Direct Spectrophotometric Observation of an Intermediate Formed from Deoxyadenosylcobalamin in Ribonucleotide Reduction†

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ABSTRACT: In the presence of dGTP, 5′-deoxyadenosylcobalamin (coenzyme B_{12}) rapidly reacts with equimolar *Lactobacillus leichmannii* ribonucleotide reductase and excess dihydrolipoate. The spectral changes in the visible and ultraviolet closely correspond to those predicted for partial conversion to cob(II)alamin, but the deoxyadenosyl moiety cannot be trapped in a pool of 5′-deoxyadenosine. This distinguishes the reaction from irreversible degradation of coenzyme to cob(II)alamin and 5′-deoxyadenosine which is 10^5 times slower. The rapid reaction has kinetics approximating first order with a rate constant of about 38 sec^{-1} at 37°, and at equilibrium 20–40% of the coenzyme appears to be converted to the cob(II)alamin-like species. The rapid reaction is completely reversed by a temperature drop from 37 to 5°. Similar sensitivity to temperature has been demonstrated for rates of exchange of the 5′ protons of the coenzyme with water, coenzyme degradation, and ribonucleotide reduction, but coenzyme degradation is not reversed by temperature decrease. Plots of the equilibrium position of the rapid reaction *vs.* temperature show a marked transition at 29° and a similar transition is observed for difference spectra of the enzyme compared at different temperatures. The implied conformation change of the enzyme at 29° probably involves movement of tryptophan residues to a less polar region or movement of charged residues in the vicinity of tryptophan. The equilibrium position for the rapid spectral change is less favorable when dATP, dCTP, or the arabino analog of ATP is substituted for dGTP, and no reaction occurs in the presence of dTTP. Monothiols will not replace dihydrolipoate except at high concentrations, but other 1,3-dithiols and the thioredoxin system give reaction rates and equilibrium positions similar to those found with dihydrolipoate. When GTP is substituted for dGTP the absorbance change rapidly reaches a maximum and then more slowly declines until a constant value is reached, the final equilibrium position depending on the GTP concentration. ATP and CTP give similar but less marked decreases after reaching a maximum, but with ITP the decrease is slight and with UTP undetectable. A similar decline is observed if a ribonucleoside triphosphate is added to the complete, equilibrated system containing dGTP. When [5′,5′-$^2H_2$]deoxyadenosylcobalamin is used the rate constant for the spectral change in the presence of dGTP is 1.4 times lower than with unlabeled coenzyme, while the rate constant for the rate of decline from the maximum change observed in the presence of GTP is 2.2 times lower than with unlabeled coenzyme. These data suggest that the ribonucleotide reductase–coenzyme complex provides a stabilized deoxyadenosyl radical which interacts with a thiol group of the enzyme or the dithiol substrate, thus providing the mechanism for the hydrogen exchange and degradation phenomena. It is postulated that in ribonucleotide reduction a hydrogen is abstracted from the radical–thiol system and transferred to the pentose with regeneration of coenzyme.

Previous studies on the 5′-deoxyadenosylcobalamin-dependent reduction of ribonucleoside triphosphates to 2′-deoxyribonucleoside triphosphates by dithiols in the presence of the reductase from *Lactobacillus leichmannii* have not succeeded in obtaining direct evidence for a reactive cobamide intermediate. Such an intermediate is nevertheless considered to be formed from the coenzyme on the basis of the following indirect evidence: (1) the hydrogens of the cobalt-bound methylene group in the coenzyme exchange with water in the presence of the enzyme, a dithiol, and an allosteric activator (Hogenkamp *et al.*, 1968); (2) the coenzyme is slowly degraded to cob(II)alamin and 5′-deoxyadenosine in the presence of reductase, a dithiol, and an allosteric activator (Hamilton *et al.*, 1971; Yamada *et al.*, 1971); and (3) there is a good deal of indirect evidence that such an intermediate is formed in the related coenzyme B_{12} dependent dehydrase and mutase reactions (Abeles, 1971).

We now present spectrophotometric evidence for the very rapid formation of such a cobamide intermediate and some data relating to its structure. A study of the factors determining the steady-state concentration of the intermediate has also given some insight into the reason that the intermediate was not detected in previous studies.

Experimental Section

Materials

Most of the materials were the same as those used previously (Hamilton *et al.*, 1971; Yamada *et al.*, 1971; Hamilton *et al.*, 1972). The reductase (specific activity 65–140 μmol of dATP formed per hr per mg) was prepared as previously described (Panagou *et al.*, 1972). For some experiments contaminating proteins were removed by preparative electrophoresis (Orr *et al.*, 1972). For a few experiments yellow contaminating proteins were also removed by fractionation on DEAE-cellu-

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lose. The enzyme was applied to a column equilibrated with 0.05 M potassium phosphate buffer, pH 7.3, and was eluted with a gradient of sodium chloride (0–0.3 M) in the same buffer. The enzyme activity overlapped the yellow protein but preceded it slightly. Colorless enzyme fractions were collected and concentrated by pressure dialysis. Since the best preparations of reductase have a specific activity of 200 μmol of dATP formed per hr per mg, the apparent molar concentration of the enzyme in reaction mixtures calculated from a molecular weight of 76,000 (Panagou *et al.*, 1972) has been corrected by decreasing it in the ratio of the observed specific activity of the preparation to 200.

Thioredoxin and thioredoxin reductase were gifts from Dr. R. Yamada and were prepared from *Escherichia coli* as previously described (Vitols *et al.*, 1967b).

Cobamides. 2′,3′-Isopropylideneadenosylcobalamin and 4-(adenin-9-yl)butylcobalamin were gifts from Dr. H. P. C. Hogenkamp. 5′-Deoxyadenosylcobalamin was obtained from Pierrel, Milan, Italy, and from Yamanouchi Pharmaceutical Co., Ltd., Tokyo, Japan. The latter product was a gift from Dr. S. Fukui. Acquocobalamin was a gift from Russell-Uclaf, Paris, France. [2,8-^{3}H]-5′-Deoxyadenosylcobalamin was prepared as follows. [2,8-^{3}H]Adenosine (1 mCi) obtained from New England Nuclear was diluted with 154 mg (0.56 mmol) of unlabeled adenosine before reaction with acetone to give 2′,3′-isopropylideneadenosine according to Mizuro *et al.* (1963). The yield was 0.28 mmol, specific activity 660 cpm per nmol. The method of preparation of [2,8-^{3}H]-5′-deoxy-2′,3′-isopropylideneadenosylcobalamin from aquocobalamin and [2,8-^{3}H]-2′,3′-isopropylideneadenosine was essentially the same as that of Hogenkamp and Pailes (1968): yield 0.16 mmol; specific activity 670 cpm per nmol. Hydrolysis yielded [2,8-^{3}H]-5′-deoxyadenosylcobalamin: 81 μmol; specific activity, 540 cpm per nmol.

Methods

Enzymic Degradation of 5′-Deoxyadenosylcobalamin and Isolation of 5′-Deoxyadenosine. Reaction mixtures containing 0.2 M sodium dimethylglutarate buffer (pH 7.3), 25 mM dihydrolipoate, 0.83 mM EDTA, 5 mM dGTP, 0.1 mM [2,8-^{3}H]-5′-deoxyadenosylcobalamin (540 cpm per nmol), and 7.5 mg of ribonucleotide reductase in a total volume of 0.5 ml were incubated at 37° in the dark under anaerobic conditions. All subsequent operations were conducted in dim light. After incubation for various predetermined periods, 0.125 ml of 10 mM 5′-deoxyadenosine was added as a carrier. After a further 1-min incubation, the reaction mixture was mixed with 0.05 ml of 35% perchloric acid and centrifuged to remove precipitated protein. The residue was washed twice with about 1.0 ml of water and the combined supernatants were adjusted to a pH between 7.0 and 7.5 with 1 N KOH and kept in the cold for 1 hr. The precipitate of potassium perchlorate was removed by centrifugation and the supernatant was concentrated to about 0.5 ml under reduced pressure, and streaked on Whatman No. 3MM paper. The chromatogram was developed with water-saturated 1-butanol–ammonium hydroxide (28%) (100:1, v/v). The ultraviolet absorbing zone was cut out and extracted with water (about 60 ml). The extract was concentrated to about 0.5 ml under reduced pressure and spotted on Whatman No. 1 paper which was developed in a two-dimensional system with 1-butanol–acetic acid–water (5:2:3, v/v) as the first solvent and 3-methyl-1-butanol–5% Na_2HPO_4 (1:1, v/v, both layers) as the second solvent. The ultraviolet absorbing area corresponding to 5′-deoxyadenosine was cut out and extracted with water. The specific radioactivity was determined for the eluted material. The recovery of 5′-deoxyadenosine was 46–53%.

Spectral Measurements (Figures 2 and 4). Spectra were measured with a Cary-14 recording spectrophotometer in cells with a 1-mm light path thermostated at 37°. The reaction mixture contained 0.2 M sodium dimethylglutarate buffer (pH 7.3), 25 mM dihydrolipoate, 0.83 mM EDTA, 5 mM dGTP, 0.2 mM 5′-deoxyadenosylcobalamin, and 7.6 mg of ribonucleotide reductase (specific activity 94 units/mg) in a total volume of 0.25 ml. The reference cell contained all components of the mixture except 5′-deoxyadenosylcobalamin. Reaction components were deoxygenated and added to cells with Hamilton syringes under nitrogen. The reaction was started by addition of dihydrolipoate to the reaction mixture in cells which had been previously incubated at 37° for 5 min in the cell holder and the cells were immediately sealed. When the spectrum was to be taken at low temperature (Figure 4), cells were taken out of the cell holder thermostated at 37° and kept in an ice bath, while the circulating water in the holder of the sample cell was changed to cold water. After the temperature of the circulating water became constant at about 10°, cells were put back in the cell holder and the spectrum was recorded while the cell compartment was flushed with dry nitrogen. The temperature of the solution was determined by means of a calibrated thermistor inserted in the solution.

Measurement of Absorbance Changes at 525 nm (Figures 1 and 3). The absorbance was measured in cells with a 1-cm light path and 4 mm width. The reaction mixture contained 11.4 mg of ribonucleotide reductase and 0.1 mM 5′-deoxyadenosylcobalamin in a total volume of 0.75 ml. The other reaction components were added in the same concentration as for the spectral measurements. 5′-Deoxyadenosylcobalamin was omitted from the reference cell. All the operations were carried out anaerobically in dim light as described above. When absorbance changes were measured at two different temperatures a Cary-14 spectrophotometer thermostated at 5° and a Gilford spectrophotometer thermostated at 37° were used. The reaction was started by addition of dihydrolipoate to the reaction mixture preincubated at 37° in a cell in place in the spectrophotometer. After 5 min at 37°, cells were incubated in a 5° bath for 2 min, and the absorbance at 525 nm was measured by the spectrophotometer thermostated at 5°. As soon as the measurement at 5° was finished, cells were replaced in the cell holder thermostated at 37° after a short period of incubation in a 37° bath. Incubation at 37° was continued and the absorbance was measured just before cells were transferred back to the 5° bath for the absorbance measurement at 5°. This process was repeated at a time interval of 10–15 min until the total incubation time at 37° reached 75 min.

Alternatively the effect of temperature on the change of absorbance at 525 mμ was measured in a Cary-14 spectrophotometer (Figure 3) by using two alternative circulating baths, one at 37° and one at 5°. Absorbance measurements and temperature readings were commenced with circulation from the 37° bath. When the temperature in the cell stabilized as measured by thermistor, circulation from the 5° bath replaced that from the 37° and absorbance and temperature readings continued until the temperature again stabilized; the circulation supply was then again drawn from the 37° bath, and so on.

Experiments with the Stopped-Flow Apparatus. Rapid absorbance changes at 525 nm were measured in a Durrum–Gibson stopped-flow apparatus thermostated at 37°. The light path was 2 cm and the dead time about 4 msec. In most

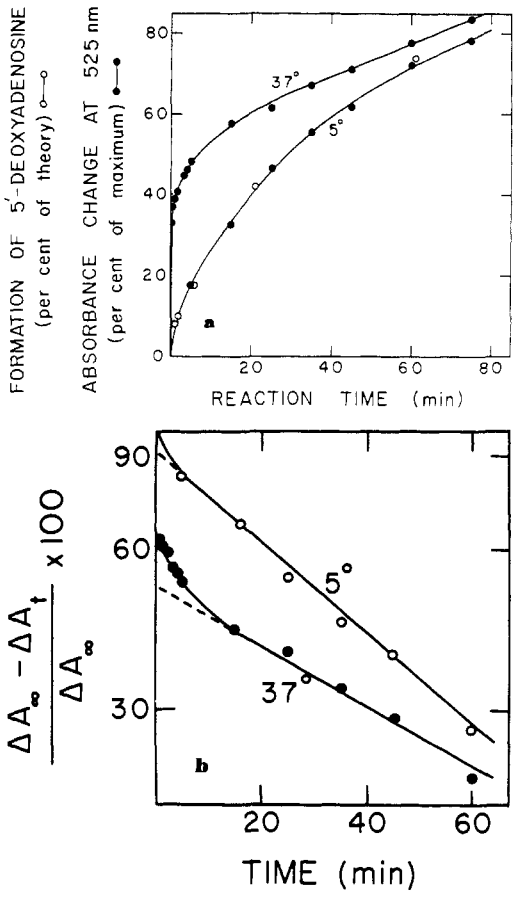


FIGURE 1: Absorbance changes at 525 nm and 5'-deoxyadenosine formation due to enzymic reactions of deoxyadenosylcobalamin. The reaction mixture contained 5'-deoxyadenosylcobalamin (100 μM), ribonucleotide reductase (80 μM) (specific activity 80 units/mg), dGTP (5 mM), dihydrolipoate (25 mM), EDTA (0.83 mM), and 0.2 M sodium dimethylglutarate buffer, pH 7.3. The reaction at 37° was commenced by addition of dihydrolipoate. Other experimental details are in the Methods section: (a) absorbance changes and deoxyadenosine formation *vs.* time; (b) semilog plot for the same absorbance changes. In calculating the values it was assumed that the molar extinction change in both rapid and slow reactions is given by the difference in the molar extinctions of deoxyadenosylcobalamin and cob(II)alamin, *i.e.*, $\Delta\epsilon = 4800$.

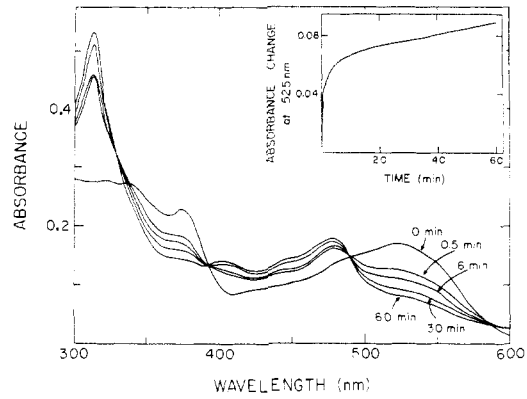


FIGURE 2: Spectral changes due to enzymic reactions of deoxyadenosylcobalamin. The reaction mixture was the same as in Figure 1 except that 160 μM reductase (specific activity 80) and 200 μM deoxyadenosylcobalamin were used. Other details are given under Methods. The reaction was commenced by the addition of dihydrolipoate and the zero time spectrum was corrected with respect to the others for absorbance of, and dilution by, dihydrolipoate.

experiments the final reaction mixture contained 0.2 M sodium dimethylglutarate buffer (pH 7.3), 25 mM dihydrolipoate, 0.83 mM EDTA, nucleoside triphosphate in the concentrations indicated, 25–200 μM 5'-deoxyadenosylcobalamin (as indicated), and ribonucleotide reductase at a concentration of 1.9–15.2 mg/ml. Usually enzyme, thiol, buffer, and nucleotide were present in one syringe and coenzyme, buffer, and nucleotide were present in the other. The reaction mixtures were thermally equilibrated in the working syringes for at least 10 min before the reaction was commenced.

Recording of Difference Spectra. Difference spectra were recorded in a Cary-15 spectrophotometer with solutions in cells of 5-mm path length and 4-mm width. The reference cell holder was thermostated at 37° and the sample compartment was maintained at lower temperatures by circulating water from a separate cooling bath.

Assay for Exchange of 5'-Hydrogen of 5'-Deoxyadenosylcobalamin with Water. This was carried out according to the method of Hogenkamp *et al.* (1968). [5'-^{3}H]-5'-Deoxyadenosylcobalamin, a gift from Dr. H. P. C. Hogenkamp, was prepared according to Gleason and Hogenkamp (1971) and used at a concentration of 18 μM. In measurements at 37° 5 μg of robonucleotide reductase was used and a 5-min incubation time. Because of the lower activity at 7°, 25 μg of ribonucleotide reductase and a 20-min incubation time were used for measurements at this temperature.

Assay for Reduction of GTP by Ribonucleotide Reductase. Reductase activity was determined colorimetrically according to Blakley (1966) with 5 mM GTP as substrate. The amounts of enzyme used in measurements at 37 and 7° were 50 and 500 μg, respectively, and the incubation times were 5 min at 37° and 20 min at 7°. In order to avoid GTP reduction during the heat treatment with chloroacetamide which was not negligible when higher concentrations of enzyme were used, enzyme was inactivated by addition of 0.1 ml of 1 N NaOH and subsequent heating for 3 min at 100°. The reaction mixture was further heated at 100° for 40 min, after a mixture of 0.15 ml of 1 N H_3PO_4 and 0.25 ml of 0.8 M chloroacetamide in 0.2 M potassium phosphate buffer (pH 7.3) had been added.

Radioactivity Determination. Radioactivity in a 0.5-ml sample was determined in 10 ml of the scintillation fluid of Bray (1960) with a Packard Model 3003 Tri-Carb scintillation spectrometer.

Results

Rapid Formation of a Cob(II)alamin-like Product. During the incubation of reductase with deoxyadenosylcobalamin, dihydrolipoate, and dGTP no cobamide product was detected except cob(II)alamin (Yamada *et al.*, 1971). From the time course of absorbance changes it originally appeared that a single reaction occurs, but closer examination of absorbance changes at short times indicated that this is not the case. Figure 1a shows that within the first minute at 37° there is a rapid change, much faster than that observed between 5 and 7 min. Negligible change occurs if any of the components of the system is omitted.

Spectral changes occurring in the first minute were determined by scanning rapidly in the Cary-14 spectrophotometer before adding thiol, 0.5 min after adding dihydrolipoate to a concentration of 25 mM, and at longer time intervals. As seen in Figure 2, the spectrum change occurring in the first half-minute was qualitatively similar to, although not so extensive as, that occurring during the slower phase of the spectrum

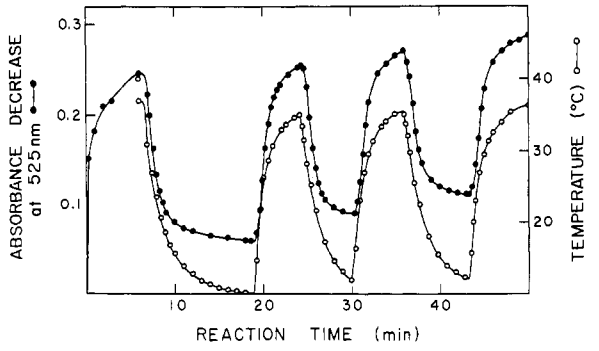


FIGURE 3: Reversible absorbance changes due to shift in the equilibrium of the enzymic reactions of deoxyadenosylcobalamin with temperature elevation and depression. The reaction mixture was the same as in Figure 1 except that 120 μM reductase (specific activity 120) was used. Other details are given in the Methods section.

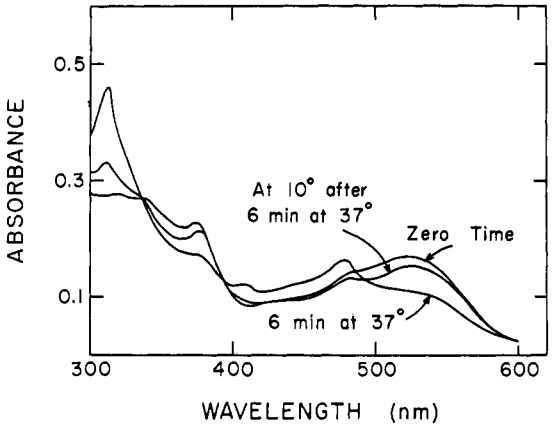


FIGURE 4: Spectral changes due to enzymic reactions of deoxyadenosylcobalamin and their reversal on cooling. The reaction mixture and other details were the same as in Figure 2.

change, and well-defined isobestic points were present through the whole period. This suggests that either both phases of the reaction produce cob(II)alamin, or the product of the initial fast reaction has a spectrum resembling that of cob(II)alamin very closely. This was confirmed by the calculation that the spectrum obtained after 1 min of incubation corresponds very closely to that of a mixture of deoxyadenosylcobalamin and cob(II)alamin in a 1:1 ratio.

When the reaction mixture is chilled to 5° before absorbance readings are taken no evidence of the initial rapid spectral change is seen (Figure 1), but the change due to the slow reaction is unaffected, so that a smooth progress curve for the slow reaction alone is now obtained. This is seen more clearly in Figure 1b where absorbance data for 37 and 5° are plotted in semilog form. The rapid reaction must therefore quickly reach an equilibrium position at 37° but have a low equilibrium constant at 5°, so that on cooling the reaction mixture the absorbance change due to the rapid reaction is reversed although the slow reaction is not. Figure 3 shows the absorbance changes occurring in a reaction mixture during several cycles of incubation at 37° followed by cooling to 5°. Rapid absorbance increases occur with each increase of temperature to 37° and the major part of each increase is reversed on cooling to 5°, observations that indicate free reversibility of the rapid reaction with temperature change. Each absorbance minimum is slightly higher than the previous one, however, because the progress of the slow reaction is delayed but not reversed by the temperature decreases.

Although the absorbance data in Figures 1 and 3 refer only to absorbance changes at 525 nm the reversal of the fast spectral change applied to the whole spectral region observable, as seen in Figure 4. Although the curve recorded at 10° after 6 min at 37° is not identical with the zero time curve because of progress of the slow reaction over the total period of almost 10 min at 37° (6 min plus recording time), it can be seen that the temperature drop from 37 to 10° caused a reversal of most of the change that had occurred during incubation over the whole of the spectral region.

The deoxyadenosyl moiety of the coenzyme is converted to 5′-deoxyadenosine by the slow reaction (Yamada *et al.*, 1971). The rate of formation of 5′-deoxyadenosine was studied by incubating deoxyadenosylcobalamin labeled with tritium in the adenine ring with other components of the enzyme system and at intervals isolating the labeled 5′-deoxyadenosine with the aid of carrier 5′-deoxyadenosine. Conversion of the deoxyadenosyl moiety to deoxyadenosine showed an identical progress curve (Figure 1) to absorbance changes due to the slow reaction (measured after chilling to 5°), a result indicating that 5′-deoxyadenosine is not released in the fast reaction.

Stopped-Flow Spectrophotometric Studies. To obtain information on the rate of this rapid reaction involving deoxyadenosylcobalamin spectrophotometric measurements were made in a Durrum stopped-flow apparatus at 525 nm, where the absorbance change is large (Figure 2) and components other than the coenzyme have negligible absorbance. The absorbance change reached an equilibrium position in about 100 msec when enzyme, dihydrolipoate, dGTP, and buffer in one syringe were mixed with coenzyme, dGTP, and buffer from the other syringe (Figure 5). In this time interval the change due to the slow (degradation) reaction is negligible so that the reaction being observed is essentially the fast reaction alone. When the absorbance change was plotted in semilog form (Figure 6) the data conformed quite closely to a straight line except at longer time intervals and the corresponding first-order apparent rate constant was 38–46 sec^{-1}. By contrast, the rate constant for the slow reaction determined from the

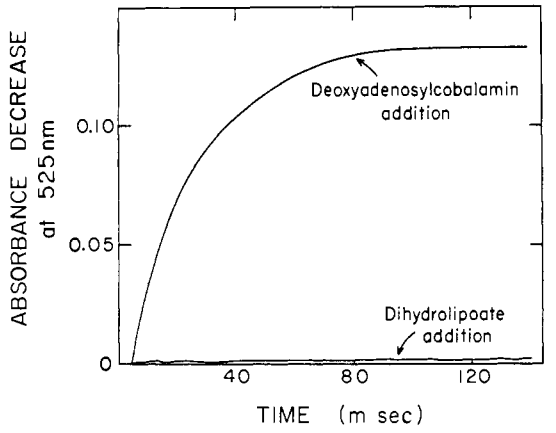


FIGURE 5: Rapid absorbance changes due to enzymic reaction of deoxyadenosylcobalamin. The final reaction mixture contained 70 μM ribonucleotide reductase (specific activity 70), 25 mM dihydrolipoate, 0.83 mM EDTA, 100 μM 5′-deoxyadenosylcobalamin, 5 mM dGTP, and 0.2 M sodium dimethylglutarate buffer, pH 7.3. In the upper curve the normal mixing procedure was used as described in the Methods section. In the lower curve one syringe contained all components except dihydrolipoate and the other all components except reductase.

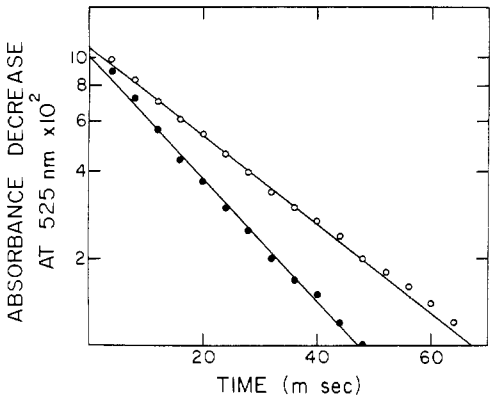


FIGURE 6: First-order plots for the absorbance changes due to enzymic reaction of deoxyadenosylcobalamin and of deuterated deoxyadenosylcobalamin. The final reaction mixture contained 26 μM ribonucleotide reductase (specific activity 105), 1 mM dGTP, and other components as in Figure 5. (●) Undeuterated coenzyme; (○) [5′,5′-$^2H_2$]deoxyadenosylcobalamin.

semilog plot of absorbance readings taken at 5° (Figure 1b) was found to be 3.3×10^{-4} sec^{-1}.

Nature of the Rapid Reaction. The possibility that the rapid reaction might represent reduction of an enzyme disulfide bridge by dihydrolipoate (Vitols *et al.*, 1967b) was eliminated by showing that when the reaction was commenced by mixing enzyme, coenzyme, dGTP, and buffer from one syringe with dihydrolipoate, coenzyme, dGTP, and buffer from the other syringe the absorbance change was extremely slow (Figure 5). Dihydrolipoate must therefore activate the enzyme by reducing a disulfide bridge at a relatively slow rate before the fast reaction can occur at the active site.

The absorbance change might be due either to binding of coenzyme to activated enzyme or to isomerization of the coenzyme–activated enzyme complex. These possibilities may be represented by the following equations[1]

$$\text{reduced reductase}\cdot\text{dGTP} + \text{dACbl} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{reduced reductase}\cdot\text{dGTP}\cdot\text{dACbl} \quad (1)$$

$$\text{reduced reductase}\cdot\text{dGTP}\cdot\text{dACbl} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \text{reduced reductase}\cdot\text{dGTP}\cdot\text{intermediates} \quad (2)$$

Linear semilogarithmic plots (Figure 6) were found as the ratio of enzyme concentration to coenzyme concentration was varied from 0.25 to 6 and the apparent rate constant remained unchanged (within experimental error) as either the enzyme concentration (Table I) or coenzyme concentration (Table II) was varied within the accessible range. Although this seems more consistent with reaction 2 it was found to be not inconsistent with reaction 1 when the kinetics of the latter were investigated with an Electronic Associates TR-20 analog computer equipped with a repetitive operation display unit (Model 34-035) and Variplotter (1130). Variation of the equilibrium constants in the range 0.5–100 did not cause much departure of the semilog plot from linearity. The greatest deviation occurred with an equilibrium constant of 100 and equimolar initial concentrations of reactants, but the actual equilibrium constant is probably about 0.5 because the absorbance change is about one-third that for complete conversion of coenzyme to cob(II)alamin. An equilibrium constant of 0.5 gave linear semilog plots for reaction 1 and negligible changes in the apparent first-order rate constant for approach to equilibrium as the ratio of coenzyme to enzyme was varied from 1 to 20. The kinetics of an isomerization (eq 2) following rapid binding also give linear semilog plots and an apparent rate constant invariant with the ratio of reactant concentrations. The kinetic data therefore do not distinguish between the two possible reactions.

Results of binding experiments undertaken to distinguish between the binding and isomerization possibilities were unsatisfactory because of practical difficulties. In the system containing dGTP and dihydrolipoate at 37°, coenzyme is completely degraded in about 1 hr and the enzyme rapidly becomes inactivated so that equilibrium dialysis is useless. The ultrafiltration method of Paulus (1969) was still not rapid enough and gave badly scattered data. The results indicated that dihydrolipoate and dGTP, the components required for the

TABLE I: Effect of Enzyme Concentration on the Rapid Enzymic Reaction of Deoxyadenosylcobalamin.[a]

Enzyme Concn (μM)	k_{obsd} (sec^{-1})	Final Absorbance Change at 525 nm
12	49.0 ± 1.6	0.0516 ± 0.0024
23	48.8 ± 1.4	0.0767 ± 0.0031
45	48.5 ± 0.8	0.144 ± 0.003
90	49.5 ± 0.53	0.200 ± 0.005

[a] The final reaction mixture contained 100 μM 5′-deoxyadenosylcobalamin, 1 mM dGTP, 25 mM dihydrolipoate, 0.83 mM EDTA, 0.2 M sodium dimethylglutarate buffer (pH 7.3), and varying amounts of ribonucleotide reductase (specific activity 90). k_{obsd} in this and succeeding tables is the observed rate constant calculated from a semilog plot of the type shown in Figure 6.

TABLE II: Effect of Deoxyadenosylcobalamin Concentration on Its Rapid Reaction in the Enzyme System.[a]

dACbl Concn (μM)	k_{obsd} (sec^{-1})	Final Absorbance Change at 525 nm
25	37.5 ± 1.2	0.0268 ± 0.0010
50	45.0 ± 2.0	0.0351 ± 0.0021
75	45.3 ± 1.9	0.0350 ± 0.0020
100	45.5 ± 1.4	0.0385 ± 0.0015
150	35.0 ± 1.6	0.0345 ± 0.0017

[a] The final reaction mixture contained 11.3 μM ribonucleotide reductase (specific activity 90), 1 mM dGTP, 25 mM dihydrolipoate, 0.83 mM EDTA, 0.2 M sodium dimethylglutarate buffer (pH 7.3), and 5′-deoxyadenosylcobalamin at the concentrations shown.

[1] The abbreviations used are: dACbl, 5′-deoxyadenosylcobalamin; *ara*ATP, the arabino analog of adenosine 5′-triphosphate; EDTA, ethylenediaminetetraacetic acid.

TABLE III: Comparison of Various Nucleotides in the Rapid Enzymic Reaction of Deoxyadenosylcobalamin.[a]

Nucleotide, Concn (mM)	k_{obsd} (sec^{-1})	Final Absorbance Change at 525 nm
dGTP, 5	37.8 ± 1.5	0.151 ± 0.006
dATP, 5	28.3 ± 1.3	0.0199 ± 0.0008
dCTP, 5	33.6 ± 1.2	0.0275 ± 0.0009
dTTP, 5		<0.005
*ara*ATP, 1	25[b]	~0.014[c]
2′-*O*-Methyl-ATP, 1		~0

[a] The final reaction mixture contained 70 μM ribonucleotide reductase (specific activity 70), 100 μM 5′-deoxyadenosylcobalamin, 25 mM dihydrolipoate, 0.83 mM EDTA, and nucleotide. The abbreviation is *ara*ATP, the arabinose analog of ATP. [b] From $t_{1/2}$ obtained directly from oscillograph trace. [c] Absorbance difference at $t = 0$ and $t = 140$ msec on oscillograph trace.

rapid reaction, are necessary for optimal binding of coenzyme, and the data therefore do not clearly distinguish between the alternatives. Nevertheless, significant binding was found to occur under conditions in which no spectral change occurs.

Significant information was obtained with analogs of 5′-deoxyadenosylcobalamin. 4-(Adenin-9-yl)butylcobalamin and 2′,3′-isopropylidine-5′-deoxyadenosylcobalamin are competitive inhibitors with respect to deoxyadenosylcobalamin having apparent K_i values of 7×10^{-6} M (Vitols, E., Blakley, R. L., and Hogenkamp, H. P. C., 1972[2]) and 3×10^{-5} M (Vitols *et al.*, 1967a), respectively. The inhibitory properties of these analogs indicate that they bind reasonably well to the active site, yet neither of them gives a rapid absorbance decrease like that occurring with deoxyadenosylcobalamin, and in fact both gave a slow increase in absorbance at 525 nm.

Spectrophotometric Changes in the Presence of Other Nucleotides. In all the experiments discussed so far the reaction mixtures contained 1 or 5 mM dGTP. Both the rate constant and the magnitude of the total change decreased only slightly as the dGTP concentration was lowered from 5 to 0.25 mM. When other deoxynucleoside triphosphates were substituted for dGTP much smaller absorbance changes were observed (Table III), although where changes did occur the apparent rate constant was of the same order as that for change in the presence of dGTP. The rather low absorbance change observed in the presence of 5 mM dATP was not due to an adverse effect of this relatively high concentration of nucleotide since a similar absorbance change was produced with 0.25 mM dATP. Presumably the differences in equilibrium concentration of the intermediate reflect different conformational states induced in the protein by the various ligands.

Ribonucleoside triphosphates produced significant effects in the system (Figure 7). Thus, when 10 mM GTP was substituted for dGTP a rapid absorbance decrease occurred at 525 nm at about the same rate as in the presence of dGTP. However, instead of proceeding to a constant maximum value the absorbance change reached a sharp maximum and then immediately commenced to decrease until a constant minimum was reached. When 1 mM GTP was used the approach to the sharp maximum was the same as for 10 mM but the subsequent

[2] Unpublished results.

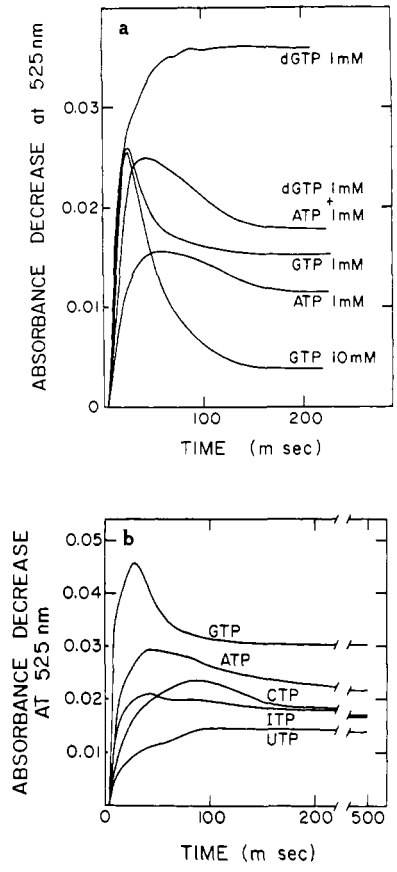


FIGURE 7: Absorbance changes due to enzymic reaction of deoxyadenosylcobalamin in the presence of ribonucleotides. In a the final reaction mixture contained 11.3 μM reductase (specific activity 90), and in b 12.5 μM reductase (specific activity 105). Nucleotides were present at the concentrations indicated in a and at 1 mM in b. Other conditions were the same as in Figure 5.

decrease was much less marked. ATP (1 mM) gave a somewhat slower initial change and reached a lower maximum but otherwise showed similar behavior to GTP. When 1 mM dGTP and 1 mM ATP were both present the maximum was greater and the subsequent decrease also greater than with ATP alone. *ara*ATP with 1 mM dGTP gave about two-thirds the absorbance change obtained with the latter alone, a rate constant of 19 sec^{-1} and no subsequent decrease in absorbance change.

In the absence of other nucleotides ITP and CTP gave total absorbance changes only a little less than that found in the presence of ATP (Figure 7b) whereas UTP gave a somewhat lower total change. The decrease in the absorbance change after reaching the maximum was small in the case of CTP, very slight in the case of ITP, and absent in the case of UTP. The rate of approach to the maximum decreased in the order GTP, ATP, ITP, CTP, and UTP.

By inspection of the curves in Figure 7 it may be seen that in all cases the initial absorbance change was significantly faster than the subsequent decrease in the change. For GTP $t_{1/2}$ for the decrease from the maximum was 18 msec (at 1 mM GTP) to 25 msec (at 10 mM GTP) and if the decline were exponential the corresponding rate constants would be 39 and 28 sec^{-1}, respectively, as compared with about 45 sec^{-1} for the initial change. In the case of 1 mM ATP $t_{1/2}$ for the decline was 61 msec in the absence of other nucleotides or 54 msec in the presence of 1 mM dGTP, corresponding to rate constants of 11 and 13 sec^{-1}.

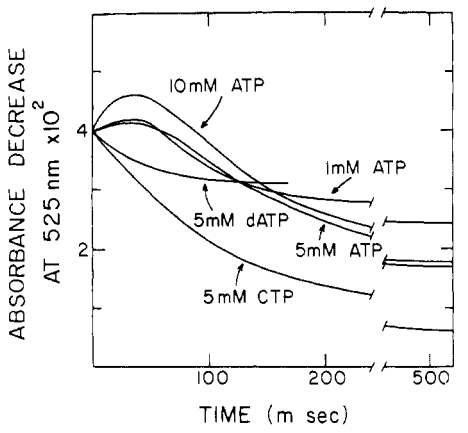


FIGURE 8: Reversal by ribonucleotides of absorbance change due to enzymic reaction of deoxyadenosylcobalamin. Reaction mixtures and experimental procedure were as indicated in Table IV.

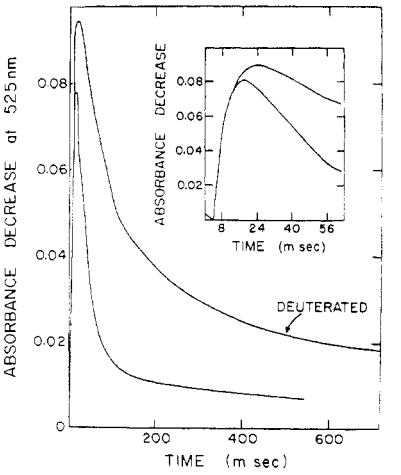


FIGURE 9: Absorbance changes due to enzymic reactions of undeuterated and deuterated deoxyadenosylcobalamin in the presence of GTP. The final reaction mixture contained 43 μM reductase (specific activity 85), 10 mM GTP, and other components as in Figure 5. The lower curve is for undeuterated coenzyme and the upper for [5′,5′-$^2H_2$]deoxyadenosylcobalamin.

The reversal of the absorbance change was also studied by rapid mixing of the ribonucleotide with a solution containing enzyme, dGTP, dihydrolipoate, and coenzyme, in which the rapid initial change had already occurred, with the results shown in Table IV. In these experiments the decline from the maximum was slower (Figure 8) than when coenzyme was added last and the extent of the decrease depended on the concentration of the ribonucleotide. Furthermore, in the case of ATP there was a distinct additional absorbance change before the latter began to decrease.

Reactions in the Presence of Deuterated Materials. As may be seen from Figure 6 the rate of absorbance change in the presence of dGTP was slightly lower when coenzyme completely deuterated in the cobalt-bound 5′-methylene group was used instead of normal coenzyme. Least-squares analysis of the data recorded up to 48 msec gave rate constants of 35.2 ± 0.5 and 49.2 ± 1.0 sec^{-1} and final absorbance changes of 0.109 and 0.102 with the deuterated and normal coenzyme, respectively. When the reaction was performed in the presence of 10 mM GTP the rate of the initial change was not perceptibly different in the presence of the deuterated and undeuterated coenzyme (Figure 9), but the maximum change was greater and the decline slower with the deuterated coenzyme. The half-times for the decrease to the constant minimum were 100 and 45 msec for the deuterated and nondeuterated coenzyme, respectively, corresponding to an isotope effect of 2.22.

Effect of Other Experimental Conditions on the Absorbance Change. Substitution of other thiols for dihydrolipoate in the reaction mixture caused decreases in the reaction rate as well as in the extent of the absorbance change (Table V). Even at 200 mM 2-mercaptoethanol produced only half the change occurring with 25 mM dihydrolipoate. It is noteworthy that 25 mM reduced glutathione gave negligible change and that the thioredoxin system gave a relatively rapid change to

TABLE IV: Reversal by Various Nucleotides of the Absorbance Change Due to the Deoxyadenosylcobalamin Reaction.[a]

Nucleotide, Concn (mM)	Absorbance Change	$t_{1/2}$ (msec)
ATP, 1	0.0157[b]	145
ATP, 5	0.0246[b]	145
ATP, 10	0.0291[b]	145
CTP, 5	0.0367	70
dATP, 5	0.0104	20

[a] The final reaction mixture contained 11.2 μM ribonucleotide reductase (specific activity 90), 100 μM 5′-deoxyadenosylcobalamin, 1 mM dGTP, 25 mM dihydrolipoate, 0.83 mM EDTA, sodium dimethylglutarate buffer (pH 7.3), and nucleotide concentrations as indicated. The reaction mixture except the ribonucleotide was placed in one syringe of the stopped-flow apparatus, and the mixture except reductase in the other. Mixing was performed after about 5 min of incubation 37°. [b] After ATP addition, the absorbance decreases up to ~40 msec and then increases. The total absorbance change is the difference between the minimum and maximum. $t_{1/2}$ is the time for half the total absorbance change.

TABLE V: Effect of Various Thiols on the Rapid Enzymic Reaction of Deoxyadenosylcobalamin.[a]

Thiol, Concn (mM)	k_{obsd} (sec^{-1})	Final Absorbance Change at 525 nm
Dihydrolipoate, 25	45.5 ± 1.4	0.0385 ± 0.0014
1,3-Dimercapto-2-propanol, 25	38.0 ± 1.2	0.0201 ± 0.0007
Mercaptoethanol, 25	18.3 ± 1.0	0.0123 ± 0.0004
Mercaptoethanol, 200	34.8 ± 2.7	0.0192 ± 0.0016
Glutathione, 25		0.004
Thioredoxin, 10 μM	35.9 ± 2.5	0.0223 ± 0.0017

[a] The final reaction mixture contained 10 μM ribonucleotide reductase (specific activity 80), 100 μM 5′-deoxyadenosylcobalamin, 1 mM dGTP, 0.2 M sodium dimethylglutarate buffer (pH 7.3), and thiol as indicated in the table. In the mixture for dihydrolipoate 0.83 mM EDTA was also present, and in that for thioredoxin 33 units/ml of thioredoxin reductase and 2 mM NADPH were also present.

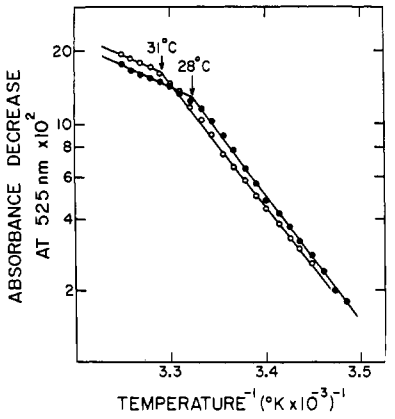


FIGURE 10: Semilog plot of absorbance change due to enzymic reaction of deoxyadenosylcobalamin *vs.* reciprocal temperature. Data are taken from the second cycle of Figure 3. The absorbance data for the warming phase (○) were corrected by subtracting an absorbance change of 0.07 attributed to irreversible degradation, while those for the cooling phase (●) were corrected by subtraction of 0.100.

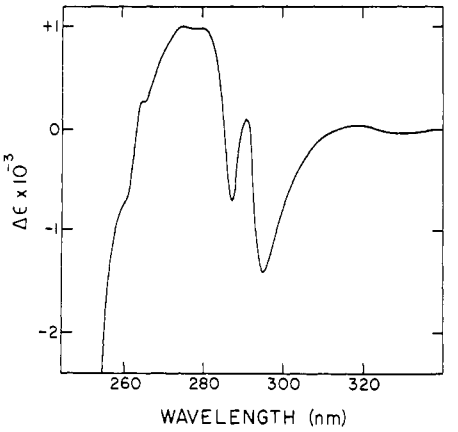


FIGURE 11: Difference spectra with temperature for ribonucleotide reductase. The cells contained 1.5 mg/ml of ribonucleotide reductase (specific activity 130) which is equivalent to a protein concentration of 1.97×10^{-5} M, in 0.2 M sodium dimethylglutarate, pH 7.3. The reference cell was maintained at 37 ± 0.5° and the sample cell at 7 ± 0.5°. Other details are given in the Methods section.

about 60% of the extent of that in the presence of dihydrolipoate.

The rate constant for the absorbance change in the presence of 5 mM dGTP and 5 mM dihydrolipoate (41.0 ± 0.8 sec^{-1}) was as great as with 25 mM dihydrolipoate (37.8 ± 1.5) and the total absorbance change (0.125 ± 0.003) was almost as great as that at the higher concentration (0.151 ± 0.006).

Both the rate constant and the extent of the absorbance change were greater at pH 7.3 than at pH 6.3 or pH 8.3 (Table VI).

Effect of Temperature on the Enzyme. As described above, the rapid absorbance changes are easily reversed by temperature decrease. The data in Table VII demonstrate large temperature quotients for three other enzymic processes, namely (1) exchange of tritium between the 5′-methylene group of the coenzyme and water, (2) degradation of the coenzyme to cob(II)alamin and 5′-deoxyadenosine, and (3) reduction of GTP to dGTP.

Data for one of the warming and cooling cycles of Figure 3 are replotted as log absorbance *vs.* reciprocal of degrees Kelvin in Figure 10. For both warming and cooling curves there are clear discontinuities at 31 and 28°, respectively, with a change in slope of 3.5. This suggests that a conformation change occurs at about 29° with a consequent change in ΔH for the reaction. However, calculation of the enthalpy changes above and below the transition have not been attempted because of the impossibility of accurately determining the equilibrium constant or deciding whether such constants apply to the isomerization only or to a combination of isomerization and binding.

Difference spectra for the reductase due to temperature difference showed marked maxima at 295 and 287 nm and a minimum in the 275–280-nm region (Figure 11). In the presence of 50 μM dGTP or 50 μM dGTP plus 25 mM dihydrolipoate the difference spectra were very similar, showing only minor differences from that in Figure 11. Somewhat similar changes for aldolase have been reported by Lehrer and Barker (1971). The plot of $\Delta\epsilon$ *vs.* temperature at two wavelengths is shown in Figure 12, and displays a clear discontinuity at 30° for enzyme alone as well as for enzyme in the presence of dGTP or dGTP plus dihydrolipoate.

Discussion

The reaction resulting in the rapid absorbance changes over the visible and near-ultraviolet requires ribonucleotide reductase, deoxyadenosylcobalamin, dihydrolipoate, and a nucleo-

TABLE VI: Effect of pH on the Rapid Enzymic Reaction of Deoxyadenosylcobalamin.[a]

Conditions	k_{obsd} (sec^{-1})	Final Absorbance Change at 525 nm
Sodium dimethylglutarate buffer, pH 6.3	30.8 ± 0.7	0.099 ± 0.002
Sodium dimethylglutarate buffer, pH 7.3	47.2 ± 1.1	0.184 ± 0.006
Tris–acetate buffer, pH 8.3	44.0 ± 0.6	0.106 ± 0.002

[a] Reaction mixtures contained 53 μM ribonucleotide reductase (specific activity 105), 100 μM deoxyadenosylcobalamin, 25 mM dihydrolipoate, 0.83 mM EDTA, 1 mM dGTP, and 0.2 M sodium dimethylglutarate buffer, pH 7.3.

TABLE VII: Effect of Temperature on Various Activities of Ribonucleotide Reductase.[a]

Activity	37°	7°	37°/7°
Tritium exchange (cpm in H_2O/nmol of enzyme per min)	69,000	770	900
Coenzyme degradation	25% in 10 min	1.4% in 2 hr	>210
Reduction of GTP (V_{max})	64	0.84	76

[a] The assay procedures are described under Methods.

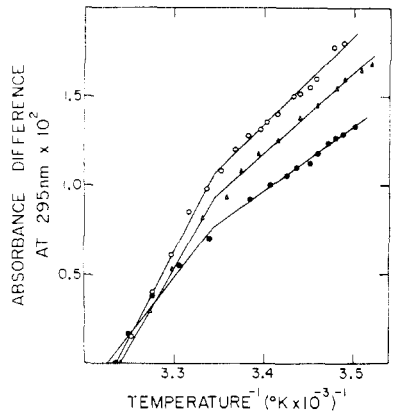


FIGURE 12: Change of absorbance of ribonucleotide reductase with temperature. The conditions were the same as for Figure 11 except that the temperature of the sample cell was decreased from 37°: (●) enzyme and buffer only; (○) plus 50 μM dGTP; (△) plus 50 μM dGTP and 25 mM dihydrolipoate.

side triphosphate—the same components that react to degrade the coenzyme to cob(II)alamin and 5′-deoxyadenosine. Yet a number of factors differentiate the rapid reaction from the degradative process: (1) these first-order processes have rate constants differing by a factor of 10^5; (2) degradation of labeled coenzyme results in the formation of 5′-deoxyadenosine which can be trapped by an unlabeled pool; the rapid reaction does not; and (3) the rapid reaction is reversed by lowering the temperature to 5° whereas degradation is irreversible. Yet, despite these distinguishing features the two reactions result in identical spectral changes, so that the rapid reaction must either produce cob(II)alamin (as does degradation) or a closely related cobalamin.

The extent of the spectral change occurring in the rapid reaction makes it unlikely that this change is due to simple binding of coenzyme to reductase, since binding of deoxyadenosylcobalamin to other enzymes results in smaller spectral changes (Babior and Li, 1969). Furthermore, when 4-(adenin-9-yl)butylcobalamin or isopropylidine-5′-deoxyadenosylcobalamin are substituted for deoxyadenosylcobalamin in the enzymic system there is no spectral change, although the K_i values for the analogs indicate that about 44 and 36% of the analogs, respectively, should have been bound. Although studies on the amount of deoxyadenosylcobalamin bound to the enzyme under various conditions gave binding constants with high standard errors, they were consistent with the preceding in that they suggested that significant binding of coenzyme occurred under conditions where no spectral change occurred. Finally, the rate constant for the spectral change is relatively small compared with those for binding of substrates to most enzymes (Hammes and Schimmel, 1970). These considerations therefore suggest that the rapid spectral change is due to isomerization of the enzyme–coenzyme complex, rather than to formation of the complex.

Product of the Rapid Reaction as Intermediate in Ribonucleotide Reduction. Several aspects of the rapid reaction, apart from the specific nature of the four reactants, suggest that the product may be a reactive intermediate in ribonucleotide reduction and perhaps in some of the other processes catalyzed by the enzyme. The rate of the spectral change exceeds by a considerable margin the rate of enzymic ribonucleotide reduction under similar conditions which is about 100–400 mol/mol of enzyme per minute at 37° depending on the substrate. Moreover, in the presence of ribonucleotides the cobamide product first accumulates, reaches a maximum concentration, and then declines to a lower steady-state concentration (Figure 7a), whereas in the presence of deoxynucleotides no decline occurs. Addition of ribonucleotide to a solution in which the cobalamin product has already accumulated in the presence of dGTP results in a decrease in its concentration as indicated by spectral changes. Both of these observations are consistent with the view that ribonucleoside triphosphates react with the cobamide intermediate to form deoxyribonucleotide and regenerate coenzyme. An alternative explanation for the decrease in the spectral change in the second type of experiment is that ribonucleotide merely displaces dGTP from an allosteric site and thereby causes the protein to adopt a conformation less favorable to formation of the cob(II)alamin-like product. Such an effect is produced by dATP which determines an equilibrium concentration of the intermediate seven times lower than observed in the presence of dGTP (Table III). When dATP is added to the equilibrated system containing dGTP the decline in intermediate concentration has a $t_{1/2}$ of only 20 msec, however, whereas after ATP addition $t_{1/2}$ for the decrease is 147 msec, clearly suggesting a different process.

It is desirable to demonstrate that the rate of disappearance of the cob(II)alamin-like species in the presence of ribonucleotide is also faster than overall ribonucleotide reduction, but the data presently available do not permit this calculation since the observed rate of disappearance represents the net difference between formation and removal of the intermediate. Nevertheless, the $t_{1/2}$ of 145 msec for net disappearance observed in the presence of ATP is of the order predicted for the rate of intermediate reaction from the overall rate of ATP reduction. It should also be noted that the greater disappearance of the cob(II)alamin-like species at higher ribonucleotide concentration is consistent with the reaction of the cobalamin with ribonucleotide.

A further aspect of the rapid reaction which suggests that it may be an integral part of overall ribonucleotide reduction is the marked effect of temperature. When the temperature is lowered from 37 to 7°, the equilibrium concentration of the cob(II)alamin-like species decreases to an undetectable level presumably with a corresponding large decrease in the rate of formation. The same temperature decrease causes overall ribonucleotide reduction to decrease 76-fold, an effect considerably greater than usually encountered for enzyme reactions, and a still greater effect is seen on the degradation reaction and on the exchange of tritium between the 5′-methylene hydrogens of the coenzyme and water. A more detailed study of the relation between temperature and the equilibrium concentration of the cob(II)alamin-like compound revealed a sharp discontinuity at about 30° (Figure 10), and a similar discontinuity was also seen in temperature-difference spectra (Figure 12). The plot of absorbance difference at 295 nm *vs.* reciprocal temperature has a slope change at about 28°. Above this temperature the apparent change in molar absorbance per degree is −43 whereas below this temperature it is −21 (both values uncorrected for volume changes with temperature). The greater change in absorbance with temperature above the transition point is presumably due to a conformation change that places one or more tryptophans in an altered environment. This change in environment could be movement to a less polar region (Lehrer and Barker, 1971), or movement of charged groups in the immediate proximity of the tryptophan (Ananthanarayanan and Bigelow, 1969). The effect of the conformation change is somewhat less in

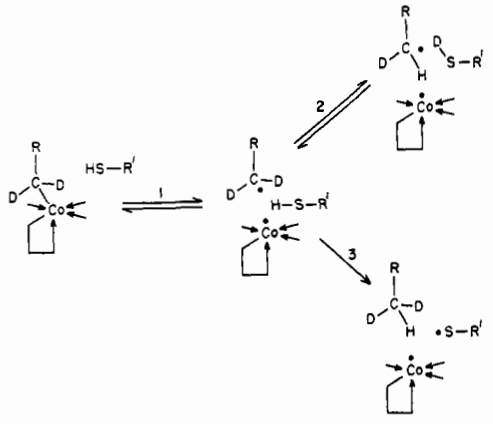
the presence of the allosteric effector dGTP, reflecting the influence of the latter on the conformation of the protein.

Mechanism of the Rapid Reaction. If it is assumed that the rapid reaction is a readily reversible isomerization of the enzyme complex that is a part of the overall mechanism of ribonucleotide reduction, it is of special interest to ascertain the nature of the reaction and the products. The spectral change corresponds to that associated with cob(II)alamin formation so closely that it is likely that the reaction involves homolytic cleavage of the cobalt–carbon bond rather than formation of cob(I)alamin as previously postulated (Hogenkamp *et al.*, 1968). This explains the inability to detect cob(I)-alamin by reaction with alkylating reagents (Yamada *et al.*, 1971). The high rate of cob(II)alamin formation and the extent of its formation from coenzyme (20–40%) make it unlikely that cob(II)alamin is a secondary product and virtually eliminate cob(I)alamin from further consideration as an intermediate.

Although the dithiol required for the reaction presumably acts as a hydrogen donor, the deoxyadenosyl moiety does not appear to be reduced to 5′-deoxyadenosine since coenzyme with a labeled deoxyadenosyl moiety does not yield labeled 5′-deoxyadenosine exchangeable with a pool of carrier, whereas the slow degradation of the coenzyme does yield both cob(II)alamin (observed spectrophotometrically) and labeled 5′-deoxyadenosine which can be trapped by a pool of carrier. It is conceivable that 5′-deoxyadenosine is formed in the fast reaction but does not exchange with free 5′-deoxyadenosine except as a result of some further change in the system that causes the slow irreversible degradation of coenzyme. However, there is no evidence for such formation of 5′-deoxyadenosine as an intermediate in the ribonucleotide reductase system. 5′-Deoxyadenosine formation from the dioldehydrase–coenzyme complex (Wagner *et al.*, 1966) and from the ethanolamine deaminase–coenzyme complex (Babior, 1970a,b), and 5′-deoxyinosine formation from the propanediol dehydrase–5′-deoxyinosylcobalamin complex (Jayme and Richards, 1971) have been reported. However, in all these cases it remains possible that 5′-deoxynucleoside formation is a result of degradation of the actual intermediate, in some cases perhaps increased or even induced by procedures used to quench the reaction. With none of these enzymes has there been demonstration that added 5′-deoxyadenosine and cob-(II)alamin or cob(I)alamin can be converted to 5′-deoxy-adenosylcobalamin, although evidence for such a reaction has been carefully sought by the use of labeled 5′-deoxy-adenosine and ribonucleotide reductase (Yamada, Tamao, and Blakley[3]). The latter enzyme binds 5′-deoxyadenosine and cob(II)alamin tightly to the active site (Yamada *et al.*, 1971), so that absence of coenzyme formation cannot be explained by the inability of the reactants to bind to the enzyme.

A stabilized 5′-deoxyadenosyl radical is the expected product from the deoxyadenosyl moiety of the coenzyme if a homolytic cleavage is assumed, but if it is the product, the 5′-methylene group must be able to interact readily with an exchangeable hydrogen of the dithiol or the enzyme so as to permit the rapid exchange with water protons that occurs in this system. Proximity to the dithiol substrate may also be inferred from the slow and irreversible reduction of the deoxy-adenosyl moiety to 5′-deoxyadenosine that occurs in the degradation reaction. Scheme I is a possible representation

SCHEME I[a]



[a] Postulated mechanism for: 1, the rapid reaction observed spectrophotometrically; 2, the exchange of deuterium between [5′,5′-$^2H_2$]-5′-deoxyadenosylcobalamin and water *via* a thiol group; and 3, irreversible degradation of deoxyadenosylcobalamin to 5′-deoxyadenosine and cob(II)alamin.

of this concept, and is consistent with the fact that the relative effectiveness of deoxyribonucleoside triphosphates in activating the enzyme-catalyzed exchange of tritium between [5′,5′-t_2]-deoxyadenosylcobalamin and water (dGTP > dATP, dCTP; Hogenkamp *et al.*, 1968) parallels the equilibrium concentration of intermediate that is formed in the presence of these nucleotides (Table III). Somewhat similar relative effectiveness was observed for these deoxynucleotides in coenzyme degradation (Hamilton *et al.*, 1971).

The concept of a deoxyadenosyl radical interacting with some other group, such as a thiol, is in general consistent with conclusions made from electron paramagnetic resonance (epr) spectra generated by the ribonucleotide reductase system (Hamilton *et al.*, 1972). These spectra were recorded after much longer times of incubation and after cooling to 77°K, so that close correlation of the data with the stopped-flow results is not possible. Nevertheless the epr results do suggest the generation in the reductase reaction of a paramagnetic system in which there is interaction between two or more components. The epr spectra did not show evidence of the simultaneous formation of cob(II)alamin, but several possible explanations for this have been presented.

The other data bearing on the nature of the rapid reaction are the isotope effects observed when [5′,5′-$^2H_2$]-5′-deoxy-adenosylcobalamin was used in place of the normal coenzyme. For the generation of the intermediate in the presence of dGTP, k_H/k_D was 1.40, and for disappearance after reaching the maximum in the presence of GTP it was 2.22. Since there may have been some exchange of the methylene deuteriums with the solvent within the reaction period (Hogenkamp *et al.*, 1968) these are minimum figures. From these data it seems clear that in the reversal of the cobamide spectrum change by ribonucleotide a primary isotope effect is involved. This provides additional evidence that the ribonucleotide substrate reacts with the intermediate formed from the coenzyme, and suggests that hydrogen is transferred from the intermediate to the 2′ position of the nucleotide. The isotope effect observed in the generation of the intermediate is rather small for a primary effect but large for a secondary isotope effect which is more usually about 1.15 and even when a carbonium ion is formed only reaches 1.29 (Richards, 1970). It may represent a primary isotope effect in a reaction which is only partially rate limiting or becomes so in the case of the deuterated coenzyme,

[3] Yamada, R., Tamao, Y., and Blakley, R. L., unpublished results.

for example, a hydrogen transfer reaction coupled to homolytic cleavage of the carbon–cobalt bond. Alternatively it may be a primary isotope effect considerably decreased by exchange of deuterium into the solvent. Although its significance cannot be interpreted at this juncture it does not seem incompatible with the type of mechanism represented in Scheme I.

Conclusion

The mechanism that is proposed may be summarized as follows. (1) Interaction of coenzyme, enzyme, and dithiol results in homolytic fission of the C–Co bond. Such homolytic cleavage utilizes the unique lability characteristic of the C–Co bond and has been postulated for several other reactions involving deoxyadenosylcobalamin–enzyme complexes (Carty *et al.*, 1971; Cockle *et al.*, 1972; Eagar *et al.*, 1972). Energy for the cleavage is presumably contributed by interaction with enzyme and dithiol and may be assisted by dissociation of the dimethylbenzimidazole base from cobalt. (2) The significance of this reaction is in the production of a stabilized adenosyl radical rather than subsequent reaction of the cobalt with substrate as postulated by other authors (Babior, 1970a; Cockle *et al.*, 1972; Eagar *et al.*, 1972). (3) The stabilized adenosyl radical is considered to be intimately associated with a thiol group and interaction with the latter is considered the mechanism for exchange of hydrogen between the 5′-methylene and water without formation of the unreactive 5′-deoxyadenosine. (4) A slow rearrangement of the radical–thiol system to yield 5′-deoxyadenosine and a sulfur radical is considered responsible for the irreversible degradation of coenzyme. (5) In rionucleotide reduction the deoxyadenosyl radical–thiol system is the immediate reducing agent with the probable involvement of a second thiol group.

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