Electron Paramagnetic Resonance Studies on Cobalamin-Dependent Ribonucleotide Reduction†

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ABSTRACT: When ribonucleotide reductase from *Lactobacillus leichmannii* is incubated with its coenzyme (5′-deoxyadenosylcobalamin), a thiol such as glutathione and certain nucleoside triphosphates, formation of paramagnetic products can be detected by electron paramagnetic resonance (epr) spectroscopy. The X-band epr spectrum is characterized by two symmetrical absorption derivative features with effective g values of 2.032 ± 0.004 and 1.965 ± 0.002 at 77°. The epr signals from the frozen reaction mixture have greatest amplitude when a ribonucleoside triphosphate is present in the reaction mixture together with the deoxyribonucleoside triphosphate which is the specific activator for enzymic reduction of that ribonucleotide. ATP with dGTP, each 5 mM, gave a particularly large signal but an even larger signal was observed when ATP was replaced by its arabino analog. Little or no signal was observed when glutathione was replaced by dithiols like dihydrolipoate which are good substrates for the enzymic reduction of ribonucleotides, but the physiological reductant, thioredoxin and its reducing system, gave a large signal. The epr spectrum was almost unchanged between 8°K and 77°K but the relative saturation behavior of the two lines was considerably altered by this temperature change. As the recording temperature was increased from 77°K to 253°K the spectrum underwent extensive changes, the g values of both peaks changing. When the epr spectrum was recorded at 35 GHz (145°K) the low-field part of the epr spectrum was unchanged but an additional line appeared in the high-field part of the spectrum. Spectral changes in the X band were produced by changing the nucleotide pairs present, but the spectrum was not altered when the reaction was carried out in the presence of D_2O, fully deuterated GTP as ribonucleotide or [5′,5′-$^2H_2$]-deoxyadenosylcobalamin. These results are considered to indicate that the epr spectrum arises from a complex interacting paramagnetic system, closely related to intermediates in the reduction of ribonucleotides.

The 5′-deoxyadenosylcobalamin-dependent ribonucleotide reductase of *Lactobacillus leichmannii* catalyzes reduction of GTP, ATP, CTP, ITP, and UTP by dithiols to the corresponding deoxyribonucleotides (Blakley, 1966b; Vitols *et al.*, 1967). The physiological reducing substrate is thioredoxin, a low molecular weight protein with two cysteine residues, but 1,3-dithiols like dihydrolipoate or 1,4-dithiols like dithiothreitol are convenient artificial hydrogen donors in the enzyme system, whereas monothiols are relatively ineffective (Vitols and Blakley, 1965; Orr and Vitols, 1966; Beck *et al.*, 1966). The reduction of ribonucleotides is specifically activated by appropriate deoxyribonucleoside triphosphates, thus dGTP specifically activates ATP reduction and this activation is considered to occur at an allosteric site or sites (Vitols *et al.*, 1967; Beck, 1967).

In the presence of a modifier (*e.g.*, dGTP) and a dithiol, the enzyme catalyzes rapid hydrogen exchange between water and both protons of the methylene group attached to cobalt in the coenzyme (5′-deoxyadenosylcobalamin) (Hogenkamp *et al.*, 1968). In the same system, but much more slowly (over 1 hr) coenzyme is converted to cob(II)alamin (B_{12r}) and 5′-deoxyadenosine (Hamilton *et al.*, 1971; Yamada *et al.*, 1971). These results have been interpreted as indicating that when dihydrolipoate and coenzyme bind to the active site of enzyme that is maintained in an appropriate conformation by the modifier, they react to form an equilibrium concentration of a reactive cobamide. This cobamide is considered to be an intermediate in both the hydrogen-exchange reaction and ribonucleotide reduction and to slowly decompose with the production of cob(II)alamin and 5′-deoxyadenosine. The intermediate has been represented as closely related to, or identical with, cob(I)alamin plus 5′-deoxyadenosine (Hogenkamp *et al.*, 1968) but all attempts to provide direct evidence for formation of these species have given negative results (Yamada *et al.*, 1971).

In further investigations of the cobamide intermediate we have now employed electron paramagnetic resonance (epr) and have detected paramagnetic species formed under various conditions that appear to be closely related to the postulated intermediate. A preliminary account of this work has been published (Hamilton and Blakley, 1969).

Materials

Most of the materials were the same as those previously described (Hamilton *et al.*, 1971). Deuterium oxide (99.7%) was obtained from the Australian Institute of Nuclear Science and Engineering and from Stohler Isotope Chemicals, Inc. 1,3-Dithiopropan-2-ol was a gift from Dr. E. Vitols and the selenium analog of lipoic acid (Bergson, 1952) was a gift from Dr. Lester J. Reed. The selenolipoate was reduced in the same manner as lipoate (Gunsalus and Razzell, 1957). The arabino analog of ATP (AraATP) and 2′-*O*-MeATP were synthesized

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as described by Follmann and Hogenkamp (1971). Partially purified thioredoxin and thioredoxin reductase from *L. leichmannii* (Orr and Vitols, 1966) were gifts from Dr. M. D. Orr. Ribonucleotide reductase was prepared as described in the preceding paper of this series (Panagou *et al.*, 1972) with omission of preparative electrophoresis except for enzyme analyzed for metals. It was approximately 70% pure as judged by analytical electrophoresis and had a specific activity of 40–130 μmol of ATP reduced per hr per mg of protein under conditions previously defined (Orr *et al.*, 1972).

Deuterated GTP. Freeze-dried deuterated algae (25 g) (Merck, Sharp and Dohme of Canada, Montreal, Canada) were extracted with 20 volumes of 2 M NaCl on a steam bath for 1 hr, the insoluble residue removed by centrifugation, resuspended in a further 10 volumes of 2 M NaCl, and heated for a further 2 hr on the steam bath. After removal of the residue by centrifugation three volumes of absolute ethanol was added to the combined supernatants and the mixture was kept at 4° overnight. The precipitate was recovered by centrifugation, redissolved in 130 ml of 1 M NaCl, and the nucleic acids were reprecipitated with 3 volumes of ethanol. The precipitate was washed five times with 85% ethanol before being dissolved in 40 ml of 0.1 M Tris-acetate (pH 8.8). A small precipitate which formed on standing, was removed by centrifugation, washed in the centrifuge cup with 10 ml of 0.1 M NaCl, and discarded. RNA in the extract was hydrolyzed by the addition of 2.5 ml of 0.3 M magnesium acetate and 10 mg of phosphodiesterase (Worthington Biochemical Corp.) and incubation at 37° with maintenance of the pH at 8.6 by a Radiometer pH-Stat. After 5 hr, when production of acid groups had ceased, the mixture was applied to Dowex 1 Cl^-, 200–400 mesh, for the separation of mononucleotides (Cohn, 1957). The yield of deuterated GMP was 69 μmol. This was converted to the triphosphate by the method of Moffat (1964). The product was shown by thin-layer chromatography to be free of significant amounts of GMP and GDP. Nuclear magnetic resonance in D_2O indicated that the content of nonexchangeable protons in the GTP was negligible.

[5′,5′-$^2H_2$]-5′-Deoxyadenosylcobalamin. This was prepared as follows. 9-(2′,3′-*O*-Isopropylidene-β-D-ribofuranosyluronic acid)adenine prepared by permanganate oxidation of 2′,3′-isopropylideneadenosine according to the method of Harmon *et al.* (1969) was esterified by diazomethane as described by Harper and Hampton (1970). The 9-(2′,3′-*O*-isopropylidene-β-D-riburonic acid methyl ester)adenine (500 mg) dissolved in 150 ml of dry dioxane–tetrahydrofuran (1:1) was added dropwise with stirring to a solution of $LiAl^2H_4$ (230 mg, 99% deuterium, Stohler Isotope Chemicals, Inc.) in 30 ml of dioxane–tetrahydrofuran (1:1) and stirred overnight. The precipitate was filtered off and the filtrate evaporated almost to dryness. The precipitate formed during evaporation was taken up in water and crystallized from water to give white crystalline [5′,5′-$^2H_2$]isopropylideneadenosine (80 mg). [5′,5′-$^2H_2$]-5′-Deoxyadenosylcobalamin was prepared from aquocobalamin and [5′,5′-$^2H_2$]isopropylideneadenosine according to Hogenkamp *et al.* (1968) except that the final purification was carried out by column chromatography on carboxymethylcellulose. The column (1.8 × 30 cm) was adjusted to pH 3 and washed with water before application of the sample and elution of the product with water.

Nucleoside Triphosphate Analogs. Arabinoadenosine obtained from Pfanstiehl Laboratories, and 2′-methyladenosine prepared according to Martin *et al.* (1968) were phosphorylated to their corresponding 5′-monophosphates according to Imai *et al.* (1969). Nucleoside 5′-monophosphates were then phosphorylated to their corresponding 5′-triphosphates according to the method of Hoard and Ott (1965). Phosphonate analogs of ATP and dGTP were obtained from Miles Laboratories, Inc.

Methods

Electron Paramagnetic Resonance Measurements. The preparation of reaction mixtures was carried out by the same general procedure used previously (Hamilton *et al.*, 1971). Humidified nitrogen was bubbled through a solution containing buffer, nucleotide and deoxyadenosylcobalamin in a quartz epr tube for 30 min at 0°. Reductase was then added and the mixture was incubated at 37° for 3 min under a stream of nitrogen. After transferring the tube to an ice bath, the thiol was added and incubation was then commenced at 37° under a stream of water-saturated nitrogen. After incubation, samples were frozen in liquid nitrogen and stored in liquid nitrogen until transfer to the spectrometer cavity. The final volume of the reaction mixture was 0.22 ml. In 35-GHz experiments incubation was carried out similarly in a small test tube and a small portion was then transferred by gas-tight syringe to the epr tube and immediately frozen. For experiments involving several different incubation times a separate epr tube was used for each time interval. When the rate of substrate reduction was measured concurrently with epr measurements, samples (0.1 ml) were removed from the reaction mixture and assayed by the colorimetric procedure (Blakley, 1966a). For experiments involving the thioredoxin system (NADPH, thioredoxin, and thioredoxin reductase) as reductant, NADPH was added after all the other components had been incubated anaerobically for 3 min at 37°. The remainder of the procedure was the same.

X-band epr spectra at liquid nitrogen temperature were recorded under the conditions previously used (Hamilton *et al.*, 1971). For the spectra at 35 GHz a Varian E-15/35 spectrometer was used. X-band spectra at liquid helium temperature were obtained with a modified Varian instrument using 100-KHz modulation (Palmer, 1967).

Reactions in Deuterium Oxide. In preparation for reactions in deuterium oxide, an aqueous solution of sodium dimethylglutarate buffer (pH 7.3), deoxyadenosylcobalamin, dGTP, and ATP in concentrations used in preparing the reaction mixtures was freeze-dried in the dark and the residue was dissolved in D_2O. The solution was freeze-dried and the process was repeated three times, the final residue being dissolved in the calculated amount of D_2O to give appropriate concentrations for preparation of the reaction mixture. Solutions of ribonucleotide reductase and of reduced glutathione were prepared in a similar fashion. These solutions were then used in the usual procedure for reaction and epr measurements except that dry nitrogen was used throughout.

Determination of Metals in the Protein. Electrophoretically pure enzyme (Orr *et al.*, 1972) was dialyzed extensively against EDTA and then against twice-distilled water, and finally freeze-dried. It was analyzed by atomic absorption by Stewart Laboratories, Knoxville, Tenn. A preliminary analysis was kindly performed by Dr. D. J. David, C.S.I.R.O., Canberra, Australia.

Results

Requirements for Appearance of an Electron Paramagnetic Resonance Signal. As previously reported (Hamilton and Blakley, 1969) an epr signal was observed at 77°K in reaction

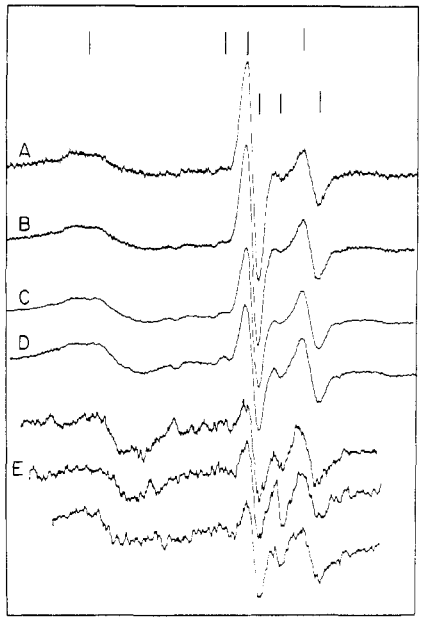

FIGURE 1: X-band epr spectrum at 12°K for the ribonucleotide reductase system. The reaction mixture contained 25 mM glutathione, 5 mM dGTP, 5 mM ATP, 0.2 mM 5′-deoxyadenosylcobalamin, 0.4 mM ribonucleotide reductase, and 0.2 M sodium 3,3-dimethylglutarate buffer (pH 7.3). The mixture was incubated anaerobically in the dark 10 min at 37° before freezing in liquid nitrogen. Conditions of epr spectroscopy were as follows: scanning rate, 200 G min^{-1}; modulation amplitude, 12.5 G; time constant, 0.5 sec; temperature (Tsai *et al.*, 1970), 12°K; microwave frequency, 9.198 GHz. The microwave power and relative amplifier gain for the five spectra were: (A) 2.7 μW and 2.5; (B) 27 μW and 1; (C) 270 μW and 0.625; (D) 2.7 mW and 0.8; (E) 30 mW and 1. The three recordings for condition E permit evaluation of noise contributions. The upper row of markers (for maxima) are at g values 2.303, 2.083, 2.046, and 1.971: the lower row of markers (for minima) are at g values 2.028, 1.999, and 1.949.

mixtures that contained 0.2 mM deoxyadenosylcobalamin, 5 mM dGTP, 5 mM ATP, 0.2 M sodium dimethylglutarate buffer (pH 7.3), 25 mM reduced glutathione, and 0.4 mM ribonucleotide reductase (calculated for molecular weight 76,000, Panagou *et al.*, 1972), and that were frozen after an incubation at 37° for 20 min. The epr spectrum centered on the mean g value of 1.998 ± 0.003 was characterized by a prominent line, symmetrical about the base line with a mean g value of 2.032 ± 0.004. (It should be noted that throughout the paper the g values quoted are for g effective rather than values of a g tensor.) A second smaller and broader line, also symmetrical about the base line has a g value of 1.965 ± 0.002. The mean line widths were 30 ± 1 and 37 ± 1 G, respectively, with a mean separation between absorption maxima of 110 ± 5 G. These two lines are referred to in the following as the doublet spectrum. In addition to the doublet, a signal of variable size relative to the doublet appeared downfield. This is presumably due to the low-field signal from enzyme-bound cob(II)alamin (B_{12r}). The latter is formed by irreversible degradation of deoxyadenosylcobalamin by the enzyme system, as previously described (Hamilton *et al.*, 1971). In many cases this cob(II)alamin signal was not sufficiently large for the hyperfine structure characteristic of the enzyme-bound cob(II)alamin to be visible, and even in some cases where it was relatively large it was broad and showed little fine structure (Figure 1). However, in other cases, especially when incubations were continued for a longer period the signal became very similar to that of enzyme-bound cob(II)alamin.

TABLE I: Requirements for Appearance of the Doublet Electron Paramagnetic Resonance Spectrum.[a]

Reaction System	Signal Amplitude (cm)
Complete system	16.0
Omissions	
Enzyme	<0.2
dA-cobalamin	<0.2
dGTP	<0.2
ATP	<0.2
Reduced glutathione	<0.2

[a] The composition of the complete system was: 0.4 mM protein, 0.20 mM deoxyadenosylcobalamin, 0.2 M dimethylglutarate (pH 7.3), 5 mM dGTP, 5 mM ATP, and 25 mM reduced glutathione. The reaction mixture was prepared and incubated anaerobically in the dark for 20 mins at 37° as described in the Methods section. Electron paramagnetic resonance recording conditions were as in Figure 2. The signal amplitude refers to the maximum to minimum height in centimeters of the g = 2.032 line (low-field doublet peak) recorded at maximum gain. The minimum signal amplitude which could be detected was 0.2 cm.

Electron paramagnetic resonance spectra with a better signal to noise ratio but with essentially the same shape were obtained at 12°K and 9.198 GHz (Figure 1), and in this case the effective g values were 2.036 and 1.960 for the two lines of the doublet, respectively.

As shown in Table I the enzyme, cobamide, dGTP, ATP, and reduced glutathione all appeared to be essential for appearance of the paramagnetic species. This is in contrast to the formation of cob(II)alamin which was formed with either nucleotide alone. No detailed experiments have been performed on the effect of oxygen on the formation of the paramagnetic species, but it was found that after preparation and incubation of the standard reaction mixture under air the doublet amplitude was not significantly diminished. Signal amplitudes were measured in reaction mixtures containing various concentrations of dGTP, and the data were analyzed by a program for weighted fit to a hyperbola (Wilkinson, 1961). A good fit was found to a hyperbolic relation between signal amplitude and dGTP concentration with half-maximum signal amplitude at a concentration of 0.14 ± 0.3 mM (±SE). In this and all other experiments where signal amplitudes were used as a measure of the concentration of the paramagnetic species the signal width did not alter significantly as the amplitude changed. This was checked in some cases by double integrations, and comparison of the double integral ratios to the signal amplitude ratios. The only exceptions were in the case of certain nucleotide combinations and of temperatures above 77°K, as described later.

Time Course of Doublet Signal Formation. Figure 2 shows the changes in doublet signal height with the period of incubation at 37° prior to freezing. As seen in Figure 2a the signal formed in reaction mixtures containing dGTP, ATP, and glutathione reached a maximum after 20 min at 37° and then slowly declined, whereas the formation of cob(II)alamin steadily progressed and by 60 min much of the cobamide was degraded to this product. During the course of these changes the doublet spectrum retains the characteristics shown in

TABLE II: Effect of Different Thiols on Doublet Signal Formation.[a]

Thiol	Cob(II)alamin Signal Amplitude (cm)	Doublet Signal Amplitude (cm)
Reduced glutathione (25 mM)	8.0	21
1,4-Dithiothreitol (1 mM)	4.0	6.5
1,4-Dithioerythritol (1 mM)	2.7	4.6
1,3-Dithiopropan-2-ol (1 mM)	2.7	2.6
L-Cysteine (25 mM)	2.8	8.3
Thioredoxin (0.3 nmol), thioredoxin reductase (10 μg), and NADPH (2.5 mM)	8.5	42.4

[a] The reaction mixture was the complete system of Table I except that the thiol was as indicated. Other details were the same as for Table I except that the incubation time was 10 min. The lines are those at $g = 2.25$ (low-field cobalamin signal) and at $g = 2.032$ (low-field doublet line).

Figure 1. Figure 2b,c indicates that if either ATP or dGTP are omitted, little signal is obtained at all time intervals from 1 to 60 min. Figure 2d,e shows that the combination dGTP, CTP was ineffective for doublet production but dATP, CTP gave significant signal with a maximum after 5 min at 37°.

Dependence of Doublet Signal on Thiol. As shown in Table II the amplitude of the doublet signal varied greatly with the nature of thiol present in the reaction mixture. Whereas 1,3- and 1,4-dithiols, which are good substrates for ribonucleotide reduction, are relatively ineffective for formation of the paramagnetic species, glutathione a very poor reducing substrate gives a large doublet signal. However, cysteine, a poor reducing substrate, was much less effective than glutathione and the thioredoxin system isolated from *L. leichmannii* which is the physiological reductant for ribonucleotides, gave a very large doublet signal. In the latter case signal production was faster, reaching a maximum at about 10 min and thereafter declining (Figure 3a). When the thioredoxin system from *Escherichia coli* was used, a large signal was also produced but not as great as found with the *L. leichmannii* thioredoxin system.

When 25 mM dihydrolipoate was used as thiol little doublet signal could be detected after incubation for periods of 2–60 min (Figure 3b). In this system degradation of coenzyme to cob(II)alamin is very rapid and might account for the nonappearance of the paramagnetic species. With 1 mM dihydrolipoate coenzyme destruction was still very rapid (Figure 3c), but a much larger doublet signal was produced.

To determine whether dihydrolipoate inhibited the formation of the paramagnetic species, or could cause the removal of the species once it had been formed, a reaction mixture containing 25 mM reduced glutathione (like that described for Figure 1) was incubated for 20 min at 37°. A portion of the reaction mixture frozen at this point showed the doublet epr spectrum. To another portion of the reaction mixture dihydrolipoate was added to give a final concentration of 25 mM, and incubation at 37° was continued for 5 min. After freezing this reaction mixture now showed no doublet spectrum. In a control experiment, addition of deoxygenated distilled water instead of dihydrolipoate solution to a duplicate portion of the reaction mixture caused no disappearance of the doublet spectrum.

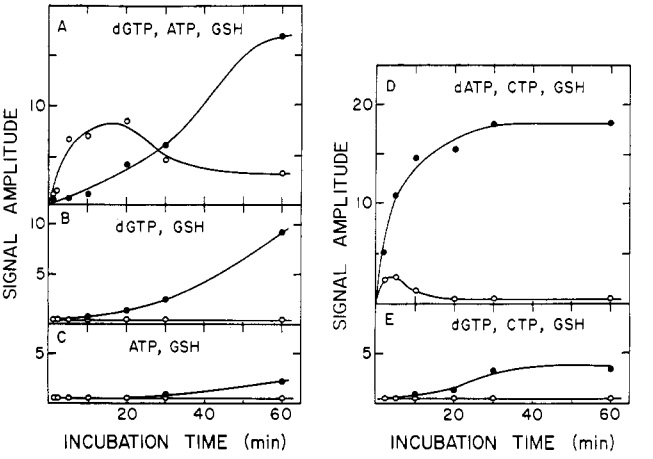

FIGURE 2: Effect of nucleotides on the amplitude of the epr signal produced by the ribonucleotide reductase system. The reaction mixtures were as in Figure 1 except for variations in the nucleotides which were present at 5 mM concentration. The incubations were at 37° and anaerobic. X-band spectra were recorded at 77°K under the following conditions: microwave frequency, 9.06 GHz; modulation frequency, 100 kHz; modulation level, 3.78 G peak to peak; microwave power, 25 mW; time constant, 1 sec; scanning rate, 100 G min^{-1}. Signal amplitude is the maximum to minimum height in centimeters of the respective lines at maximum recorder gain: open circles, line at $g = 2.032$ (low-field doublet line); closed circles, line at $g = 2.25$ (low-field cob(II)alamin signal).

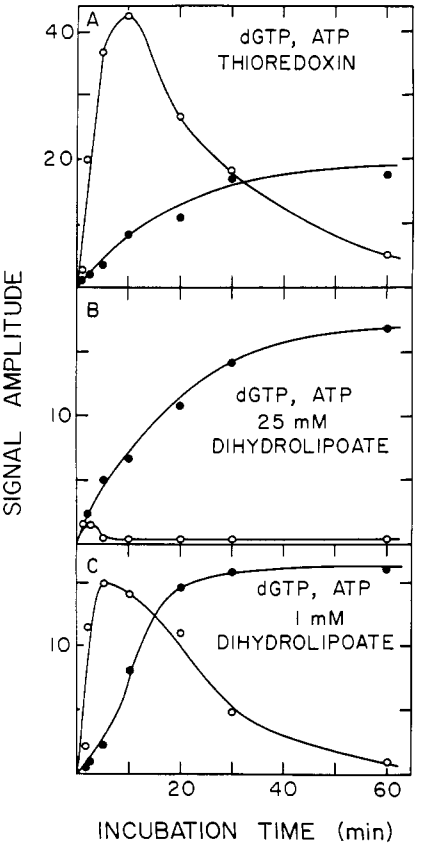

FIGURE 3: Epr spectra of the ribonucleotide reductase system with thioredoxin or dihydrolipoate as reducing substrate. The reaction mixtures were the same as in Figure 1 except that glutathione was replaced by dihydrolipoate at the concentrations shown or by the thioredoxin system which consisted of 10 μg of thioredoxin reductase, 0.3 nmol of thioredoxin, and 2.5 mM NADPH. Recording conditions and notation as in Figure 2. Open circles, low-field doublet line; closed circles, low-field cob(II)alamin line.

TABLE III: Doublet Signal Obtained in the Presence of Various Nucleotides.[a]

Substrate	ATP		CTP		GTP		ITP		UTP	
	Cob(II)-alamin	Doublet	Cob(II)-alamin	Doublet	Cob(II)-alamin	Doublet	Cob(II)-alamin	Doublet	Cob(II)-alamin	Doublet
Activator										
dATP	0.4	<0.2	*12.0*	*0.5*	3.9	<0.2	0.8	0.5	3.2	<0.2
dCTP	8.3	<0.2	6.2	<0.2	7.0	0.4	3.6	<0.2	*2.8*	*5.4*
dGTP	*8.0*	*13.0*	3.5	<0.2	1.0	1.2	1.4	3.0	5.6	<0.2
dTTP	0.7	<0.2	6.9	<0.2	12.2	1.6	*10.1*	*7.6*	1.4	<0.2
None	0.2	<0.2	5.9	<0.2	*2.6*	*3.4*	0.2	0.5	1.3	<0.2

[a] The reaction mixtures were the same as the complete system of Table I except for nucleotides. Values are signal amplitudes in cms and the lines are as in Table I. The values in italics are for nucleotide combinations giving maximum rates of ribonucleotide reduction.

Temporal Relation between Appearance of Doublet Signal and Substrate Reduction. Since the failure of the signal to appear in the presence of 25 mM dihydrolipoate is not easily explained solely in terms of coenzyme degradation, the possibility was examined that nonappearance or disappearance of signal is due to rapid conversion of ATP to dATP under these conditions. In the presence of 25 mM dihydrolipoate, 5 mM ATP was completely reduced to dATP within 5 min, so that the small, short-lived signal seen under these conditions (Figure 3b) might be due to rapid exhaustion of ribonucleotide necessary for the appearance of the signal (Table I).

On the other hand, disappearance of doublet signal under other conditions was not primarily due to ribonucleotide exhaustion. Thus when GTP or ATP was reduced by glutathione, mercaptoethanol or thioredoxin the doublet signal began to decrease when only 20–30% of the ribonucleotide had been reduced (Figure 4). Under conditions of slow reduction of the ribonucleotides, decrease in the doublet signal was, in most cases, correlated quite well with extensive degradation of the coenzyme (Figures 2, 3a,b, and 4). However, in some cases where doublet decreased sooner than anticipated from either the extent of substrate reduction or coenzyme degradation (Figures 4b,c) denaturation of enzyme may have also contributed to decrease in concentration of the paramagnetic species.

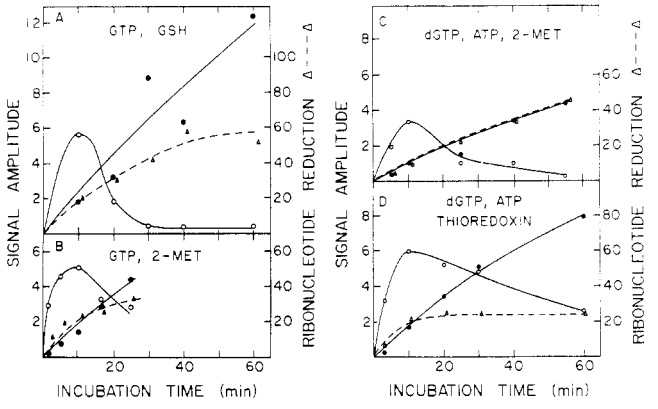

FIGURE 4: Time course of doublet epr signal appearance and of ribonucleotide reduction. Reaction mixtures all contained 0.4 mM ribonucleotide reductase and 0.2 M dimethylglutarate buffer (pH 7.3). Other components were as follows: (A) 0.3 mM deoxyadenosylcobalamin, 5 mM dGTP, and 25 mM glutathione; (B) 0.3 mM deoxyadenosylcobalamin, 5 mM GTP, and 25 mM 2-mercaptoethanol; (C) 0.3 mM deoxyadenosylcobalamin, 0.2 mM dGTP, 5 mM ATP, and 25 mM 2-mercaptoethanol; (D) 0.2 mM deoxyadenosylcobalamin, 0.2 mM dGTP, 5 mM ATP, 20 μg of thioredoxin reductase, 0.6 nmol of thioredoxin, and 2.5 mM NADPH. Other experimental conditions, epr spectroscopy and notation as for Figure 2. The broken line indicates per cent reduction of the ribonucleotide present.

Role of Ribonucleotide in Formation of Paramagnetic Species. Data presented above demonstrate that a ribonucleotide appears to be necessary for appearance of the doublet spectrum (Table I) and that a specific modifier accelerating reduction of the ribonucleotide greatly increases the doublet signal. Thus dGTP increases doublet signal in presence of ATP for which it is a reduction activator, but does not increase doublet production from CTP, reduction of which is unaffected by dGTP (Figure 2). dATP, the specific activator of CTP reduction, enhances the doublet signal produced in presence of CTP. GTP which does not require an activator for reduction gives a relatively good doublet signal in absence of modifiers (Figure 4).

These results have been extended to all the combinations of readily accessible deoxyribonucleoside and ribonucleoside triphosphates with the results shown in Table III, and it is evident that the specific activator for reduction of a given ribonucleotide always produces the maximum doublet signal formed in presence of that ribonucleotide. On the other hand degradation to cob(II)alamin, while occurring rapidly under conditions producing maximum doublet signal sometimes occurs even more rapidly with other combinations of nucleotides. A combination of two deoxyribonucleotides (*e.g.*, dATP and dGTP) gave no doublet signal, although considerable cob(II)alamin formation occurred.

A possible explanation for the appearance of strong doublet signal when a ribonucleotide and the appropriate activator are both present is that a strong doublet signal is produced only when rapid ribonucleotide reduction is occurring. This is not completely consistent with Table III since GTP, the substrate reduced at the maximum rate gives a much smaller doublet signal than either ATP (with dGTP) or ITP (with dTTP). However, there was some variation in signal amplitude from one experiment to another, perhaps due to variations in the state of the enzyme or other experimental details, so that this inconsistency alone is inconclusive. The effects of nucleotide analogs on doublet signal production were therefore investigated.

TABLE IV: Doublet Signals in the Presence of Nucleotide Analogs.[a]

Nucleotides	Cob(II)alamin Signal Amplitude (cm)	Doublet Peak Signal Amplitude (cm)
dGTP, AOPOPCP	0.6	<0.2
dGTP, AOPCPOP	1.5	<0.2
dGTP, AMP	4.8	<0.2
dGTP, ADP[b]	5.3	<0.2
dGOPOPCP, ATP	4.0	14.9
dGTP, ATP	7.9	10.7

[a] The reaction mixtures were the same as the complete system of Table I except for nucleotides which were present at a concentration of 5 mM. Other details as in Table I. [b] Also showed no doublet signal at incubation times of 5, 15, and 25 min.

Data in Table IV indicate that whereas a strong doublet signal was observed with ATP and either dGTP or a phosphonate analog of dGTP, combinations of dGTP with AMP, ADP, phosphonate analogs of ATP or 2′-*O*-MeATP gave no doublet signal. In these cases doublet signal appears only in the cases where ribonucleotide reduction occurs. However, in the case of AraATP a most important exception to this parallelism occurs, for although this analog is not reduced by the reductase (Follmann and Hogenkamp, 1971), it gives an even larger doublet signal than ATP (Table V). The possibility was investigated that some reduction of AraATP might occur in the presence of the substrate amounts of enzyme used in these experiments. No reduction of AraATP could be detected under similar conditions to those of Table V when the thioredoxin system was present, but under the same conditions rapid reduction of ATP was readily demonstrated. This clearly demonstrates that it is not necessary to have reduction of the pentose ring occurring for the doublet signal to be formed.

Whereas there is negligible doublet signal with ATP as the only nucleotide (Table III), AraATP alone does give a small signal which is greatly increased in the presence of dGTP but not in the presence of other deoxyribonucleotides (Table V).

It may be noted in Table V that negligible doublet signal was formed with dGTP and AraATP as nucleotides in the presence of 25 mM dihydrolipoate although a significant signal was formed in the presence of 1 mM dihydrolipoate. Absence of signal in the presence of 25 mM dihydrolipoate was partly due to extensive degradation of coenzyme that occurred in the 20-min incubation period, but even at shorter time intervals when degradation of deoxyadenosylcobalamin was less extensive the doublet signal was still small (Table VI).

The general shape and characteristics of the epr signal obtained with dGTP and AraATP were the same as those obtained with dGTP and ATP (Figure 5), but with other pairs of nucleotides there were significant differences. The recording of spectra were difficult in these cases because of the small signal obtained but from Figure 5 it is clear that the combinations dATP plus CTP or dCTP plus UTP give a different epr spectrum with the lines closer together and overlapping. The ratio of the amplitudes of the two lines also varied from 2 to almost 1. Several of the smaller lines in spectrum A are discernible in other spectra and are probably due to traces of adventitious Mn^{2+}, while others may be due to the relatively large cob(II)alamin signal.

TABLE V: Doublet Signal in the Presence of AraATP.[a]

Nucleotides	Thiol	Cob(II)-alamin Signal Amplitude (cm)	Doublet Signal Amplitude (cm)
dGTP, ATP	25 mM Glutathione	6.7	9.7
dGTP, AraATP	25 mM Glutathione	7.4	21.0
dATP, AraATP	25 mM Glutathione	1.0	0
dCTP, AraATP	25 mM Glutathione	6.7	6.5
dTTP, AraATP	25 mM Glutathione	1.7	0
AraATP	25 mM Glutathione	2.9	2.5
dGTP	25 mM Glutathione	1.6	0
dGTP, AraATP	25 mM Dihydrolipoate	18.0	0
AraATP	25 mM Dihydrolipoate	18.0	0
dGTP	25 mM Dihydrolipoate	12.1	0
dGTP, AraATP	1 mM Dihydrolipoate	15.1	13.0
AraATP	1 mM Dihydrolipoate	15.5	0
dGTP	1 mM Dihydrolipoate	9.1	0

[a] The reaction mixtures were the same as the complete system of Table I except as indicated. Nucleotides were present at a concentration of 5 mM. Other details as in Table I.

Characteristics of the Doublet Electron Paramagnetic Resonance Signal. The general shape of the signal obtained with dGTP and ATP or AraATP has been described above. No additional fine structure was observed for either line at 12°K or 77°K at X-band frequency. As the recording temperature is raised from 77°K to 253°K the amplitude of the signal decreases, the signal shape alters and the separation between the lines descreases significantly due to movement of both lines (Figure 6). The decreased signal amplitude at these higher temperatures makes interpretation of these changes difficult.

When the first derivative amplitudes, calculated from the Gaussian and Lorentzian line-shape equations described by Poole (1967a), were plotted on the experimental first derivative

TABLE VI: Doublet Signal in the Presence of AraATP and 25 mM Dihydrolipoate.[a]

Incubn Time (min)	Cob(II)alamin Signal Amplitude (cm)	Doublet Signal Amplitude (cm)
0.5	1.9	1.5
2.0	7.6	4.4
5.0	13.1	4.8
10.0	14.9	5.3

[a] The reaction mixture was the same as in Table I except that AraATP replaced ATP, dihydrolipoate replaced reduced glutathione, and the time of incubation at 37° was as shown. Other details as in Table I.

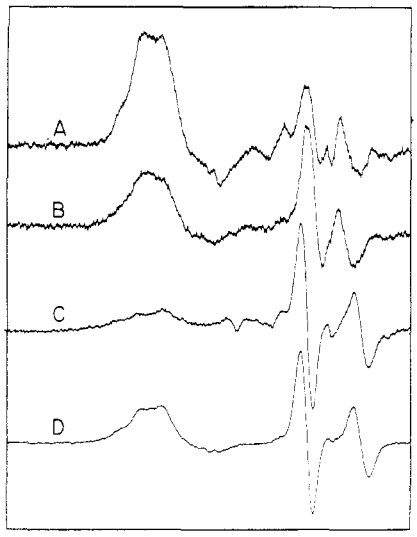

FIGURE 5: Effect of nucleotides in the reaction mixture on the epr signal shape. The reaction mixture was the same as in Figure 1 except that the nucleotides (present at 5 mM concentration) were as follows: (A) dATP and CTP; (B) dCTP and UTP; (C) dGTP and ATP; (D) dGTP and AraATP. The time of anaerobic incubation at 37° was: (A) 5 min, (B) 15 min, (C) 10 min, and (D) 20 min. Epr recording conditions were as used for Figure 1.

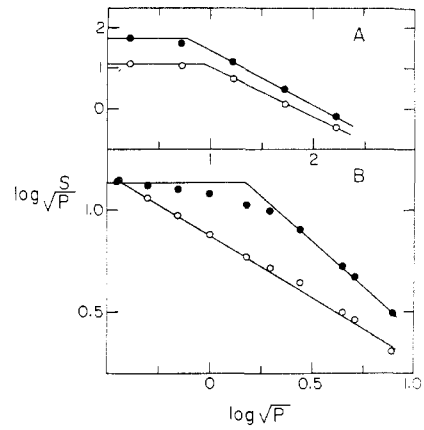

FIGURE 7: Epr doublet signal saturation. The ordinate shows the logarithm of maximum to minimum signal amplitude (S) after correction to the same recorder gain, and division by the root of the microwave power (P, expressed in μW in A and mW in B). The abscissa shows the logarithm of the root of the microwave power. (A) Data from Figure 1. (B) Data obtained under the recording conditions used in Figure 2 (except for microwave power) on a reaction carried out as in Figure 1. Closed circles, line with $g = 2.032$; open circles, line with $g = 1.965$.

X-band curves obtained at 77°K, the close agreement for both peaks with the Gaussian curves indicated that the line shape was definitely Gaussian under these recording conditions. Double integration was then carried out with the use of the equations for Gaussian line shape (Poole, 1967b), and the assumption that a paramagnetic species containing a single unpaired electron produced the signal. Copper sulfate was used as an intensity signal. For maximum recorder gain and a recording speed of 100 G/min, a signal amplitude of 20 cm for the line centered on $g = 2.032$ corresponded to an approximate concentration of 6 μM for the paramagnetic species.

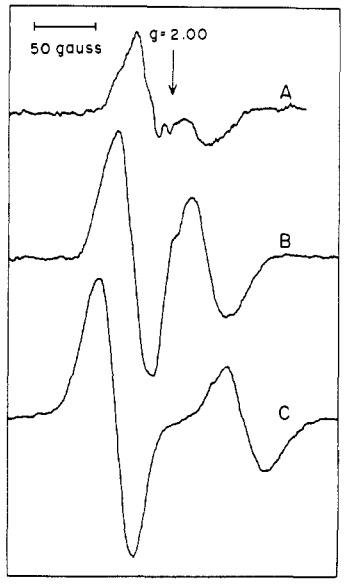

FIGURE 6: Effect of increasing temperature on the X-band epr signal. The reaction mixture and conditions were the same as in Figure 1, except that the incubation time was 20 min. Recording conditions were as in Figure 2, except that the temperature was as follows: (A) 253°K, (B) 188°K, and (C) 77°K.

This line had approximately twice the area of the line centered on $g = 1.965$ at 77°K and X-band.

The saturation behavior of the epr spectrum at 12°K is shown in Figure 1. Saturation of the low-field line was more marked than that of the high-field line as the incident microwave power was increased from 2.7 μW to 27 mW. The difference in saturation is seen more clearly in Figure 7A where the measured signal amplitude was divided by the gain to give the effective signal amplitude (S) and this was divided by the square root of the microwave power, $(P)^{1/2}$. Log $[S/(P)^{1/2}]$ was then plotted against log $(P)^{1/2}$ (Beinert and Orme-Johnson, 1967), to give $P_{1/2}$ the power at half-saturation where the break in the line occurred. $P_{1/2}$ for the line with $g = 2.036$ was approximately 23 μW and for the line with $g = 1.960$, 69 μW. At 77°K the saturation behavior of the two lines was considerably altered (Figure 7b) the line with $g = 2.032$ now having $P_{1/2}$ of about 2.5 mW while that with $g = 1.965$ had $P_{1/2}$ below the accessible range (<0.2 mW).

The slopes of the curves were then calculated in the saturation region ($P > P_{1/2}$) and compared to the theoretical values of the slopes for the limiting cases of inhomogeneously broadened (IHB) and homogeneously broadened (HB) lines. The theoretical slopes were calculated using the epr saturation theories developed by Castner (1959) and Portis (1953) and parameterized to fit the "Vänngård plot" described by Beinert and Orme-Johnson (see Figure 1). The results are: $\Delta \log (S/(P)^{1/2})/\Delta \log (P)^{1/2} = -1$ or -4 for IHB or HB lines, respectively. The 12°K saturation data are in very good agreement with IHB giving slope values of -1.2 and -1.3, respectively, for the $g = 1.960$ and 2.036 lines, respectively. The 77°K saturation data are in poorer agreement with the IHB theoretical slope, but are still farther from the HB expected values.

The doublet spectrum at 12°K is therefore well described as an inhomogeneously broadened spectrum, as indicated by the similar saturation behavior (consistent with IHB) and similar values of $P_{1/2}$ for each of the components of the doublet. This implies that the spin packets comprising the IHB line shape each have similar relaxation rates (T_1T_2) since $P_{1/2} = (4\pi\gamma_e{}^2 \cdot T_1T_2)^{-1}$.

The simple model of inhomogeneous broadening (*e.g.*, each

epr "line" is the envelope of a single distribution function for spin packets) fails at 77°K and higher temperature. Not only do the slopes of the saturation curve deviate from the IHB value, but the vastly different values of $P_{1/2}$ at 77°K show that the $g = 1.960$ line relaxes much faster than the other line. This fact, in addition to the tendency for the doublet spectrum to coalesce near 253°K (Figure 6) shows that the higher temperature spectrum has some of the properties of a multiple site system with an increasing jumping rate giving rise to both relaxation anomalies and line-shape coalescence. A multiple site interpretation is also consistent with the observation that the relative heights of the two components of the 77°K, X-band doublet are seen to vary from preparation to preparation, as well as with the extra structure resolved in the 35-GHz spectrum at 77°K (Figure 8).

The 35-GHz epr spectrum obtained at about 1.5°K with dGTP and AraATP as nucleotides is shown in Figure 8. Essentially the same spectrum was obtained with a reaction mixture containing ATP instead of AraATP, incubated for 10 min at 37° before freezing. The most obvious difference in the spectrum is seen in the high-field region, where as compared to the X-band spectrum an additional small line has appeared in the trough at about $g = 1.992$.

The comparison of the 35- and 9-GHz spectra shows conclusively that the doublet splitting is of hyperfine origin since the splitting between the components of the doublet is found to be frequency independent. It follows that the average value of the g tensor is approximately equal to the average value of the g_{eff} found for each line of the doublet.

Examination of the Reductase for Metals. An epr signal of the type observed might conceivably arise from a metal ion tightly bound to the protein, and the necessity for a thiol to be present for appearance of the doublet signal might then be interpreted as due to reduction of the metal ion to a paramagnetic species. However, atomic absorption spectrum indicated that only trace amounts of metals were present. Actual estimates in terms of μg per g of protein were as follows: Cu, 2.5; Co, 0.2; Fe, 2.1; Mn, 0.3; Mo, <0.04; Zn, 7.3; Ca, 20; Mg, 22. The only elements found in the protein ash in significant amounts were Al, 360; Si, 220; and Na, 545.

Electron Paramagnetic Resonance Signal in Presence of D_2O, Deuterated GTP, Deuterated Coenzyme, and Reduced Selenolipoate. One possible explanation for a signal of the doublet type is that a signal resonance centered on $g = 2$ has been split by hyperfine interaction with a nucleus of $I = {}^1/_2$, such as a proton. In order to determine whether hyperfine splitting was due to any proton readily exchanging with water, exchangeable protons of the enzyme and substrate were replaced by deuterium and the incubation of the resulting materials was carried out in deuterium oxide. Apart from the replacement of water with D_2O as described in the Methods section, the reaction was carried out as described in Figure 1. Electron paramagnetic resonance spectroscopy was performed as described in Figure 2 and the signal observed was identical with that shown in Figure 6C. In other experiments the standard conditions were used except that deuterated GTP or [5′,5′-$^2H_2$]deoxyadenosylcobalamin was used. In both cases the doublet spectrum was the same as that obtained with the usual components.

In other experiments the reaction was carried out with 1 mM reduced selenolipoate as reductant instead of glutathione. In the presence of 1 mM reduced selenolipoate the rate of ATP reduction was about 3% of the rate in presence of 1 mM dihydrolipoate under standard conditions. The epr spectrum observed on freezing an incubation mixture containing 5 mM dGTP, 5 mM ATP, 0.2 mM deoxyadenosylcobalamin, 0.6 mM reductase, and 1 mM reduced selenolipoate did not differ significantly from that obtained with 1 mM dihydrolipoate or other thiols.

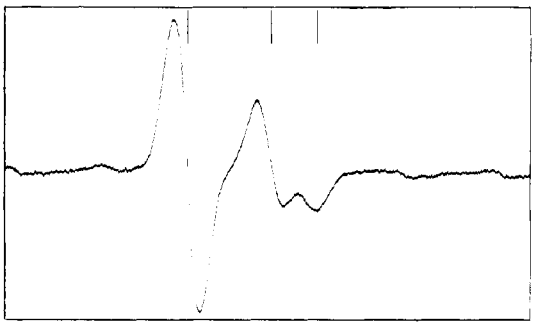

FIGURE 8: 35-GHz epr spectrum for the ribonucleotide system. The reaction mixture and incubation conditions were the same as for Figure 1 except the 5 mM AraATP replaced ATP and incubation was for 20 min. Conditions of epr spectroscopy were as follows: scanning rate, 200 G min^{-1} over a range of 1000 G; modulation amplitude, 16 G; time constant, 0.3 sec; microwave frequency 35.166 GHz; microwave power 0.5 mW. The temperature was approximately 145°K. The three markers indicate g values of 2.012, 1.998, and 1.992.

Discussion

Paramagnetic Material as Intermediate. Evidence suggesting that the paramagnetic species may be an intermediate of ribonucleotide reduction is as follows. (1) The paramagnetic signal appears only in the presence of the complete ribonucleotide reductase system: enzyme, coenzyme, thiol, ribonucleoside triphosphate (or the arabino analog), and appropriate allosteric activator. (2) Under most conditions the rate of appearance of the doublet epr signal is much more rapid than the degradative conversion of coenzyme to 5′-deoxyadenosine and cob(II)alamin, so that the species does not appear to be associated with the latter process. (3) The doublet signal appears in the presence of the highly specific thioredoxin system. Further, an epr signal very similar to the doublet has been observed by Cockle *et al.* (1972) when glycerol dehydrase is incubated with deoxyadenosylcobalamin and propane-1,2-diol and the reaction mixture then frozen and examined at 115°K. The species is therefore not formed through adventitious reaction of the substrates with the cobamide.

A difficulty for identification of the doublet species as an intermediate in ribonucleotide reduction is that the rate of its appearance is very slow compared to ribonucleotide reduction, reaching a maximum in 5–20 min. However, the observed rate of accumulation of the species may be the difference between much faster rates of formation and disappearance. Similar considerations may account for the low concentrations of the paramagnetic species accumulated (up to 20 μM, and more frequently in the range 1–5 μM). However, it would not be surprising if the true equilibrium concentration of a reactive intermediate were low, even under the most favorable conditions, since formation of such an intermediate from the relatively stable coenzyme is probably an endergonic reaction. A further very important consideration is that the epr signals were examined with frozen reaction mixtures at low temperatures and cooling the reaction mixture from 37 to 0°, freezing and cooling further to 77°K or below may have significantly altered the concentrations of intermediates.

Effect of Thiols and Nucleotides on the Paramagnetic Material. The most striking feature of the effect of thiols is that the 1,3- and 1,4-dithiols that are effective reducing substrates for ribonucleotide reduction gave weak doublet signals at 1 mM concentration and none at higher concentrations, whereas very poor reducing substrates like glutathione gave a strong epr signal. The inefficiency of dihydrolipoate and other dithiols in promoting formation of the doublet signal may be due in part to promotion of faster exhaustion of ribonucleotide substrate and faster degradation of coenzyme, but the data in Table II do not seem explicable solely in these terms. Thus dithiothreitol or dithioerythritol gave small doublet peaks even when cob(II)alamin formation was less than in the presence of glutathione or thioredoxin and when ATP was present at five times the concentration of the thiol. Further, dGTP plus AraATP gave a much smaller doublet signal with 25 mM dihydrolipoate than with 25 mM glutathione, even at time intervals too short for coenzyme degradation to have become extensive. The explanation of these data must await further information, but it seems probable that the 1,3- and 1,4-dithiols participate in two reactions, one leading to formation of the species with the doublet spectrum and others causing its disappearance.

There is a remarkable increase in the paramagnetic species when a ribonucleotide and the allosteric activator for reduction of that ribonucleotide are both present. This is not due to the rapid rate of ribonucleotide reduction occurring under these conditions, since dGTP plus AraATP (which is not enzymically reduced) also gives a strong doublet signal. We have previously obtained evidence that ribonucleoside and deoxyribonucleoside triphosphates determine specific conformations of the enzyme–cobamide complex (Hamilton *et al.*, 1971), which are correlated with changes in parameters (A_Z and G_Z) associated with the axial coordination positions of cobalt (Pilbrow and Winfield, 1972). Appropriate pairs of nucleotides (or GTP alone) may therefore specify more rigidly those conformations of the enzyme–coenzyme complex that are particulary efficient both for reduction of the ribonucleotide and also for formation of the doublet species. These parallel effects also suggest that the paramagnetic species can be identified with, or is formed from, an intermediate in the reductive process. It seems likely that changes in the epr spectrum with the nucleotide pair present (Figure 6) also reflect conformation changes of the enzyme–cobalamin complex.

Molecular Structure of the Paramagnetic Species. The observation of a similar epr signal in the deoxyadenosylcobalamin-dependent glycerol dehydrase system (Cockle *et al.*, 1972) and in the ethanolamine deaminase system (Babior *et al.*, 1972) suggests that in the case of all three enzymes the signal arises primarily from paramagnetic species produced from the coenzyme. Cockle *et al.* (1972) suggested that initially cob(II)alamin and a deoxyadenosyl radical are formed by homolytic cleavage of the carbon–cobalt bond of the coenzyme, although they postulated that in the case of the dehydrase other organic radicals might be formed subsequently. Homolytic cleavage of this bond has also been postulated for ethanolamine deaminase (Carty *et al.*, 1971) and glutamate mutase (Eagar *et al.*, 1972), but the source of the energy for such homolytic cleavage has not been clarified in any of these cases. In the case of ribonucleotide reductase a mechanism involving fission of this bond would be consistent with the exchange of the 5′-methylene protons with water in the presence of enzyme, dihydrolipoate, and dGTP and with the ultimate degradation of the coenzyme to cob(II)alamin and 5′-deoxyadenosine in the presence of the same reactants.

No part of the epr spectrum is due to transition metals present in the enzyme, since the latter contained only trace amounts of the likely metals.

If the production of paramagnetic species in the ribonucleotide reductase system does result from homolytic fission of the cobalt–carbon bond in the coenzyme, then two superimposed signals should be observed, one corresponding to cob(II)alamin and the other to a deoxyadenosyl radical. The characteristic epr spectrum of reductase-bound cob(II)alamin is well known (Hamilton *et al.*, 1971), but although it frequently was found to accompany the doublet spectrum, a study of the course of the rise and fall of the two signals (Figures 2–4) clealy indicated that most, if not all, the cob(II)alamin is formed by a different process to that producing the doublet. Further, under suitable conditions the doublet signal could be obtained with little if any cob(II)alamin signal present. In the epr spectrum obtained with glycerol dehydrase by Cockle *et al.* (1972) the cob(II)alamin signal is also very small. It is clear that in the ribonucleotide reductase system cob(II)alamin arises mainly as a product of the known irreversible cleavage of the coenzyme with 5′-deoxyadenosine as the other product (Hamilton *et al.*, 1971; Yamada *et al.*, 1971), but the possibility cannot be entirely excluded that it is formed stoichiometrically with the doublet species.

If C–Co homolytic fission is assumed, this apparent absence of the cob(II)alamin spectrum could be explained in either of two ways. The cob(II)alamin may react with substrate (or enzyme) to yield a radical and a cobalamin containing Co^{3+}, or the Co^{2+} may exist in such an altered environment that the epr spectrum is greatly modified. As regards the first possibility, reaction of cob(II)alamin with the substrate to form an alkylcobalamin and an organic radical has been postulated for the glycerol dehydrase reaction (Cockle *et al.*, 1972) and the ethanolamine deaminase reaction (Babior, 1970) but in the case of the ribonucleotide reductase steric hindrance would make it impossible for cobalt to react with the ribose ring of the nucleotide substrate. Moreover the doublet is produced with the nonreacting AraATP present instead of substrate. Reaction with the thiol substrate is sterically possible, but would produce a short-lived hydrogen atom. While reaction of cob(II)alamin with a disulfide would produce a more stable sulfur radical, no substrate or protein disulfide is known to be involved in the reaction and the observed epr spectrum does not resemble published spectra of sulfur radicals (Ayscough, 1967). To turn to the alternative explanation, divalent cobalt in an environment considerably different from cob(II)alamin, two possibilities must be considered: the presence of high-spin divalent cobalt; or cob(II)alamin, perhaps base-off, with one or both axial positions occupied by sulfur ligands. In connection with the latter McGarvey (1967) shows A_{Co} values of the order of 5×10^{-4} cm^{-1} for Co^{2+} in a host lattice of ZnS or CdS. A hyperfine coupling constant of this order would probably not permit resolution of hyperfine structure with the line width of 37 G observed and would therefore account for the absence of the expected hyperfine structure in the observed signal, but it is uncertain whether the environment provided by the enzyme could decrease A_{Co} to this value from that observed for cob(II)alamin ($A_{Co} = 103 \times 10^{-4}$ cm^{-1}).

As regards the possibility of high-spin cobalt in the enzyme–coenzyme complex, no evidence for its presence is currently available, and there have been few cases where its existence has been postulated in other structures. Furthermore, the general

pattern of the doublet spectrum is not of the type to be expected for an $S = {}^3/_2$ system, since even for general rhombic symmetry the spin Hamiltonian includes quadratic zero-field splitting terms.

Cockle *et al.* (1972) suggest that the doublet signal arises from a single paramagnetic species (presumably an organic radical) and is split by scalar or dipole interaction with the spin of one or more nuclei, the predominant interaction being with a nucleus with spin $^1/_2$, probably a proton. The frequency independence of the splitting between the two major lines of the doublet shows the presence of a field-independent operator in the effective spin Hamiltonian for the magnetic energy and excludes the possibility that the two lines arise from independent paramagnetic species. However, it is difficult to attribute the splitting to hyperfine splitting by an ordinary σ-bonded hydrogen. The separation of the peaks (110 G at 77°K) is larger than expected for hyperfine interaction with a proton (Lefebvre and Maruani, 1965). Further, the intensities of the lines are always unequal, the ratio usually being about 2 for ribonucleotide reductase but almost 5 for glyceroldehydrase (Cockle *et al.*, 1972). Although the results of Lefebvre and Maruani (1965) suggest that restricted rotation could cause a line intensity ratio of 2 it is not clear how restricted rotation could produce two symmetrical lines of unequal intensity. Finally, replacement of hydrogen by deuterium in the solvent and the exchangeable positions of the enzyme, coenzyme, and substrates, or in all positions of the nucleotide or in the 5′-methylene of deoxyadenosylcobalamin did not change the signal shape as it should have if the nucleus of hydrogen in any of these positions was interacting with the unpaired electron.

The change of the X-band epr spectrum with recording temperature, the effect of temperature on saturation behavior and the changes in X-band spectrum with the nucleotides present are especially interesting and together suggest a complex interacting paramagnetic system the nature of which remains to be elucidated.

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