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Degradation of 5′-Deoxyadenosylcobalamin by Ribonucleoside Triphosphate Reductase and Binding of Degradation Products to the Active Center*

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ABSTRACT: In the presence of ribonucleotide reductase, dihydrolipoate, and dGTP, deoxyadenosylcobalamin undergoes a spectrum change consistent with formation of cob(II)alamin (B_{12r}). No other cobamide accumulates in significant amounts. After removal of dihydrolipoate and exposure to air, aquocobalamin and 5′-deoxyadenosine were identified as reaction products. Treatment of the reaction mixture with iodoacetamide, methyl iodide, and acetylene yielded carboxamidomethylcobalamin, methylcobalamin, and vinylcobalamin, respectively. Yields were never greater than expected from reaction with cob(I)alamin formed from accumulated cob(II)alamin. Iodoacetamide also yielded 3-carboxamidomethyl-5,6-dimethylbenzimidazolyl-*Co*-5′-deoxyadenosylcobamide. Cob(II)alamin formed from aquocobalamin and dihydrolipoate was poorly bound to ribonucleotide reductase except in the presence of both dGTP and 5′-deoxyadenosine (dissociation constant 10.5 ± 1.9 μM). This accounts in part for the high resolution of the electron spin resonance spectrum of cob(II)alamin observed only in presence of enzyme, 5′-deoxyadenosine, and dGTP. dGTP and cob(II)alamin enhance the binding of 5′-deoxyadenosine to the enzyme severalfold. Binding of both cob(II)alamin and 5′-deoxyadenosine to the site for 5′-deoxyadenosylcobalamin is indicated by linear competitive inhibition of ribonucleotide reduction by 5′-deoxyadenosine and cob(II)alamin *vs.* 5′-deoxyadenosylcobalamin. $K_{i,app}$ for 5′-deoxyadenosine in the presence of 10 μM cob(II)alamin is 14 μM; $K_{i,app}$ for cob(II)alamin in the presence of 50 μM 5′-deoxyadenosine is 3 μM.

Recently it has been shown (Hamilton *et al.*, 1971) that 5′-deoxyadenosylcobalamin is slowly degraded by the ribonucleoside triphosphate reductase of *Lactobacillus leichmannii* in the presence of the reducing substrate (*e.g.*, dihydrolipoate) and a nucleoside triphosphate activator such as dGTP. One of the degradation products is a paramagnetic cobamide with an electron spin resonance (esr) spectrum identical with that of cob(II)alamin, except that it shows unique resolution. On this evidence the cobamide degradation product was tentatively identified as enzyme-bound cob(II)alamin, presumably formed by degradation of a reactive cobamide intermediate. It is postulated that this intermediate is formed by the interaction of the reducing substrate and deoxyadenosylcobalamin at the active center of the enzyme.

The unique resolution of the cob(II)alamin esr spectrum is interpreted to mean that the unpaired electron on the cobalt atom experiences a relatively constant magnetic field, firstly because the cob(II)alamin is bound to the active center, and secondly because the conformation of the active center is determined by the nucleoside triphosphate activator. Supporting evidence was provided by the observation that although all nucleoside triphosphates determined a similar highly resolved high-field part of the cob(II)alamin spectrum, different nucleotides determined somewhat different spectra in the low-field region. Furthermore, cob(II)alamin formed nonenzymically gave the high-resolution esr spectrum when added to the reductase provided dihydrolipoate, a nucleoside triphosphate, and 5′-deoxyadenosine were also present. It is assumed the 5′-deoxyadenosine causes the appearance of the highly resolved cob(II)alamin spectrum because it binds to the active center with consequent determination of the conformation of that part of the protein. The requirement for added 5′-deoxyadenosine for high resolution with nonenzymically formed cob(II)alamin but not with cob(II)alamin formed by degradation of deoxyadenosylcobalamin suggests that 5′-deoxyadenosine is formed in the latter degradation.

In this report further evidence is presented that the paramagnetic cobamide is indeed cob(II)alamin, that 5′-deoxyadenosine is the other degradation product, and that dGTP and 5′-deoxyadenosine increase binding of cob(II)alamin to the enzyme but probably also determine the conformation of the active site.

Materials

Most of the materials were the same as those previously described (Hamilton *et al.*, 1971). *Lactobacillus leichmannii* was obtained as a frozen paste from Grain Processing Corp., Muscatine, Iowa, and early stages of the preparation of ribonucleotide reductase were carried out on a large scale

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TABLE I: Thin-Layer Chromatography of Some Cobamides.[a]

Substance	R_F Values Solvent A[b]	Solvent B	Solvent C
5′-Deoxyadenosyl-cobalamin	0.25	0.55	0.10
Aquocobalamin	0.05 (0.33)[c]	0.05	0.05 (0.11)
Carboxyamido-methylcobalamin	0.22	0.57	0.08
Carboxymethyl-cobalamin	0.22	0.49	0.14
Methoxycarbonyl-methylcobalamin	0.34	0.63	0.14
Methylcobalamin	0.36	0.64	0.17
Ethylcobalamin	0.36	0.64	0.20

[a] Results were obtained with Eastman Kodak precoated silica gel sheets 6060. [b] Composition of solvent mixtures is given in the Methods section. [c] One or sometimes two minor spots were observed in aquocobalamin. They were possibly autoxidation products of aquocobalamin (Schrauzer and Lee, 1970) or some other products formed from aquocobalamin on standing, since these spots were not obtained with a freshly prepared solution of crystalline aquocobalamin.

at the New England Enzyme Center, Boston, Mass. The final preparations had a specific activity of 60–120 units/mg and were estimated to be 30–60% pure. [^{3}H]5′-Deoxyadenosine was prepared by hydrogen exchange with tritiated water (1 Ci/g) (Shelton and Clark, 1967). The specific radioactivity was 11 μCi/μmole, when concentration was calculated on the assumption of the same molar absorbance at 259 nm as for 2′-deoxyadenosine. [^{3}H]Aquocobalamin was prepared according to an unpublished method of Dr. H. P. C. Hogenkamp by hydrogen exchange between tritiated water and methylcobalamin, which was subsequently photolyzed to aquocobalamin. Direct incorporation of tritium into aquocobalamin by this method is unsuccessful because of extensive degradation during heating at 100° (H. P. C. Hogenkamp, 1971, unpublished observation). Crystalline methylcobalamin (10 mg), was heated in 0.5 ml of tritiated water (1 Ci/g) at about 100° in the dark for 24 hr. The solution was freeze-dried, residual tritiated water and tritium exchangeable at room temperature were removed by dissolving three times in water (0.5 ml each time) and freeze-drying. The labeled methylcobalamin was photolyzed and the resultant aquocobalamin was purified and crystallized (Hogenkamp and Rush, 1968). The yield was 6–8 mg and the specific radioactivity was 10–15 μCi/μmole. Crystalline carboxamidomethylcobalamin was prepared by the partial synthesis of alkylcobalamins of Müller and Müller (1962) using chloroacetamide as alkylating agent. Thin-layer chromatographic behavior of the cobalamin compared with those of other alkylcobalamins, which were gifts from Dr. H. P. C. Hogenkamp, is given in Table I. The absorption spectrum of carboxamidomethylcobalamin had maxima at 262, 342, 372, 404, and 527 nm in neutral solutions, and at 266, 286, 325, 424, and 454 nm in strongly acidic solutions (0.5 N HCl). The pK_a value for the protonation of the dimethylbenzimidazole moiety, which is associated with the pH-dependent spectral change, was estimated as 2.3 by determining A_{351}/A_{527} at different pH values (Ladd *et al.*, 1961).

Methods

Thin-Layer and Paper Chromatography. Typical data for thin-layer chromatography (tlc) of cobamides are shown in Table I. Paper chromatography was performed on Whatman No. 1 or No. 3MM paper by the ascending method unless otherwise stated. Solvents used in chromatography were as follows: (A) 1-butanol–2-propanol–water (10:7:10, v/v), (B) 2-propanol–ammonium hydroxide (28%)–water (7:1:2, v/v), (C) water-saturated 2-butanol–acetic acid (100:1, v/v), (D) 3-methyl-1-butanol–5% Na_2HPO_4 (1:1 v/v, both layers), (E) 1-butanol–water (43:7, v/v), (F) 1-butanol–acetic acid–water (4:1:5, v/v, upper layer), (G) water-saturated 2-butanol, (H) 2-propanol–water–ammonium hydroxide (28%) (85:15:1.3, v/v), (I) 1-butanol–acetic acid–water (5:2:3, v/v), (J) 3-methyl-1-butanol–10% Na_2HPO_4 (1:1, v/v, both layers), (K) 2-butanol water–acetic acid (137:50:1, v/v), (L) 2-butanol–water–ammonium hydroxide (50:18:7, v/v).

Radioactivity Determinations. Radioactivity of samples was determined in 10 ml of the scintillation fluid of Bray (1960) with a Packard Model 3003 Tri-Carb scintillation spectrometer.

Spectrophotometric Experiments on the Enzyme–Cobamide Complex. Spectra were measured with a Cary 14 recording spectrophotometer thermostatted at 37°, in cells with 1-mm light path. These permitted concentrations of reactants similar to those previously employed (Hamilton *et al.*, 1971). The enzyme was freed of colored proteins by preparative electrophoresis (Orr *et al.*, 1971) and had specific activity 87 units/mg. A unit is defined as that amount causing reduction of 1 μmole of ATP/hr under standard conditions (Hamilton *et al.*, 1971).

Reaction components were deoxygenated and kept anaerobic before addition to the sample cell with Hamilton syringes under nitrogen. A mixture of the enzyme, dGTP, and sodium dimethylglutarate buffer (pH 7.3), in about 90% of the final reaction volume, was incubated in the cell under a nitrogen stream for at least 7 min. After measurement of the absorbance spectrum either dihydrolipoate or deoxyadenosylcobalamin was added and the solution mixed by inversion of the cell. After 3 min under nitrogen and recording of the spectrum the final component was added and mixed immediately before recording the spectrum. Scanning was repeated at intervals during continued incubation under nitrogen.

Enzymic Degradation of 5′-Deoxyadenosylcobalamin and Isolation of Products. A reaction mixture containing 0.2 M sodium dimethylglutarate buffer (pH 7.3), 25 mM dihydrolipoate, 0.83 mM EDTA, 2 mM dGTP, 0.25 mM 5′-deoxyadenosylcobalamin, and 400 mg of ribonucleotide reductase (specific activity 115 units/mg) in a total volume of 20 ml was incubated for 2 hr in the dark at 37° under anaerobic conditions. All subsequent operations were conducted in the dark or in dim light. Exposure of cob(II)alamin solutions to air in the presence of dihydrolipoate results in irreversible conversion of cob(II)alamin into unknown yellow substances. Dihydrolipoate was therefore removed from the reaction mixture by applying it under a nitrogen stream to a column (2.6 × 33 cm) of Dowex 1-X2 (acetate form, 100–200 mesh) which had been washed with deoxygenated water. The column was then washed with deoxygenated water until substances absorbing at 525 and 260 nm had been eluted. The eluate (about 500 ml) was concentrated under reduced pressure (bath at 35°), and the concentrate was streaked on Whatman No. 3MM paper, which was developed with solvent D (descending). Two cobamides (A and B) and two ultraviolet-absorbing zones (uv-A

and uv-B) were present. Three zones containing cobamide A, mainly cobamide B plus uv-A and uv-B, respectively, were cut out and extracted with water. To each extract three volumes of 95% ethanol was added and precipitated Na_2HPO_4 was removed by centrifugation. Extracts were concentrated and rechromatographed with solvent E on Whatman No. 3MM paper prewashed with this solvent. This effectively separated cobamide B, uv-A, and residual cobamide A in the second extract. The cobamides and ultraviolet-absorbing compounds were extracted with water.

Cobamide A was further purified on CM-cellulose (Na^+) (pH 6, 1.2 × 6 cm) by elution with 0.1 M sodium phosphate buffer (pH 6.8); cobamide B on CM-cellulose (pH 3, 1.6 × 15 cm) by elution with water; uv-A on Dowex 1 (OH^-) (1.6 × 10 cm), by washing with 120 ml of water and subsequent elution with methanol; uv-B on Dowex 50 (pH 3, 0.9 × 1.0 cm), by washing with 50 ml of water and subsequent elution with 0.05 M sodium phosphate buffer (pH 7).

Treatment of the Enzyme–Coenzyme with Alkylating Agents and Isolation of Cobamides. All operations were conducted in the dark or in dim light.

A. Treatment with iodoacetamide. A reaction mixture containing 0.3 M sodium dimethylglutarate buffer (pH 7.3), 20 mM dihydrolipoate, 0.67 mM EDTA, 2 mM dGTP, 0.39 mM 5′-deoxyadenosylcobalamin, and 590 mg of ribonucleotide reductase (specific activity 61 units/mg) in a total volume of 20 ml was incubated at 37° under anaerobic conditions with gentle stirring. After 80 min, 4 ml of deoxygenated 0.38 M iodoacetamide was added and after a further 5 min, 1.6 ml of deoxygenated 0.5 N NaOH was added to neutralize liberated HI. After incubation for a further 60 min at 37°, any residual dihydrolipoate was removed by the method described above, before desalting the cobamide solution by phenol extraction. The resulting solution was applied to CM-cellulose (Na^+) (1.6 × 20 cm, pH 7) and most of the cobamides eluted with water. The eluate was applied to CM-cellulose (Na^+) (1.5 × 20 cm, pH 3). Two cobamide bands were successively eluted with water (cobamides C and D), and two more (E and F) with 0.003 M sodium phosphate buffer (pH 7.0). The cobamides retained on the first CM-cellulose column were eluted with a linear gradient of 0–0.033 M sodium phosphate buffer (pH 7, total volume 400 ml). Three cobamides (G, H, and I) were eluted, but the latter two incompletely separated. Fractions containing cobamides H and I were combined, desalted by phenol extraction, and applied to CM-cellulose (Na^+) (1.6 × 41 cm, pH 7). A linear gradient of 0.033–0.083 M sodium phosphate buffer (pH 7, total volume 700 ml), eluted cobamides H and I separately. All the solutions of cobamides were desalted by phenol extraction, and cobamide C was crystallized by addition of acetone after concentration under reduced pressure.

B. Treatment with methyl iodide. A reaction mixture containing 0.2 M sodium dimethylglutarate buffer (pH 7.3), 25 mM dihydrolipoate, 0.83 mM EDTA, 2 mM dATP, 0.2 mM 5′-deoxyadenosylcobalamin, and 87 mg of ribonucleotide reductase (39.3 units/mg) in a total volume of 2.5 ml was incubated for 3 min at 37° under anaerobic conditions. Deoxygenated 0.2 M methyl iodide in ethanol (0.125 ml) was added to the reaction mixture under nitrogen, and the incubation was continued for a further 3 min. After removal of dihydrolipoate as described above, the solution containing cobamides were desalted by phenol extraction, concentrated under reduced pressure, and the cobamides identified by paper chromatography.

C. Treatment with acetylene. Acetylene was bubbled through a solution (volume 2.93 ml) of sodium dimethylglutarate buffer (1 mmole, pH 7.3), dGTP (10 μmoles), and 5′-deoxydenosylcobalamin (1 μmole) for 20 min at 0°. After addition of 0.25 ml of 0.5 M dihydrolipoate containing 16.7 mM EDTA and 1.82 ml of reductase solution (387 mg, 39 units/mg), the reaction mixture was shaken at 37° under a stream of acetylene for 5 min, and the mixture then allowed to stand 26 min at 37° under acetylene. After removal of dihydrolipoate as described above and desalting by phenol extraction the cobamide solution was concentrated under reduced pressure. The cobamides present were identified by paper chromatography in the solvent systems previously described.

Determination of the Binding of Cob(II)alamin to Enzyme. For comparison with kinetic and esr results (Hamilton *et al.*, 1971) binding was determined at 37°, but at this temperature the enzyme was not stable over the period required for equilibrium dialysis (1–2 days). Most of the determinations were therefore carried out by ultrafiltration, the apparatus and procedure being essentially as reported by Paulus (1969). A minor change was that the ultrafiltration membranes were Sartorius cellulose filters (SM 11536) supported by nylon cloth rather than Diaflo membranes which incompletely retained protein and bound large amounts of cobamide. Before use the membrane was soaked in 0.2 M sodium dimethylglutarate buffer (pH 7.3) for at least 1 hr. Samples containing [^{3}H]aquocobalamin, the enzyme, dihydrolipoate, and the other components were incubated anaerobically for 6 min at 37° for reduction of aquocobalamin to cob(II)alamin and attainment of equilibrium between the free and bound cob(II)alamin. The samples were then transferred with oxygen-free Hamilton syringes to the channels of the upper block of the apparatus, through which nitrogen was passing and which had previously been warmed to 37°. After sealing the sample ports the lower part of the apparatus was wrapped with a thin plastic sheet to collect radioactive filtrate, placed in a water bath at 37°, and nitrogen pressure was applied (100 psi). Filtrations were usually finished in 1.5–2 hr, but 3 hr were allowed for each experiment. After rinsing the membranes and transferring them to vials containing 1 ml of water as described by Paulus, the vials and their contents were shaken mechanically for at least 3 hr to effect solution of protein and cobamide. After addition of 1 ml of 1 M methanolic Hyamine hydroxide they were shaken overnight before addition of scintillation fluid for determination of radioactivity. The specific radioactivity of two [^{3}H]aquocobalamin preparations used was 485,000 and 700,000 cpm per μmole, respectively, when determined under the same conditions. It was determined that in absence of protein a small volume of solution (about 1.2 μl) was retained by the membranes. Appropriate blank corrections were made for this retention of the solution of the labeled cob(II)alamin by the membrane. The amount of bound cob(II)alamin and the concentration of free cob(II)alamin were calculated from the corrected radioactivity associated with the membrane, the specific activity of the aquocobalamin and the total amount present in the reaction solution.

Binding was also determined by ultrafiltration at 4° so that a comparison could be made between results obtained by ultrafiltration and those obtained by equilibrium dialysis. For ultrafiltration at 4° samples were cooled with ice–water for about 5 min before applying to the apparatus which was maintained at 4°. The filtration was almost finished in 4 hr but was allowed to continue for a further 3 hr.

Equilibrium dialysis was carried out in cells (Interscience, Philadelphia) holding 0.1 ml of fluid on each side of a circular

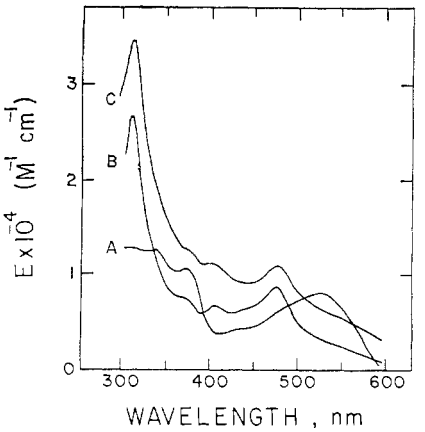

FIGURE 1: The ultraviolet-visible spectra of authentic 5′-deoxyadenosylcobalamin, cob(II)alamin, and the cobamide formed from 5′-deoxyadenosylcobalamin by incubation with ribonucleotide reductase. (A) 0.184 mM 5′-deoxyadenosylcobalamin in 0.2 M sodium dimethylglutarate buffer (pH 7.3) containing 0.1 M mercaptoethanol. It was confirmed that the presence of mercaptoethanol made no difference to the spectrum. (B) The same solution as used in A was illuminated (15-W lamp at 3 cm for 15 min) to convert 5′-deoxyadenosylcobalamin into cob(II)alamin. (C) A solution containing 0.172 mM 5′-deoxyadenosylcobalamin 5 mM dGTP 0.2 M sodium dimethylglutarate buffer (pH 7.3), 2.5 mM dihydrolipoate, and 6.8 mg of ribonucleotide reductase in a total volume of 0.235 ml was incubated for 140 min at 37°. The reference cell contained the same concentrations of dGTP, the buffer, and the enzyme as the sample.

membrane (Sartorius SM 11536). The membrane was cut into disks 17 mm in diameter and soaked in 0.2 M sodium dimethylglutarate buffer (pH 7.3) for at least 1 hr before use. After flushing nitrogen through the chambers *via* the sample ports, a deoxygenated solution (0.1 ml) containing the enzyme and the other components was added to one chamber with a Hamilton syringe, and an identical solution except for absence of enzyme and presence of [^{3}H]aquocobalamin was added to the other chamber. The cells were sealed with cellophane tape and rotated on a drum at 4° for 48 hr. Each chamber contained a glass bead 1 mm in diameter (Walter Stern Inc., Port Washington, N. Y.) which effected stirring during rotation. This procedure was found to achieve equilibration without significant inactivation of the enzyme in the time period used. Although [^{3}H]aquocobalamin was not reduced instantly to cob(II)alamin by dihydrolipoate at 4°, the reduction was complete in a few hours, so that the delay in completing reduction probably did not seriously influence the equilibrium attained after 48 hr. Samples (0.05 ml) from each chamber was transferred to vials containing 1 ml of 1 M methanolic Hyamine hydroxide. After standing for 30 min, 10 ml of scintillation fluid was added for determination of the radioactivity. The [^{3}H]aquocobalamin used had a specific radioactivity of 1,300,000 cpm/μmole under the same conditions. The difference in the radioactivity of the solutions from a pair of chambers gave the amount of cob(II)alamin bound to the enzyme, and the radioactivity of the solution lacking the enzyme indicated the amount of free cob(II)alamin in the solution in each chamber.

Determination of 5′-Deoxyadenosine Binding. This was carried out at 37° by the ultrafiltration technique described above except that protein on membranes was dissolved with 0.5 ml of water and 0.5 ml of 1 M methanolic Hyamine hydroxide. The [^{3}H]5′-deoxyadenosine had a specific activity of 2.46×10^6 cpm per μmole under the conditions used.

Inhibition Studies on Ribonucleotide Reductase. Ribonucleotide reductase activity was determined colorimetrically according to Blakley (1966). The standard enzymic reaction mixture containing 1 M sodium acetate, 10 mM ATP, 0.05 M potassium phosphate buffer (pH 7.3) 30 mM dihydrolipoate, 1 mM EDTA, and 50–70 μg of ribonucleotide reductase (specific activity 65 units/mg) in a total volume of 0.5 ml was incubated under nitrogen at 37° for 10 min. Cobalamins and nucleosides were added in varying concentrations as indicated for each experiment. Aquocobalamin was reduced to cob(II)alamin by the dihydrolipoate within 60 sec after the immersion of the tubes in the 37° bath. Nucleosides other than 2′-deoxyadenosine did not inhibit the color development nor develop any significant color in the range of concentration used.

Absorbance values were corrected by subtraction of a blank obtained with reaction mixtures from which 5′-deoxyadenosylcobalamin was omitted. Except in the case of 2′-deoxyadenosine nucleosides were also omitted from blank mixtures.

Double-reciprocal plots were fitted to each series of points at a particular inhibitor concentration by a computer program for weighted least-squares fit (Cleland, 1963). When inspection of the resultant family of plots indicated that the inhibition was competitive, the whole of the data was fitted by means of a linear competitive computer program (Cleland, 1963) to the optimum family of lines intersecting on the ordinate. These are the lines drawn in the figures. Values of the variance indicated a reasonably good fit of the data to the corresponding rate equation. The K_{is} values obtained from the program are reported as apparent K_i values together with standard error.

Results

Spectral Changes of Enzyme-Bound 5′-Deoxyadenosylcobalamin. Incubation of 5′-deoxyadenosylcobalamin with a molar excess of ribonucleotide reductase in the presence of dGTP and dihydrolipoate at 37° under conditions similar to those used for the formation of a paramagnetic cobamide (Hamilton *et al.*, 1971) resulted in a marked change in the spectrum of 5′-deoxyadenosylcobalamin. A spectrum taken after incubation for 140 min is shown in Figure 1 together with the spectra of authentic 5′-deoxyadenosylcobalamin and cob(II)alamin. After that time no further spectral change was observed. No spectral changes occurred over this period in absence of enzyme. The spectrum of the reaction product, having absorption maxima at 313, 402, and 475 nm closely resembles that of authentic cob(II)alamin (maxima at 312, 403, and 473 nm). This provides strong confirmatory evidence that the cobamide product is cob(II)alamin. Some difference in extinction over the whole wavelength range was probably due to turbidity in the reaction mixture which often appeared during prolonged incubation.

Although turbidity in the reference solution made it difficult to compare precisely successive spectra obtained during cob(II)alamin formation, spectra taken at the early stage of the reaction were found to have the same isosbestic points (Figure 2) as those of the spectra of authentic 5′-deoxyadenosylcobalamin and cob(II)alamin (Figure 1), so that other cobamide species, such as cob(I)alamin, were probably not formed in significant amounts. To minimize errors due to turbidity, per cent cob(II)alamin formation was calculated from the difference in absorbance at 522 nm (where 5′-deoxyadenosylcobalamin has an absorption peak) and 588 nm (where one of the isosbestic points occurred) and the corresponding difference for authentic 5′-deoxyadenosylcobalamin

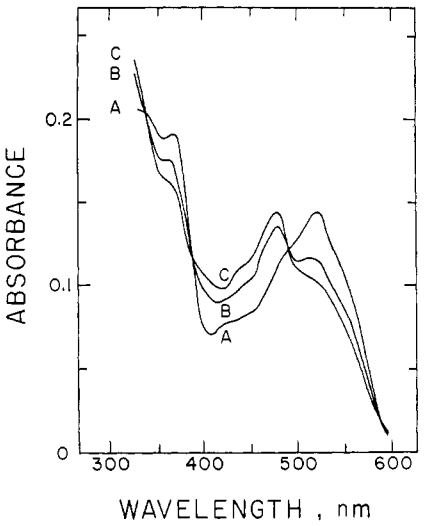

FIGURE 2: Spectral change of 5′-deoxyadenosylcobalamin incubated with ribonucleotide reductase. The sample solution was as described in Figure 1C, except that the concentration of deoxyadenosylcobalamin was 0.18 mM and the total volume was 0.225 ml. The reference solution was the same as the sample except that it lacked 5′-deoxyadenosylcobalamin. The reaction was initiated by addition of 0.005 ml of 112 mM dihydrolipoate to the sample at 37°. (A) Difference spectrum after addition of 5′-deoxyadenosylcobalamin to the sample cell. (B and C) Spectra at 2.5–4.1 and 8.2–9.8 min, respectively, after the addition of dihydrolipoate. Spectra B and C were corrected for dilution by the dihydrolipoate solution.

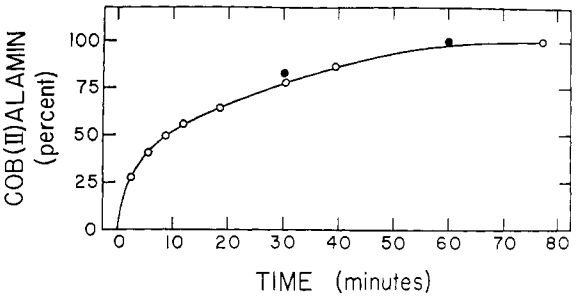

FIGURE 3: Time course of cob(II)alamin formation from 5′-deoxyadenosylcobalamin incubated with ribonucleotide reductase. Open circles: calculated from the spectra of the experiment described in Figure 2, from absorbance difference at 522 and 588 nm. Closed circles: calculated from the peak height of esr spectra of samples containing the same components as described in Figure 2. Esr spectra were recorded as previously (Hamilton *et al.*, 1971).

and for cob(II)alamin. The resulting time course for cob(II)alamin formation is shown in Figure 3.

Cob(II)alamin formation, like formation of the paramagnetic signal (Hamilton *et al.*, 1971), was complete in 1 hr, despite the fact that the dihydrolipoate concentration used in the spectrophotometric experiments was 2.5 mM whereas it was 25 mM in the previous esr measurements. Estimates from the esr signal of the amount of paramagnetic species in the reaction mixture used for the spectrophotometric experiments gave results consistent with the spectral data (Figure 3).

Degradation Products of 5′-Deoxyadenosylcobalamin. In order to investigate the fate of the 5′-deoxyadenosyl moiety during degradation of deoxyadenosylcobalamin and to show that cob(II)alamin can be oxidized to a known cobalamin, 5′-deoxyadenosylcobalamin was incubated with the enzyme and other reaction components, and cobamides and ultraviolet-absorbing substances were isolated as described in the Methods section. The absorption spectra of cobamides A and B were identical with those of aquocobalamin (λ_{max} 274, 351, 410, and 525) (Hogenkamp and Rush, 1968) and 5′-deoxyadenosylcobalamin (λ_{max} 260, 315, 375, and 522 nm) (Barker *et al.*, 1960), respectively. Thin-layer chromatographic behavior of cobamides A and B was identical with that of aquocobalamin and 5′-deoxyadenosylcobalamin, respectively (Table I), with which they are considered identical. The yield of aquocobalamin based on $\epsilon_{525} = 8.75 \times 10^3$ M^{-1} in neutral solution (Hogenkamp and Rush, 1968) was 2.40 μmoles (48%), and the recovery of 5′-deoxyadenosylcobalamin was 0.92 μmole (18%) based on $\epsilon_{522} = 8.0 \times 10^3$ M^{-1} cm^{-1} (Barker *et al.*, 1960). Uv-B behaved identically with authentic adenine in tlc (Table II) had an absorption peak at 260.5 nm, at pH 7 and is considered identical with adenine. The yield calculated from $\epsilon_{260} = 13.3$ M^{-1} cm^{-1} at pH 7 after correction by using A_{250}/A_{260} of 0.76 (Beaven *et al.*, 1955) was 0.43 μmole (8.6%).

Uv-A had an absorption peak at 259 nm and A_{250}/A_{260} of 0.80, values identical with those for 5′-deoxyadenosine. On paper electrophoresis in 0.5 M sodium borate (pH 9.3), uv-A migrated to the anode at the same rate as adenosine, demonstrating the presence of vicinal hydroxyl groups, and precluding identification with 2′-deoxyadenosine or 3′-deoxyadenosine. Uv-A exhibited behavior in tlc similar to that of 5′-deoxyadenosine and 4′,5′-didehydro-5′-deoxyadenosine.

Positive identification of uv-A, was obtained by periodate oxidation. A solution of adenine nucleoside (0.1 ml, 0.2 μmole) was treated with 0.1 ml of 0.1 M $NaIO_4$ (pH about 5) and the mixture immediately frozen in a tube in liquid nitrogen. Sodium bisulfite (2.67 ml, 1 mg/ml) was placed in a second tube and frozen, and the tubes were then connected to two ends of a Y-shaped glass tube. While the solutions were kept in liquid nitrogen, the gas pressure was reduced to 20 mm through a stopcock on the third arm. The stopcock was then closed and the solutions were allowed to thaw and remain at room temperature overnight for completion of the reaction and diffusion of acetaldehyde into the bisulfite. Samples of the bisulfite solution were used for duplicate determination of trapped acetaldehyde (Barker and Summerson, 1941). As shown in Table III, uv-A and 5′-deoxyadenosine gave nearly quantitative yields of acetaldehyde, while 4′,5′-didehydro-5′-deoxyadenosine and adenosine gave none. Although periodate

TABLE II: Identification of Ultraviolet-Absorbing Substances by Thin-Layer Chromatography.[a]

Substance	R_F Values		
	Solvent H	Solvent I	Solvent J
Uv-A	0.46	0.69	0.45
Uv-B	0.36	0.67	0.24
Adenine	0.36	0.67	0.24
Adenosine	0.32	0.64	0.41
5′-Deoxyadenosine	0.46	0.69	0.45
4′,5′-Didehydro-5′-deoxyadenosine	0.46	0.69	0.48
5′,8′-Cyclicadenosine	0.31	0.57	0.18
Adenosine-5′-aldehyde	Streaking	0.66	Streaking

[a] Results were obtained with Eastman Kodak precoated cellulose sheets 6065.

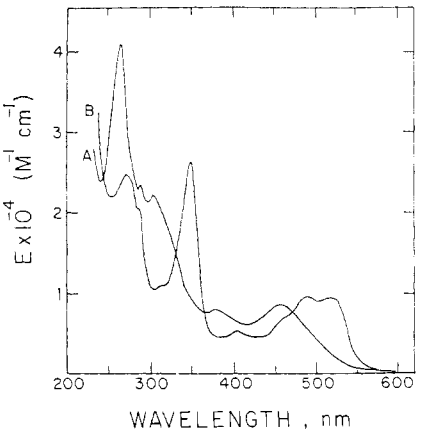

FIGURE 4: Ultraviolet-visible absorption spectra of A, the yellow cobamide produced by the reaction of 5′-deoxyadenosylcobalamin with iodoacetamide in water (pH 5.1, λ_{max} 263, 288, 304, 380, and 457 nm); B, the cobamide produced by the photolysis of the yellow cobamide in 0.1 M acetate buffer (pH 5.1, λ_{max} 270, 349, 409, 492, and 519 nm).

TABLE IV: Cobamides Obtained from Enzyme–Coenzyme Complex Treated with Iodoacetamide.

Substance	Identity	Yield[b] (%)
Cobamide C	Carboxamidomethylcobalamin	17
Cobamide D Cobamide G[a]	5′-Deoxyadenosylcobalamin	28
Cobamide F Cobamide H[a]	Aquocobalamin	13
Cobamide E[a] Cobamide I[a]	3-Carboxamidomethyl-5,6-dimethylbenzimidazolyl-Co-5′-deoxyadenosylcobamide	10

[a] Tentative identification. See the text. [b] Determined based on $\epsilon_{384} = 9.8 \times 10^3$ M^{-1} cm^{-1} (Firth *et al.*, 1967) for dicyanocobalamin after conversion of the cobamides to dicyanide in 0.1 M KCN. The total recovery was 68%.

oxidation of sugars terminating in a methyl group to yield acetaldehyde is well known (Nicolet and Shinn, 1941), this reaction does not appear to have been described previously for 5′-deoxyadenosine. Presumably lactaldehyde is an intermediate and is further oxidized in the manner described for other dicarbonyl compounds (Clutterbuck and Reuter, 1935). It is concluded from these results that uv-A is 5′-deoxyadenosine derived from the deoxyadenosyl moiety of the coenzyme. The yield was 1.85 μmoles (37%) assuming that ϵ_{259} is the same as for 2′-deoxyadenosine (15.0×10^3 M^{-1} cm^{-1}).

It seemed likely that adenine formation was due to the action on 5′-deoxyadenosine of a nucleosidase contaminating the reductase preparation. In similar experiments in which less pure enzyme was used (82.7 and 48.5 units per mg) and the reaction mixture was incubated for 24 hr, the yield of 5′-deoxyadenosine was less than 26 and 0%, respectively. This further suggested that adenine is a secondary product of the enzymic hydrolysis of 5′-deoxyadenosine. This was confirmed when enzyme of low specific activity (46.3 units/mg) and 5′-deoxyadenosine was incubated for periods of 1–24 hr and the products subjected to paper chromatography in solvent D. An ultraviolet-absorbing product with R_F identical with that of adenine (0.48) was present after 1 hr and increased throughout the incubation period.

Cobamides Produced by Treatment of Enzyme–Coenzyme Complex with Iodoacetamide. To further identify the primary cobamide product from enzymic degradation of 5′-deoxyadenosylcobalamin, the enzyme–deoxyadenosylcobalamin complex was treated with various alkylating agents. Details of these experiments are given in the Methods section. In the first experiment 5′-deoxyadenosylcobalamin was incubated with the enzyme under conditions allowing the degradation of the coenzyme. At the end of the incubation iodoacetamide was added to the reaction mixture, and the resulting cobamides were then isolated as described in the Methods section with the results summarized in Table IV. The total recovery of cobamides was 68%. Cobamides C, D, and F were identified with carboxamidomethylcobalamin, 5′-deoxyadenosylcobalamin, and aquocobalamin, respectively, by their absorption spectra (Methods section; Barker *et al.*, 1960; Hogenkamp and Rush, 1968), and by their chromatographic behavior in thin-layer sheets as described in the Methods section. Cobamides G and H had spectra similar to those of 5′-deoxyadenosylcobalamin and aquocobalamin, respectively, but no further evidence was obtained to confirm this identification because of the small amount available.

Cobamide E was a light-sensitive, yellow compound, and cobamide I resembled E in spectral properties. A control experiment without enzyme gave no carboxamidomethylcobalamin or aquocobalamin but did give a 41% yield of cobamide E presumably from reaction of free 5′-deoxyadenosylcobalamin with iodoacetamide. Cobamide E (spectrum Figure 4) was photolyzed and the products separated into an orange cobamide and a colorless substance having an absorption peak at about 260 nm. This result together with the spectrum strongly suggests that a deoxyadenosyl group is attached to cobalt in cobamide E. The uv-absorbing product was washed through a CM-cellulose column (Na^+) (pH 5.1) with water whereas the cobamide was eluted subsequently with 0.1 M sodium acetate buffer (pH 5.1). The cobamide product (spectrum Figure 4) was distinguished from aquocobalamin and diaquocobinamide by tlc in solvents K and B to which were added 0.1% KCN. In the former solvent, diaquocobinamide and the cobamide photolysis product gave dicyano derivatives whereas aquocobalamin gave the red monocyano derivative. On paper electrophoresis at pH 2.7 cobamide E migrated to the cathode 1.2 times faster than 5′-deoxyadenosyl-

TABLE III: Formation of Acetaldehyde by Periodate Degradation of Adenine Nucleosides.[a]

Substance Degraded	Amt of Substance (nmoles)	Acetaldehyde Obtained (nmoles)
Uv-A	78	69
5′-Deoxyadenosine	75	62
Adenosine	75	0
4′,5′-Didehydro-5′-deoxyadenosine	75	0

[a] Method is described in the text.

cobalamin, and in 0.03 M potassium phosphate buffer (pH 8.0), cobamide E behaved as a cation, whereas deoxyadenosylcobalamin was neutral. These data suggest the presence of a modified dimethylbenzimidazole moiety not coordinated to cobalt. This is confirmed by the absorption maximum at 288 nm for cobamide E, which corresponds to that at 284 nm for protonated 5′-deoxyadenosylcobalamin and is due to the dimethylbenzimidazolyl moiety. A distinct shoulder also appears at 288 nm in the spectrum of the photolysis product but is not found for diaquocobinamide (Bernhauer *et al.*, 1962), and is less distinct for aquocobalamin. Iodoacetamide presumably reacts nonenzymically with N-3 of the dimethylbenzimidazole moiety of deoxyadenosylcobalamin to form 3-carboxamidomethyl-5,6-dimethylbenzimidazolyl-*Co*-5′-deoxyadenosylcobamide.

The formation of carboxamidomethylcobalamin in the preceding experiment can be explained by reactions 1–4.

$$\text{deoxyadenosylcobalamin} + \text{dihydrolipoate} \xrightarrow[\text{reductase}]{\text{dGTP}} \text{cob(II)alamin} + \text{5′-deoxyadenosine} + \text{lipoate} \quad (1)$$

$$2\text{cob(II)alamin} \longrightarrow \text{cob(I)alamin} + \text{aquocobalamin} \quad (2)$$

$$\text{aquocobalamin} + \text{dihydrolipoate} \longrightarrow \text{cob(II)alamin} + \text{lipoate} \quad (3)$$

$$\text{cob(I)alamin} + ICH_2CONH_2 \longrightarrow \text{carboxamidomethylcobalamin} + I^- \quad (4)$$

The disproportion of cob(II)alamin according to reaction 2 has been previously reported (Yamada *et al.*, 1968) and the equilibrium would be driven further to the right by coupling with reactions 3 and 4. However, if reaction 1 proceeds through an intermediate in which cob(I)alamin or a closely related cobamide is bound to the active center of the enzyme, carboxamidomethylcobalamin should also be generated by direct attack of iodoacetamide on this intermediate. Since formation of carboxamidomethylcobalamin in this way might be expected to proceed much faster than reactions 1–4, the rate of carboxamidomethylcobalamin by reactions 2, 3, and 4 was investigated.

Formation of carboxamidomethylcobalamin was investigated in a reaction mixture containing the same components as previously described (see Methods section) except that enzyme was omitted, 5′-deoxyadenosylcobalamin was replaced by 0.4 mM aquocobalamin and the total volume was 10 ml. After 6-min anaerobic incubation at 37° aquocobalamin was completely reduced to cob(II)alamin by dihydrolipoate. The mixture was treated with iodoacetamide as in the previous experiment. After incubation for a further 10 or 60 min at 37°, dihydrolipoate was removed, cobamides were extracted with phenol and separated by paper chromatography (Whatman No. 3MM, solvent L). The cobamides identified were carboxamidomethylcobalamin (R_F 0.08) and aquocobalamin (R_F 0.02); the yields were 37 and 21%, respectively, after 10-min incubation and 46 and 15%, respectively, after 60-min incubation.

These results indicate the carboxamidomethylcobalamin formation in this control experiment was at least as great as in the experiment utilizing enzyme and deoxyadenosylcobalamin. Hence carboxamidomethylcobalamin formation by the action of iodoacetamide on the enzyme–deoxyadenosylcobalamin system can be accounted for by reactions 1–4, without assuming attack of iodoacetamide on a reactive intermediate.

Cobamides Produced by Treatment of Enzyme–Coenzyme Complex with Other Alkylating Agents. The apparent absence of direct alkylation of the postulated reactive cobamide by iodoacetamide might be due to inability of this reagent to penetrate to the active center. Alkylation by methyl iodide, a less bulky reagent, was therefore investigated.

A short incubation period was used to avoid methylation of the enzyme and degradation of the coenzyme to cob(II)alamin (see Methods section). Paper chromatography (solvent K, Whatman No. 3MM) indicated that almost all the cobamide isolated was 5′-deoxyadenosylcobalamin with only a trace of methylcobalamin present. In a control in which enzyme and 5′-deoxyadenosylcobalamin were absent but other components and 0.2 mM aquocobalamin and 0.2 mM 5′-deoxyadenosine were present the yield of methylcobalamin isolated was about 40% in the same period.

The lack of any evidence for direct attack by iodoacetamide or methyl iodide on the enzyme–cobamide complex might be explained by rapid alkylation of thiol groups at the active groups at the active center of the enzyme, with consequent disappearance of the reactive cobamide. This seems less likely in the experiment with methyl iodide where the time period (3 min) should have been long enough for generation of the reactive cobamide but short for methylation of thiol groups. Nevertheless Rochat *et al.* (1970) report 95% methylation of cysteine residues of *Adroctonus australis* toxin in 15 min at 40° by a saturated solution of methyl iodide. Accordingly a further experiment was conducted with acetylene as alkylating reagent. This small molecule is unlikely to react with the protein and its only disadvantage is that it reacts more slowly with cob(I)alamin. However, Johnson *et al.* (1963) reported significant reaction in a few minutes and an 80% yield of product in 3 hr.

When the solution of the enzyme–coenzyme complex was saturated with acetylene, and the cobamides were isolated as described in Methods section, paper chromatography (solvent K, Whatman No. 3MM) showed that the major part of the cobamides isolated consisted of 5′-deoxyadenosylcobalamin (R_F 0.19) and aquocobalamin (R_F 0.14, 0.23). Vinylcobalamin (R_F 0.39) was present only in a trace amount. In a control nonenzymic experiment in which 2 mM aquocobalamin and 2 mM 5′-deoxyadenosine replaced deoxyadenosylcobalamin, vinylcobalamin was again formed in trace amounts. Reaction with acetylene therefore gave no indication of the formation of cob(I)alamin or its equivalent at the active site of the enzyme.

Effect of Nucleosides and Nucleotides on the Binding of Cob(II)alamin to Enzyme. Cob(II)alamin gives an unusually well-resolved esr spectrum in the presence of the ribonucleotide reductase, a nucleoside triphosphate, and 5′-deoxyadenosine (Hamilton *et al.*, 1971). It was therefore of interest to investigate the effect of nucleotides and nucleosides on the binding of cob(II)alamin to the enzyme. Experiments were carried out as described in the Methods section and the dissociation constant, K_D, for the enzyme–cob(II)alamin complex was obtained from the data by least-squares analysis according to the equation

$$\frac{1}{r} = \frac{1}{n}\frac{K_D}{[A]} + \frac{1}{n} \quad (1)$$

This equation, modified from Klotz (1953), by use of a dissociation constant K_D is valid when an enzyme has n independent identical binding sites for a ligand A, r is the average number of ligand molecules bound per enzyme molecule, and

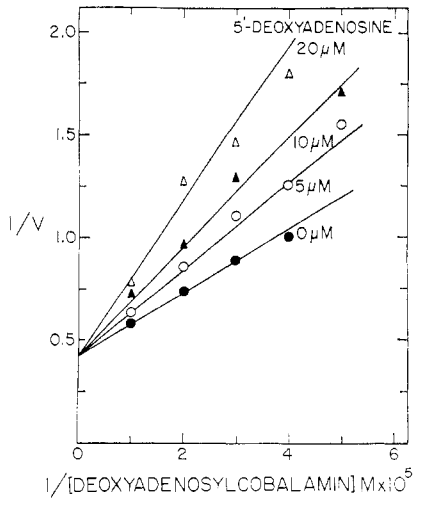

FIGURE 5: The competitive inhibition of ribonucleotide reduction with respect to 5'-deoxyadenosylcobalamin by 5'-deoxyadenosine in the presence of cob(II)alamin. Standard assay conditions were used in the presence of 10 μM cob(II)alamin and the indicated concentrations of 5'-deoxyadenosine.

[A] is the free-ligand concentration. As shown in Table V, the K_D values for the complex at 4° obtained by ultrafiltration and by equilibrium dialysis were in reasonably good agreement. It was therefore assumed that dissociation constants obtained by ultrafiltration under other conditions could be considered accurate within the experimental error of the method.

At 37° binding was considerably greater than at 4° and there was a marked effect of nucleoside triphosphates on the binding of cob(II)alamin (Table V). ATP or dGTP decreased K_D to about 0.01 the value obtained in absence of nucleotide. K_D was also decreased significantly (20 times) by the addition of 5'-deoxyadenosine to the system, and a similar effect was produced by 4',5'-didehydro-5'-deoxyadenosine and adenosine, whereas 2'-deoxyadenosine had no effect.

Binding of 5'-Deoxyadenosine to Reductase. The remarkable effect of 5'-deoxyadenosine on the binding of cob(II)alamin to the enzyme, despite the fact that the nucleoside is not a substrate or activator, raised the question of how well it is bound to the enzyme. Although results obtained by the ultrafiltration method were subject to large standard errors, they indicated that the binding is relatively strong (K_D about 13 μM) in the presence of both cob(II)alamin (formed from aquocobalamin and dihydrolipoate) and dGTP but is decreased about fourfold when either dGTP or cob(II)alamin is omitted.

Inhibition of ATP Reduction by Cob(II)alamin and 5'-Deoxyadenosine. The relatively strong binding of cob(II)alamin and 5'-deoxyadenosine to the reductase, and the mutual enhancement that each ligand produces on the binding of the other, suggest that binding of both these compounds are binding at the site for 5'-deoxyadenosylcobalamin. To obtain evidence for this the inhibition of the ribonucleotide reductase by cob(II)alamin and 5'-deoxyadenosine was investigated.

5'-Deoxyadenosine at concentrations up to 100 μM did not cause inhibition in the absence of cob(II)alamin, despite the relatively strong binding found for 5'-deoxyadenosine. However, 5'-deoxyadenosine showed marked linear competitive inhibition with respect to 5'-deoxyadenosylcobalamin in the presence of a fixed concentration of cob(II)alamin (Figure 5).

TABLE V: Effect of Some Nucleosides and Nucleotides on Binding of Cob(II)alamin to Ribonucleotide Reductase.

Components	Dissociation Constant K_D (μM)
A. Ultrafiltration at 37°[a]	
Complete system	10.5 ± 1.9
−5'-Deoxyadenosine	172 ± 47
−5'-Deoxyadenosine + 2'-deoxyadenosine	317 ± 181
−5'-Deoxyadenosine + 4',5'-didehydro-5'-deoxyadenosine	8.0 ± 2.8
+5'-Deoxyadenosine + adenosine	8.6 ± 3.4
−dGTP	1070 ± 1870
−dGTP + ATP	10.0 ± 6.8
B. Ultrafiltration at 4°[a]	
Complete system	63.7 ± 6.9
C. Equilibrium dialysis at 4°[b]	
Complete system	75.5 ± 32.2

[a] The complete system contained 0.2 mM 5'-deoxyadenosine, 2 mM dGTP, 25 mM dihydrolipoate, 0.2 M sodium dimethylglutarate buffer (pH 7.3), 20 μM ribonucleotide reductase (assuming mol wt 76,000, specific activity 90 units/mg), and [^{3}H]cob(II)alamin at concentrations in the range 16–280 μM in a total volume of 0.2 ml. When present other nucleosides were at a concentration of 0.2 mM and ATP at 2 mM. [b] Concentrations of the components were the same as used in ultrafiltration experiments except for enzyme (40 μM).

The apparent K_i was 1.4 ± 0.11 × 10^{-5} M. Cob(II)alamin was a linear competitive inhibitor with respect to 5'-deoxyadenosylcobalamin even in the absence of 5'-deoxyadenosine, with an apparent K_i of 3.7 ± 0.2 × 10^{-5} M. This inhibition was considerably enhanced in the presence of 5'-deoxyadenosine, the apparent K_i becoming 3.0 ± 0.23 × 10^{-6} M (Figure 6). These results are consistent with binding of cob(II)alamin and 5'-deoxyadenosine at the 5'-deoxyadenosylcobalamin site.

When adenine nucleoside were compared for their ability to cause inhibition in the presence of cob(II)alamin, 5'-deoxyadenosine was much the most active (Figure 7); adenosine also had significant activity, but 3'-deoxyadenosine, 2'-deoxyadenosine, and 4',5'-didehydro-5'-deoxyadenosine had no significant inhibitory effect.

Discussion

The observed spectral changes confirm the conclusion reached from esr studies (Hamilton *et al.*, 1971) that in presence of a nucleotide modifier and a dithiol substrate ribonucleotide reductase degrades 5'-deoxyadenosylcobalamin to cob(II)alamin.

The spectrum of the cobamide product closely resembles that of authentic cob(II)alamin, and in particular the maximum at 313 nm excludes the possibility that the product is a form of the coenzyme in which the nucleotide base is not coordinated to cobalt. The general agreement between the rate of spectral change and of formation of the paramagnetic species, the identification of the oxidized product as aquo-

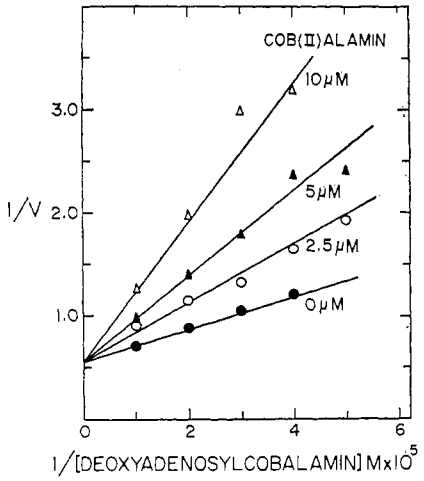

FIGURE 6: The competitive inhibition of ribonucleotide reductase reaction with respect to 5′-deoxyadenosylcobalamin by cob(II)-alamin in the presence of 5′-deoxyadenosine. Standard assay conditions in the presence of 50 μM 5′-deoxyadenosine and the indicated concentration of cob(II)alamin.

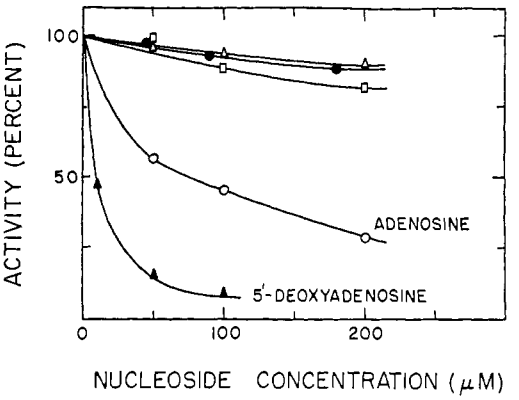

FIGURE 7: Inhibitory effects of adenine nucleosides on ribonucleotide reduction in the presence of cob(II)alamin. Standard assay conditions in the presence of 50 μM cob(II)alamin, 5 μM 5′-deoxyadenosylcobalamin, and the indicated concentrations of nucleosides. (□) 2′-Deoxyadenosine, (△) 3′-deoxyadenosine, (●) 4′-5′-didehydro-5′-deoxyadenosine, (○) adenosine, and (▲) 5′-deoxyadenosine.

cobalamin and the demonstration that alkylating agents are able to yield alkylcobalamin provides additional evidence.

As the previous results of esr experiments with nonenzymically formed cob(II)alamin and nucleosides have suggested, the high resolution of the esr spectrum of cob(II)alamin formed by the enzymic degradation of the coenzyme is found to be dependent on the presence of the other degradation product from the coenzyme 5′-deoxyadenosine, which has been unambiguously identified. The results of binding experiments strongly suggest that one means by which 5′-deoxyadenosine and the nucleotide modifier enhance the resolution of the esr spectrum is by inducing tighter binding of cob(II)-alamin to the enzyme. Under the conditions previously used in esr experiments (0.3 mM enzyme, 0.2 mM cob(II)alamin, 0.2 mM 5′-deoxyadenosine, and 5 mM dGTP or ATP), it was calculated from the K_D value in Table V that 93% of the cob(II)alamin was bound to the enzyme. The poorly resolved esr spectrum of cob(II)alamin in the presence of enzyme but the absence of either 5′-deoxyadenosine or a nucleotide modifier is consistent with the much smaller fraction of cobalamin bound to enzyme under these conditions, as calculated from the appropriate dissociation constants for enzyme–cob(II)-alamin complex (Table V).

Furthermore, the fact that adenosine and 4′,5′-didehydro-5′-deoxyadenosine produce the same effect as 5′-deoxyadenosine, on both the binding of cob(II)alamin (Table V) and the resolution of its esr spectrum, whereas 2′-deoxyadenosine affects neither property, provides additional evidence.

Several facts suggest, however, that the enhancement of resolution by 5′-deoxyadenosine and the nucleotide modifier is not solely due to the effect on binding. Calculation from the data in Table V indicates that 53 and 20% of cob(II)alamin is bound to the enzyme in the reaction mixtures lacking 5′-deoxyadenosine and nucleotide, respectively, whereas esr spectra of these samples do not show any enhancement of resolution as compared with the spectrum for unbound cob(II)alamin. Furthermore, the different specific effects of nucleotides and some nucleosides on the detailed structure of the esr spectra (Hamilton *et al.*, 1971) cannot be explained in terms of the extent of binding. These facts suggest that the nucleotide and nucleoside not only enhances the binding of cob(II)alamin but also specifically determines the conformation of the cob(II)alamin enzyme complex.

The mutual enhancement that 5′-deoxyadenosine and cob(II)alamin each has on the binding of the other to the enzyme, as well as the effect that 5′-deoxyadenosine apparently has on the conformation of the cob(II)alamin–enzyme complex, can be more readily understood if both 5′-deoxyadenosine and cob(II)alamin bind to the site on the enzyme normally occupied by 5′-deoxyadenosylcobalamin. Evidence to indicate that this is the case is provided by the kinetic experiments which showed that 5′-deoxyadenosine and cob(II)alamin are both linear competive inhibitors with respect to 5′-deoxyadenosylcobalamin, and that the two were together much more inhibitory than either alone. Although it was only possible to obtain apparent K_i values these agreed reasonably well with the dissociation constants obtained in the binding experiments.

The identification of 5′-deoxyadenosine as one of the degradation products is consistent with the view (Hamilton *et al.*, 1971) that the species undergoing degradation is some reactive enzyme–coenzyme complex which is closely associated with the mechanism of ribonucleotide reduction and with the hydrogen exchange between water and the 5′-methylene group of the coenzyme. It is also in accord with the postulate that the structure of the complex is closely related to 5′-deoxyadenosine and cob(I)alamin (Hogenkamp *et al.*, 1968), and makes it unlikely that the mechanism of ribonucleotide reduction involves formation of 4′,5′-didehydro-5′-deoxyandenosine as proposed by Schrauzer and Sibert (1970) for related enzymic reactions involving deoxyadenosylcobalamin.

The well-defined isosbestic points in the spectrophotometric experiments indicate that no cobamide is detectable other than cob(II)alamin and deoxyadenosylcobalamin during the degradation of the coenzyme. One interpretation of this result is that the reactive complex, although possessing a different spectrum from cob(II)alamin and deoxyadenosylcobalamin has a very low steady-state concentration. If the cobamide in the reactive complex is closely related to cob(I)alamin as previously suggested (Hamilton *et al.*, 1971) then some such explanation must be invoked for the spectral results. Alternatively, the cobamide in the reactive complex may be more

closely related to cob(II)alamin and give rise to a spectrum indistinguishable from that of the latter. The negative results in all experiments designed to trap cob(I)alamin or its equivalent with alkylating agents is consistent with the latter explanation although it might simply be due to the low steady-state concentration of the complex, and the stereochemical consequences of tight binding of the cobamide to the enzyme.

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Mass Spectrometry of Trifluoroacetyl Derivatives of Nucleosides and Hydrolysates of Deoxyribonucleic Acid*

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ABSTRACT: Mass spectra of the trifluoroacetyl derivatives of ribonucleosides and deoxyribonucleosides have been examined in detail and shown to be useful for the determination of a number of structural features. Reaction conditions were employed in which sugar hydroxyls and amino or alkylated amino groups in the base were trifluoroacetylated. The most useful ions for determination of molecular mass and composition were a series of elimination reactions from the molecular ion involving trifluoroacetyl moieties. Breakage of the glycosidic bond with retention of charge in the base produced major ions representing the base and its substituents. Specific fragmentation reactions were found which permit recognition of N,N-dimethylation, and permit differentiation between purines methylated at N-1 and N^6. Trifluoroacetylated, unfractionated hydrolysates of DNA can be examined for the presence of minor deoxyribonucleosides. Exact mass values derived from photographically recorded high resolution mass spectra provide an independent and objective means of identifying components of a hydrolysate, which is not dependent upon ultraviolet absorbance or chromatographic mobility.

In recent years a number of studies have demonstrated the potential role of mass spectrometry for dealing with structural problems of nucleic acids and their analogs (McCloskey, 1971). The majority of these have involved the structure determination of modified nucleosides isolated from transfer RNA (*e.g.*, Baczynskyj *et al.*, 1968; Carbon *et al.*, 1968; Armstrong *et al.*, 1970) which were examined as free (chemically underivatized) compounds. However, since constituents of nucleic

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