination site. However, ligand substitution reactions on the biologically important B_{12} coenzymes 5′-deoxyadenosylcobalamin and methylcobalamin have not been studied to our knowledge. We have chosen reduced glutathione to study 5,6-dimethylbenzimidazole displacement because this thiol is a peptide which best simulates the type of interaction which may be expected to occur in proteins. Furthermore, earlier studies with B_{12} derivatives have established that reduced glutathione will coordinate to cobalt.[11–14]

Experimental Section

5′-Deoxyadenosylcobalamin was synthesized and purified by the procedure of Law, *et al.*[15] Aquocobalamin was obtained by photolysis of methylcobalamin under aerobic conditions, and diaquocobinamide was synthesized by photolysis of methylaquocobinamide (Penley, *et al.*[16]). Reduced glutathione was purchased from Nutritional Biochemicals. For experiments conducted under anaerobic conditions, solutions were degassed with O_2-free argon. All experiments with alkylcobalamins were carried out in subdued light.

Methods. Uv–visible spectra were recorded using a Perkin-Elmer-Coleman 124 spectrophotometer. The percentage of each species present in a spectrum was calculated by using a least-squares program on B_{12} compounds developed for an IBM 1800 computer. Anaerobic cuvettes were set up by continuously evacuating and flushing with O_2-free argon. Esr experiments were carried out with a Varian V-4502 spectrometer operating at X-band frequency. Co^{2+} esr spectra were recorded at 85°K, and g values were calculated from the known frequency obtained by using a Hewlett-Packard 5420-A 12.4-GHz digital frequency meter. Samples for Co^{2+} esr experiments were transferred under anaerobic conditions to quartz esr tubes *via* a gas tight syringe. After transfer these samples were immediately frozen in liquid N_2. 1,2-Propanediol was added to each esr tube (1:4 v/v) in order to enhance the esr signal.

Results

5′-Deoxyadenosylcobalamin–Glutathione Complex. When 1.0 ml of 5×10^{-5} M 5′-deoxyadenosylcobalamin is mixed with 0.5 ml of 0.2 M reduced glutathione at pH 7.5, there is a shift in the visible spectrum to a typical "base-off" cobalamin (Figure 1). The λ_{max} shifts from 525 to 465 nm. The complex with a λ_{max} at 465 nm is still photolabile, and upon photolysis in the presence of air, the spectrum shifts to give that of the aquocobalamin–glutathione complex which has been previously reported.[11] The titration of 5′-deoxyadenosylcobalamin with reduced glutathione was carried out by adding 0.2 ml of various concentrations of reduced glutathione to 1.0-ml aliquots of 5′-deoxyadenosylcobalamin (2×10^{-5} M) in 0.05 M KH_2PO_4 buffer at pH 7.4. For each reaction the spectrum was recorded from 420 to 650 nm, and the concentrations of the glutathione complex *vs.* free 5′-deoxyadenosylcobal-

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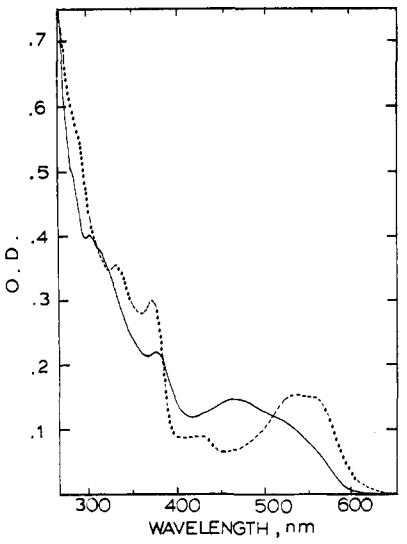
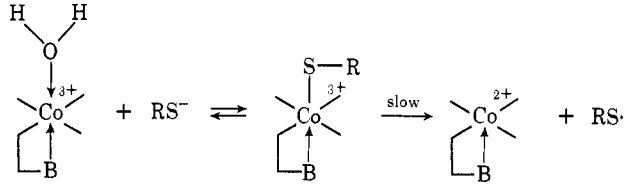


Figure 1. Uv-visible spectrum of the reduced glutathione–5′-deoxyadenosylcobalamin complex at pH 7.4 (——) and the reduced glutathione–aquocobalamin complex at pH 7.4 (- - -).

amin was determined by the computer program described under Methods. The concentration of reduced glutathione to form a complex with 2×10^{-5} *M* 5′-deoxyadenosylcobalamin was 1.2×10^{-1} *M* (6000 times the concentration of the alkylcobalamin) (Figure 2). The formation of the glutathione complex depends on the absolute concentration of reduced glutathione rather than the relative concentration. For this titration an isosbestic point was obtained at 475 nm. When a similar titration was carried out with aquocobalamin, 2.5×10^{-4} *M* reduced glutathione was required to form a complex with 2×10^{-5} *M* aquocobalamin (ten times the concentration of aquocobalamin) (Figure 2). Apparent log K_f values for the glutathione complexes formed between 5′-deoxyadenosylcobalamin and aquocobalamin were 0.5 and 4.8, respectively. The log K_f for the aquocobalamin–glutathione complex was previously reported as 4.9.[17] A very high concentration of reduced glutathione relative to 5′-deoxyadenosylcobalamin is required to displace 5,6-dimethylbenzimidazole, and this is not too surprising since the log K_f for the formation of monocyanocobalamin from aquocobalamin with cyanide ion is 5.6, but the displacement of benzimidazole with a second cyanide to give dicyanocobalamin gives a log K_f of 2.7 at pH 7.4.

Schrauzer[18] has reported that in the presence of thiol, aquocobalamin undergoes the following equilibrium.



When 1.0 ml of 10^{-5} *M* aquocobalamin in 0.05 *M* KH_2PO_4 buffer at pH 7.4 was mixed with 0.5 ml of 0.2 *M* reduced glutathione, the reduced glutathione aquocobalamin complex was formed (Figure 3). When the

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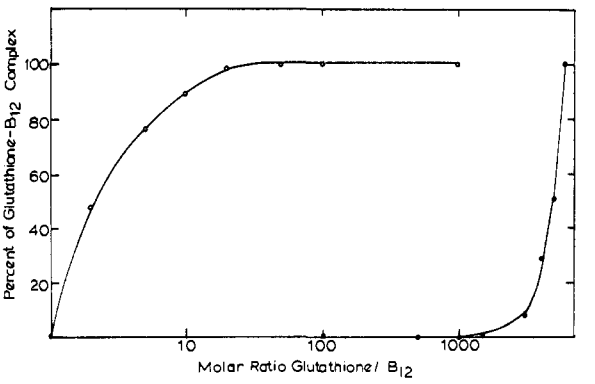

Figure 2. Complex formation at different reduced glutathione concentrations with 2×10^{-5} *M* solutions of aquocobalamin (○) and 5′-deoxyadenosylcobalamin (●) at pH 7.4.

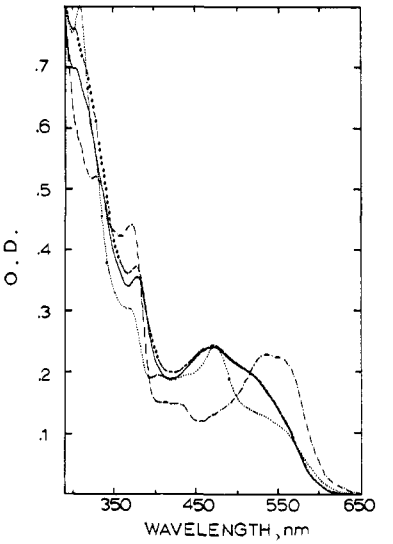

Figure 3. The instability of the reduced glutathione–aquocobalamin complex to heat. Uv-visible spectrum taken under anaerobic conditions of 10^{-5} *M* reduced glutathione–aquocobalamin complex at pH 7.4 before heating (——·), and after heating to 100° for 1 min (· · ·). The stability of the reduced glutathione–5′-deoxyadenosylcobalamin complex to heat (10^{-5} *M*) before heating (——) and after heating at 100° for 1 min (- - -).

solution containing this complex is degassed with argon and heated at 100° for 1 min, then the reduced glutathione–aquocobalamin spectrum collapses to give cob(II)alamin (Figure 3). These data support Schrauzer's proposed scheme. When the same reactions were conducted and examined by esr, then upon heating the reduced glutathione–aquocobalamin complex, cob(II)alamin and a sulfhydryl radical signal were observed. When the same reaction was repeated with 5′-deoxyadenosylcobalamin in place of aquocobalamin, then little change in the uv-visible spectrum was observed (Figure 3).

The pK_a for the reduced glutathione sulfhydryl group is 9.7.[17] Therefore, in the pH range of 2–7, this group is protonated. The coordination of reduced glutathione to alkylcobalamins is therefore independent of the dissociation of the sulfhydryl group to give the thiolate anion. When reduced glutathione coordinates in place of 5,6-dimethylbenzimidazole, then it is to be expected that protonation and displacement of benzimidazole should promote complexation with reduced

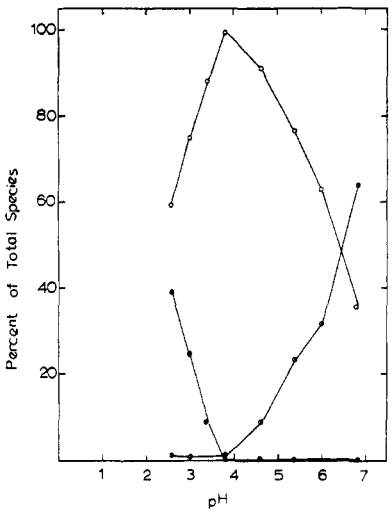

Figure 4. The pH dependence for the formation of the reduced glutathione–5′-deoxyadenosylcobalamin complex. Different pH values were adjusted in 0.1 *M* citrate-phosphate buffer: reduced glutathione–5′-deoxyadenosylcobalamin complex (%) (○), "base-on" 5′-deoxyadenosylcobalamin (%) (◑), and "base-off" 5′-deoxyadenosylcobalamin (%) (●).

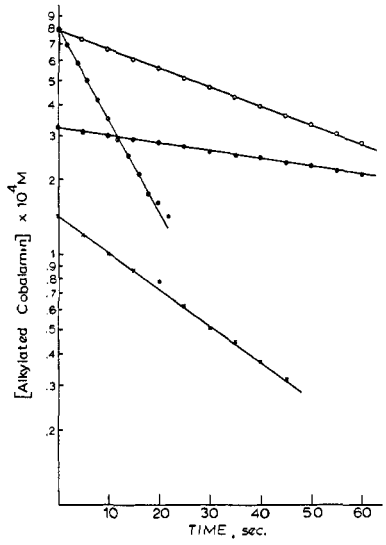

Figure 5. Photolysis rates for the reduced glutathione spin-labeled alkylcobalamins compared with spin-labeled alkylcobalamins. Reduced glutathione spin-labeled 5′-deoxyadenosylcobalamin complex (×), spin-labeled 5′-deoxyadenosylcobalamin complex (●), reduced glutathione spin-labeled methylcobalamin complex (◑), and spin-labeled methylcobalamin complex (○). Experimental methods are given in the text.

glutathione. Figure 4 shows the amount of reduced glutathione complex formed with 5′-deoxyadenosylcobalamin as the pH is decreased. Due to the large number of acid–base equilibria to be considered in both reduced glutathione and 5′-deoxyadenosylcobalamin it is difficult to determine a pK_a value for the displacement of reduced glutathione. However, from this pH profile it is clear that reduced glutathione forms a much more stable complex with 5′-deoxyadenosylcobalamin than 5,6-dimethylbenzimidazole.

Methylcobalamin–Glutathione Complex. Methylcobalamin reacts with reduced glutathione in an identical way to 5′-deoxyadenosylcobalamin. The reaction of methylcobalamin with reduced glutathione was studied at pH 4.0 because at pH values greater than 8.7, the methyl group is transferred from cobalt to the sulfhydryl group.[19] When methylcobalamin (5×10^{-5} *M*) is mixed with reduced glutathione (0.15 *M*), a spectrum resembling "base-off" methylcobalamin is obtained; there is a shift in the λ_{max} of the α band from 530 to 490 nm. The resulting reduced glutathione–methylcobalamin complex is stable to heat but unstable to light. Photolysis of this complex in the presence of air yields a product with an identical spectrum to reduced glutathione–aquocobalamin (Figure 1). Furthermore, the reduced glutathione–methylcobalamin complex shows similar pH dependency to the reduced glutathione–5′-deoxyadenosylcobalamin complex (Figure 4).

Stability of the Co–C σ Bond in the Reduced Glutathione–Alkylcobalamin Complexes. The photolability of the Co–C σ bond in the reduced glutathione–alkylcobalamin complexes was compared to that of their corresponding alkylcobalamins by using covalently spin-labeled corrinoids labeled in the B ring.[20] Esr was used to monitor the interaction between the unpaired electrons on cobalt and piperidine-*N*-oxyl during homolytic cleavage of the Co–C bond with light. The reduced glutathione is slowly oxidized by the piperidine-*N*-oxyl derivative to glutathione sulfate.[21] However, the rate of disappearance of the nitroxide esr signal is much slower for the oxidation–reduction reaction than it is for the interaction between the unpaired electrons generated by photolysis. In these photolysis experiments the spin-labeled alkylcobalamin solutions were degassed with argon and transferred anaerobically to the quartz esr cells. A projector with a 250-W tungsten filament lamp was placed 80 cm from the esr cavity. The rate of photolysis was determined by recording the change in the intensity of a single nitrogen hyperfine esr signal using the method of Law, *et al.*[15] Figure 5 shows the relative rates of homolytic cleavage with light of the Co–C bond for methylcobalamin and 5′-deoxyadenosylcobalamin compared to their corresponding reduced glutathione complexes. The relative rates for the photolysis of the glutathione complexes of methylcobalamin and 5′-deoxyadenosylcobalamin, respectively, compared to the uncomplexed alkylcobalamins were 3.8 and 4.6 times faster. Similar results were obtained spectrophotometrically for this photolysis reaction.

Reduced Glutathione–Diaquocobinamide Complex. Diaquocobinamide (5×10^{-5} *M*) reacts with reduced glutathione to give a complex. The reaction occurs smoothly at concentrations of reduced glutathione less than 6×10^{-4} *M* at a pH of 7.4. The resulting complex has λ_{max} at 495, 420, and 351 nm which differ markedly from the reduced glutathione–aquocobalamin complex (λ_{max} 510, 420, 375, and 330 nm). The reduced glutathione–diaquocobinamide complex is heat labile, but no reduction to Co^{II} occurs upon raising the temperature as is the case for the reduced glutathione–aquocobalamin complex. Upon heating the reduced glutathione–diaquocobinamide complex, the uv-visible spectrum collapses to give a λ_{max} at 351 nm and a broad

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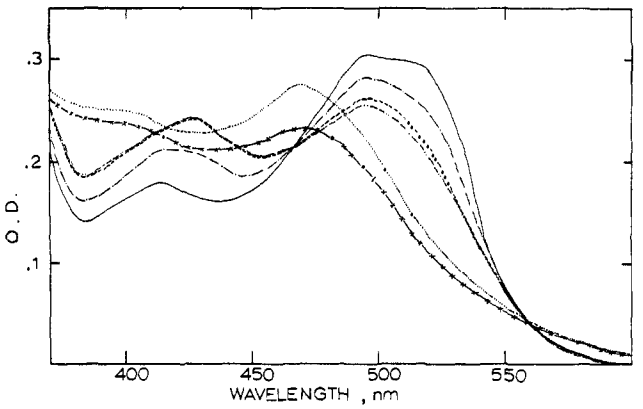

Figure 6. The reduction of diaquocobinamide with reduced glutathione. Spectra represent: (a) diaquocobinamide (5×10^{-5} *M*) at pH 7.4 (——); (b) reduced glutathione added to (a) at a concentration of 5×10^{-6} *M* (——·), 2.5×10^{-4} *M* (– – –), 5×10^{-4} *M* (—··), 10^{-3} *M* (×), and 2×10^{-3} *M* (···).

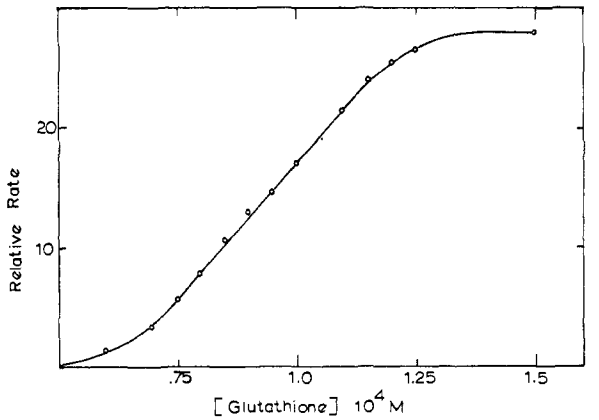

Figure 7. Rate of reduction of diaquocobinamide by reduced glutathione at pH 7.4.

absorption maximum at 530–440 nm. This spectral change was accompanied by a sharp decrease in extinction of the chromophore. The dissociation constant of the reduced glutathione–diaquocobinamide complex was determined to be 4.6×10^{-7} *M* compared to the reduced glutathione–aquocobalamin complex (1.3×10^{-5} *M*), and therefore the cobinamide complex is much more stable. These data suggest that reduced glutathione is coordinated at the sixth coordination site. When diaquocobinamide (5×10^{-5} *M*) reacts with concentrations of reduced glutathione in excess of 6×10^{-4} *M*, a second species is formed (Figure 6). This species has a λ_{max} at 470 nm which indicates that the original reduced glutathione–diaquocobinamide complex has been reduced to Co^{II}. The formation of this Co^{II} species is not instantaneous, and its rate of formation can be measured by the change in extinction at 510 nm after the addition of reduced glutathione. This transition was shown to be dependent on the concentration of reduced glutathione added to reaction mixtures (Figure 7). When an oxygen electrode was used to determine the O_2 concentration in the reaction mixture during this reduction reaction, it was found that O_2 removal had a direct correlation with the rate of the reaction. The reduction of the reduced glutathione–diaquocobinamide complex is shown to be dependent on the removal of O_2 from the solution by uncomplexed reduced glutathione. Potentiometric titrations have shown that aquocobalamin is reduced more easily than diaquocobinamide.[22] The standard electrode potentials for these reactions are as follows

	E_0, V
aquocobalamin $\underset{e}{\overset{e}{\rightleftharpoons}}$ cob(II)alamin	−0.03
diaquocobinamide $\underset{e}{\overset{e}{\rightleftharpoons}}$ cob(II)inamide	−0.74

Also

$$\text{glutathione} + 2e + 2H^+ \rightleftharpoons 2 \text{ reduced glutathione} \quad +1.9$$

Use of the relationship between the emf of the cell and the reaction of free energy ($\Delta G = -nFE_0$) makes it clear that reduced glutathione will not reduce either aquocobalamin or diaquocobinamide. At a concentration of 0.2 *M*, reduced glutathione does not reduce aquocobalamin (Figure 2). The fact that reduction of diaquocobinamide does occur at 10^{-3} *M* reduced glutathione indicates the formation of a reduced glutathione–aquocobinamide complex is a prerequisite for reduction of the cobalt atom. Coordination of reduced glutathione to the sixth coordination site would raise $E_0 > +0.19$ which would make the cobalt atom more electrophilic. These data help to explain why the reduced glutathione–alkylcobalamins are more photolabile.

Co^{II} Electron Spin Resonance Studies with the Reduced Glutathione Complexes. Co^{II} corrinoids were generated in different ways from the different reduced glutathione complexes: (1) a Co^{II} species was produced by heating the reduced glutathione–aquocobalamin complex at 100° for 1 min, (2) the reduced glutathione–alkylcobalamin complexes were converted to Co^{II} by photolysis of the Co–C σ bond under anaerobic conditions; (3) for the reduced glutathione–diaquocobinamide complex an excess of reduced glutathione (0.05 *M*) to cobinamide (5×10^{-3} *M*) was sufficient to generate Co^{II}. The Co^{II} esr spectrum of (1) was a typical "base-on" spectrum,[23] with a shoulder at $g = 2.52$ (Figure 8a). The shoulder is interpreted as the sulfur radical which would be formed when reaction 1 proceeds.

When the Co^{II} esr spectrum from reaction 2 was examined, it was also shown to be a typical "base-on" spectrum (Figure 8b). This result shows that upon generation of Co^{II} by photolysis, the reduced glutathione dissociates and 5,6-dimethylbenzimidazole recoordinates. Values for $g_\perp$, $A_\parallel^{Co}$, and $A_\parallel^{N}$ of cob(II)alamin were determined in the presence and absence of reduced glutathione. With no reduced glutathione $g_\perp = 2.32$, $A_\parallel^{Co} = 100(10^{-4})$ cm^{-1}, and $A_\parallel^{N} = 17.3$ (10^{-4}) cm^{-1} over a pH range of 6.0–10.0.[24] With reduced glutathione $g_\perp = 2.33$, $A_\parallel^{Co} = 104(10^{-4})$ cm^{-1}, and $A_\parallel^{N} = 17.1(10^{-4})$ cm^{-1}. These values recorded with reduced glutathione are similar to those published by Cockle, *et al.*,[24] of spectra taken in 0.8 *M* reduced glutathione. Cockle, *et al.*,[24] found $g_\perp = 2.32$, $A_\parallel^{Co} = 103(10^{-4})$ cm^{-1}, and $A_\parallel^{N} = 17.1(10^{-4})$ cm^{-1}.

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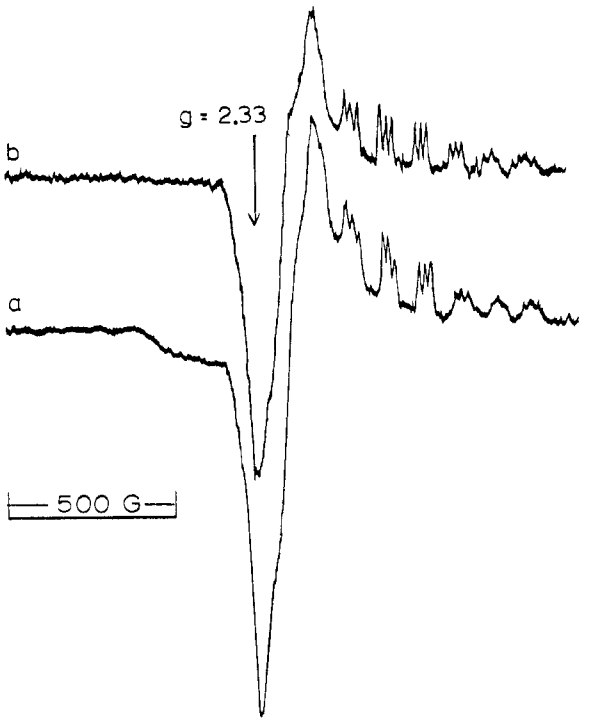

Figure 8. Esr spectra of cob(II)alamin. (a) Spectrum obtained by heating the reduced glutathione–aquocobalamin complex at 100° for 1 min. (b) Spectrum obtained by photolysis of the reduced glutathione–5′-deoxyadenosylcobalamin complex under anaerobic conditions.

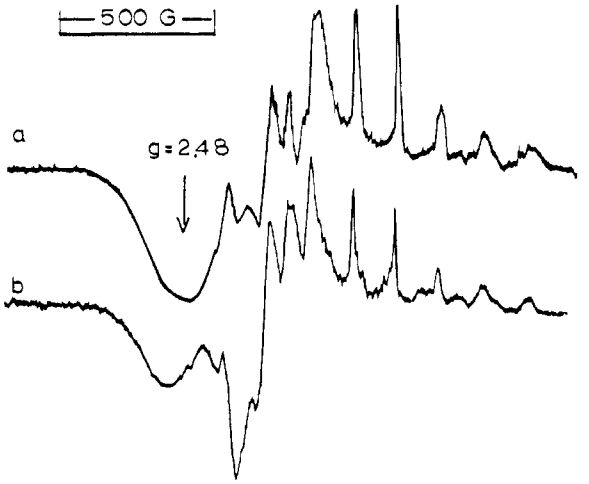

Figure 9. Esr spectra of cob(II)inamide. (a) Spectrum obtained by the reduction of diaquocobinamide with excess reduced glutathione. (b) Spectrum obtained by the reduction of diaquocobinamide with 3 *M* sodium formate.

These data help to confirm that reduced glutathione is displaced by 5,6-dimethylbenzimidazole in the Co^{II} species. Since 5,6-dimethylbenzimidazole has a pK_a of 2.5 in cob(II)alamin, and a pK_a of -2.4 in aquocobalamin, it is possible that the pK_a for coordination of reduced glutathione has increased by several pH units.

The Co^{II} spectrum from reaction 3 was similar to a "base-off" spectrum (Figure 9a). The eight $g_\parallel$ multiplets were resolved. The values for $g_\parallel = 2.003$, $g_\perp = 2.48$, and $A_\parallel^{Co} = 135(10)^{-4}$ cm^{-1}. Electrolytically reduced diaquocobainamide give $g_\parallel = 2.005$, $g_\perp = 2.58$, and $A_\parallel^{Co} = 133(10)^{-4}$ cm^{-1}.[25] These data indicate that cob(II)inamide generated with reduced glutathione does not have reduced glutathione coordinated to it. The difference in $g_\perp$ for the above values may be due to the presence of different anions in reaction mixtures, because when diaquocobinamide is reduced with sodium formate at pH 7.0, then different $g_\perp$ values were determined at different concentrations of sodium formate (*i.e.*, with 0.1 *M*, $g_\perp = 2.58$; 1.0 *M*, $g_\perp = 2.52$; 3 *M*, $g_\perp = 2.50$; and 6 *M*, $g_\perp = 2.48$). Also at 3 *M* sodium formate a sharp signal is observed at $g = 2.33$. This signal is identical with the one formed when thiolate replaced formate as the reducing agent.[24] The $A_\parallel^{Co} = 135(10)^{-4}$ cm^{-1} at high concentrations of sodium formate is similar to that value obtained by electrolytic reduction. These data indicate that no direct interaction between formate and Co^{II} occurs.

Discussion

Coordination of reduced glutathione to 5′-deoxyadenosylcobalamin and methylcobalamin provides the first evidence for a possible role for sulfhydryl groups in reactions catalyzed by B_{12} enzymes. In a recent publication Toraya, *et al.*,[26] have shown the importance of sulfhydryl groups in the binding of 5′-deoxyadenosylcobalamin to 1,2-propanedioldehydrase apoenzyme. Although high concentrations of reduced glutathione are required to facilitate the displacement of 5,6-dimethylbenzimidazole, the proximity effect of cysteine residues in B_{12} enzymes could satisfy this condition. When reduced glutathione displaces 5,6-dimethylbenzimidazole, then the Co–C σ bond is labilized, and homolytic cleavage would be promoted.[27-29] In methionine synthetase apoenzyme if the sulfhydryl groups are blocked, then the protein is unable to bind cobalamins.[11,23] However, in methionine synthetase, enzyme-bound methylcobalamin is much more stable to light, and from our studies if sulfur is coordinated to the cobalt in this enzyme, then it is to be expected that the Co–C bond should be more photolabile. Diol dehydrase holoenzyme is not inactivated by sulfhydryl reagents which suggests that coordination of sulfur to Co in this enzyme protects against inactivation.[5]

Our observation that the reduced glutathione–aquocobinamide complex is reduced to Co^{II} by excess reduced glutathione raises the question as to whether the Co^{II} esr spectrum observed for B_{12} enzymes really represents an active intermediate.[30,31] In some B_{12}-catalyzed reactions large quantities of thiols are added to reaction mixtures, and a reaction between thiol and alkylcobalamin in these enzymes could explain many of the esr observations. The ease with which the methionine synthetase holoenzyme reacts with propyl iodide suggests the presence of a Co–S intermediate.[5]

In conclusion the position of sulfhydryl groups, in B_{12} enzyme catalysis should not be ignored, and more

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emphasis should be placed on studying the involvement of cysteine residues in the mechanisms of interaction between B_{12} apoenzymes and their coenzymes.

Acknowledgment. This research was supported by a grant from the United States Public Health Service (USPH AM-12599).

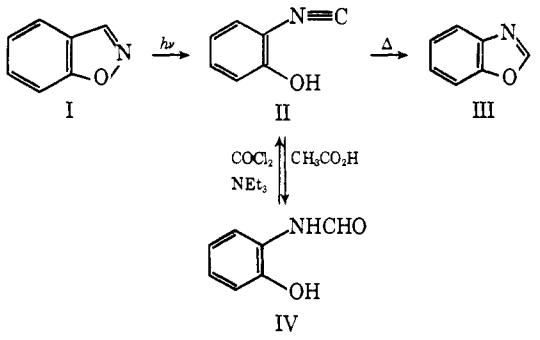
Communications to the Editor

Mechanism of the Photoisomerization of Isoxazoles and 2-Cyanophenol to Oxazoles

Sir:

The photochemical rearrangement of isoxazoles to oxazoles is usually assumed to proceed *via* an azirine intermediate.[1-4] In this communication, we present evidence for the formation of isonitrile intermediates in this photoisomerization. In addition, some evidence has been obtained for the direct photochemical conversion of a nitrile to an isonitrile.

Benzoxazole (III) and 2-cyanophenol are the photoproducts of indoxazene (I) at room temperature.[5] When indoxazene is photolyzed at −77 and −196°[6] with a 254-nm light source in a KBr matrix or neat film, ir bands are observed at 3350 (-OH), 2220 (-C≡N), and 2130 (-N≡C) cm^{-1}.[7] The bands at 3350 and 2130 cm^{-1}, assigned to II, disappear on warming in the dark, and a band at 1065 cm^{-1}, an intense band in the ir spectrum of benzoxazole, appears. The 2220-cm^{-1} band, assigned to the cyano grouping in 2-cyanophenol, does not change in intensity in the dark reaction. The presence of benzoxazole and 2-cyanophenol was confirmed by tlc and uv. Azirine infrared absorption was not observed.[3,8]



Irradiation of I in glacial acetic acid yields IV (14%) along with benzoxazole (10%) and 2-cyanophenol (10%).[10] The acid-catalyzed hydration of isonitriles is a well-documented reaction.[11] Reaction of IV with phosgene at −78° followed by rapid work-up gave a product with absorption at 2130 cm^{-1}. This absorption decreased in intensity on warming, and benzoxazole (III) was isolated from the crude reaction mixture.[12]

The uv spectrum of II was observed (λ_{max} 288 nm) when a 10^{-4} *M* solution of I was irradiated for 10 min at −77° in 95% ethanol. The characteristic sharp uv maxima of benzoxazole at 269 and 276 nm were observed when the solution was allowed to warm to room temperature. Irradiation of a 10^{-4} *M* solution of 2-cyanophenol for 1 hr at −77° in methanol–water (3:2 by volume) resulted in a shift of the uv maximum from 295 to 293 nm. The intensity of the absorption at 293 nm decreased markedly and the characteristic uv absorption of benzoxazole at 269 and 276 nm was observed when the solution was allowed to warm to room temperature. These data suggest that II is an intermediate in the photochemical conversion of cyanophenol to benzoxazole.[13]

Indoxazene (I) is converted to 2-cyanophenol *via* the triplet manifold. This was demonstrated by quenching studies using piperylene, cyclohexene,[14] and biacetyl as quenchers (Table I). Quenching by exciplex formation was ruled out because similar quenching efficiency was observed with three structurally different quenchers and because quenching was still observed at low (0.01 *M*) piperylene concentrations.[15]

The conversion of indoxazene (E_s = 94, E_t = 82 kcal/mol)[16] to benzoxazole appears to proceed from the singlet excited state. The reaction is sensitized by benzene (E_s = 109, E_t = 82 kcal/mol) but not acetone ($E_s \gtrsim 92$, E_t = 79 kcal/mol).[17] The formation of 2-methylbenzoxazole from 3-methylindoxazene (E_s = 92 kcal/mol, E_t could not be determined) is sensitized by

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(8) Azirines exhibit moderate to strong ir bands in the 1740–1800 cm^{-1} region.[3,9] (The complete spectrum of an azirine is reproduced in ref 9a.) A band of this intensity would have been detected in our studies.

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