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Cobamides and Ribonucleotide Reduction. VII. Cob(II)alamin as a Sensitive Probe for the Active Center of Ribonucleotide Reductase*

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ABSTRACT: Incubation of deoxyadenosylcobalamin with a molar excess of ribonucleotide reductase from *Lactobacillus leichmannii*, dihydrolipoate, and a nucleoside triphosphate results in the formation of a paramagnetic cobamide. The reaction does not occur if any component of the system is omitted, is relatively slow under optimal conditions, and is believed due to oxidative degradation of a reactive cobamide intermediate. Electron spin resonance spectra of the cobamide product recorded with frozen solutions at low temperature indicate that the cobamide is cob(II)alamin, but the spectra show unique hyperfine and superhyperfine structure. Similar cob(II)alamin electron spin resonance spectra were obtained when cob(II)alamin was formed: (1) from hydroxocobalamin and dihydrolipoate in the presence of reductase, a nucleoside triphosphate and 5′-deoxyadenosine or certain other closely related nucleosides; (2) by photolysis of deoxyadenosylcobalamin in the presence of dihydrolipoate, a nucleoside triphosphate and reductase; or (3) by photolysis of other cobamides in the presence of a nucleoside triphosphate, 5′-deoxyadenosine, dihydrolipoate, and reductase. The details of the spectra, especially in the low-field region, vary significantly with the identity of the nucleoside triphosphate present, the nucleoside present, and the conditions used for recording the spectra. The data are interpreted to mean that when cob(II)alamin is bound to the active center of the reductase the unpaired electron of the cobalt atom is subject to a relatively constant magnetic environment provided a nucleoside triphosphate is bound to the allosteric site of the enzyme and a nucleoside such as 5′-deoxyadenosine is bound to the active center.

It is assumed that this relatively constant magnetic environment depends on the conformation of the active center and of the ligands bound there, and that it is for this reason that the spectrum varies with the particular nucleoside triphosphate at the allosteric site and with the nucleoside at the active center.

In the reduction of ribonucleotides by the reductase of *Lactobacillus leichmannii* deoxyadenosylcobalamin assists hydrogen transfer by a mechanism similar to that for dioldehydrase and various mutases and deaminases (Hogenkamp *et al.*, 1968; Hogenkamp, 1968). These other reactions involve a specific intramolecular hydrogen transfer and also hydrogen transfer from substrate to the Co-bound 5′-methylene of deoxyadenosylcobalamin. The ribonucleotide reductase reaction differs from the others in that the donated hydrogen comes from thiol groups and is, therefore, in equilibrium with water. Consequently the reductase catalyses a unique hydrogen exchange between the 5′-methylene group of deoxyadenosylcobalamin and water. Also, hydrogen transfer is intermolecular in the reductase reaction and intramolecular in the others.

It has been assumed that in these reactions a reactive intermediate is formed by donation of a hydride ion to the 5′-methylene of the cobamide, and the the intermediate is closely related to cob(I)alamin, a powerful nucleophile that attacks water at neutral pH, especially in the presence of buffers (Tackett *et al.*, 1963; Das *et al.*, 1968). Although this postulated intermediate has not been detected directly, the results reported here indicate that cob(II)alamin, a likely product of its degradation, is slowly accumulated in the reductase system.

The cob(II)alamin formed from the coenzyme by ribonucleotide reductase, presumably at its active center, becomes a probe capable of relaying information through changes in its electron spin resonance spectrum related to changes in the conformation of the active center.

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A preliminary report of some of these results has been published previously (Hamilton *et al.*, 1969).

Materials and Methods

Nucleotides, the common nucleosides and adenine were obtained from P. L. Biochemicals; DL-lipoic acid from Calbiochem and from Sigma; dithiothreitol and dithioerythritol from Calbiochem; reduced glutathione from Sigma; 2-mercaptoethanol from Eastman; L-cysteine from Fluka; 5′-deoxyadenosylcobalamin from Pierrel, Milan, Italy; hydroxocobalamin from Glaxo-Allenburys (Melbourne, Australia) Pty Ltd.; N^6-dimethyladenosine from Zellstofffabrik Waldhof, Mannheim, Germany. Dihydrolipoate was prepared according to Gunsalus and Razzell (1957).

2′,5′-Dideoxyadenosylcobalamin, 4-(adenin-9-yl)butylcobalamin and diaquocobinamide were synthesized according to Hogenkamp and Oikawa (1964), Hogenkamp *et al.* (1970), and Pailes and Hogenkamp (1968), respectively. Some of the deoxyadenosylcobalamin and the 2′,3′-isopropylidene-5′-deoxyadenosylcobalamin were synthesized according to Morley and Blakley (1967). 5′-Deoxycytidylcobalamin was a gift from Dr. L. Mervyn. 5′-Deoxyadenosine was synthesized according to the method of Wagner *et al.* (1966) except that the treatment of 5′-thioethyladenosine with Raney nickel was continued until conversion into 5′-deoxyadenosine was almost complete. Separation of residual 5′-thioethyladenosine from the product by chromatography on G-25 was then unnecessary, and material of satisfactory purity was obtained by cyrstallization, 6-Amino-9-(5-deoxy-β-D-*erythro*-pent-4-enofuranosyl)purine (4′,5′-didehydro-5′-deoxyadenosine) was synthesized by the method of McCarthy *et al.* (1967); 9-(4′-hydroxybutyl)adenine by the method of Ikehara *et al.* (1961); isoadenosine by the method of Leonard and Laursen (1965). 9β-Arabinofuranosyladenine and 9β-D-xylofuranosyladenine were gifts from Dr. H. Follmann and were synthesized by the methods of Glaudemans and Fletcher (1963) and of Baker and Hewson (1957), respectively.

Ribonucleotide reductase was purified from extracts of *L. leichmannii* by a method to be described (M. D. Orr and R. L. Blakley, unpublished data). The preparations had a specific activity of 80–110 μmoles of ATP reduced per hr per mg of protein under standard assay conditions (1.0 M sodium acetate, 0.05 M potassium phosphate buffer, pH 7.3, 1 mM EDTA, 10 mM ATP, 8 μM deoxyadenosylcobalamin, and 30 mM dihydrolipoate incubated for 10 min at 37° in the dark under nitrogen). From polyacrylamide gel electrophoresis it was estimated that the preparations were about 50–75% pure. A preparation from which all contaminating proteins had been removed by preparative electrophoresis on acrylamide gel (M. D. Orr, R. L. Blakely, and D. Panagou, 1970, unpublished data) was a gift from Miss D. Panagou. Activity of enzyme in reaction mixtures was determined by the spectrophotometric method (Vitols *et al.*, 1967).

Thioredoxin and thioredoxin reductase used in most experiments were a gift from Dr. M. D. Orr and were highly purified from *L. leichmannii* (M. D. Orr and R. L. Blakley, 1970, unpublished data). For determination of ribonucleotide reductase activity the thioredoxin and thioredoxin reductase used were prepared from *Escherichia coli* as previously described (Vitols *et al.*, 1967).

Enzyme reactions were routinely performed in quartz electron spin resonance tubes, and unless otherwise indicated anaerobic conditions and very dim lighting were used. Solutions of the reaction components except enzyme and thiol were added to the tube with gas-tight Hamilton syringes. The solutions were mixed and deoxygenated by bubbling water-saturated, oxygen-free nitrogen through the solution for 30 min at 0°. Immediately before addition of the enzyme, the nitrogen stream was removed from the solution, but it was allowed to continue flushing through the quartz tube during subsequent steps. After addition of enzyme (previously stored at least 48 hr under nitrogen) the solution was mixed by repeatedly drawing it slowly into, and expelling it from, an oxygen-free, gas-tight Hamilton syringe. It was then incubated 3 min at 37°, and again placed in an ice bath. Thiol was added, and the solution again mixed by syringe before incubation at 37°. Alterations in the order of addition of reactants made no no difference to the spectra eventually obtained. After incubation, the mixture was frozen and the tube immediately stoppered and stored in liquid nitrogen until transfer to the cavity of the electron spin resonance spectrometer.

Electron spin resonance spectra were determined with Varian V-4501 and V-4502 spectrometers at microwave frequencies 9.05–9.25 GHz and a modulation frequency of 100 kHz. In most of the work a "fieldial" was used for direct calibration of field strength. The spectra were recorded as first derivative traces generally at an incident power of 6 or 25 mW, with an integration time constant of 1 or 3 sec. Concentrations of unpaired spins were calculated by double integration (Poole, 1967), by comparison with $CuSO_4 \cdot 5H_2O$ as the intensity standard or by comparison with samples of cob(II)alamin prepared nonenzymically from hydroxocobalamin, the concentration of which was determined spectrophotometrically with the aid of the published extinction coefficient (Firth *et al.*, 1967). Electron spin resonance spectra were recorded with the sample in liquid nitrogen (77°K) unless indicated; otherwise, a variable-temperature, nitrogen-cooled probe was used.

Results

Conditions for the Appearance of a Paramagnetic Species. Deoxyadenosylcobalamin was stable in the presence of an equimolar concentration of reductase when no substrates were present. However, when a nucleoside triphosphate and a dithiol was also present, incubation at 37° resulted in the formation of a paramagnetic species which was detected by freezing the reaction mixture and recording the electron spin resonance spectrum of the frozen solution of 77°K. The characteristic spectrum obtained in the presence of dGTP is shown in Figure 1, curve A. Omission of the cobamide, enzyme, nucleotide, or dithiol prevented any formation of the paramagnetic substance over a period of 1 hr at 37° (Table I). Formation of the compound required about 1 hr (Figure 2), and during this period there was no inactivation of the enzyme. In a system that contained 5 mM ATP and other components as in Figure 1A the rate of ATP reduction was more than ten times faster than the rate of generation of the paramagnetic species. Although reductase used in these experiments contained 25–50% of contaminating proteins, the use of enzyme free of contaminants gave the same rate of production of the paramagnetic species and the same electron spin resonance spectrum.

The paramagnetic species is formed when the reaction mixture contains either a ribonucleoside triphosphate (that is being reduced) or a deoxyribonucleoside triphosphate (Tables I and II). While monophosphates are inactive, GDP and ADP appeared to permit some formation of the compound, but less than with corresponding triphosphates, and

TABLE I: Requirement for Appearance of the Cob(II)alamin Electron Spin Resonance Signal.[a]

Reaction System	Signal Peak Height (cm)
Complete system	18
Omissions	
Enzyme	<0.2
dA-cobalamin	<0.2
dGTP	<0.2
Dihydrolipoate	<0.2

[a] The composition of the complete system was as the same as in the experiment described in Figure 1A, and the period of anaerobic incubation at 37° was 60 min. Electron spin resonance recording conditions were as in Figure 1. The peak height of 18 cm was equivalent to 0.2 mM cob(II)alamin.

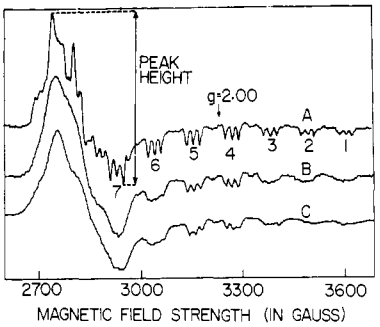

FIGURE 1: Electron spin resonance spectra of cob(II) alamin formed under various conditions. (A) From deoxyadenosylcobalamin and enzyme. The reaction mixture containing approximately 0.3 mM ribonucleotide reductase (28 mg of protein per ml), 0.2 M sodium dimethylglutarate buffer (pH 7.3), 0.2 mM deoxyadenosylcobalamin, 5 mM dGTP, and 25 mM dihydrolipoate was incubated 40 min. (B) From photolysis of deoxyadenosylcobalamin in the presence of dimethylglutarate buffer, dGTP, and dihydrolipoate. Further details appear in the text. (C) From reaction of hydroxocobalamin and dihydrolipoate. Hydroxocobalamin (0.2 mM) was incubated with 0.2 mM sodium dimethylglutarate buffer (pH 7.3) and 25 mM dihydrolipoate at 37° for 40 min under nitrogen. Recording conditions were as follows: microwave frequency, 9.049 GHz; modulation amplitude 3.78 gauss; microwave power, 25 mW; integration time constant, 1 sec; scanning rate, 100 gauss per min; temperature, 77° K. In A the portion of the spectrum used in measuring peak height is indicated (see Figures 2 and 6).

contaminating triphosphates may have been responsible. The $\beta\gamma$- and $\alpha\beta$-phosphonate analogs of ATP gave about the same rate of formation as ATP.

The thiol requirement was satisfied by dihydrolipoate, 1,4-dithiothreitol, 1,4-dithioerythritol, 1,3-dithiopropan-2-ol, 2-mercaptoethanol, glutathione, or cysteine. However, 1,3- and 1,4-dithiols permitted rates of formation several times faster than with monothiols. Thioredoxin kept in the reduced state by thioredoxin and NADPH (Orr and Vitols, 1966) was also active.

No electron spin resonance signal could be observed when methylcobalamin, 2′,3′-isopropylidene-5′-deoxyadenosylcobalamin, or 4-(adenin-9-yl)butylcobalamin were used instead of deoxyadenosylcobalamin in the reaction mixture, and with 5′-deoxycytidylcobalamin the rate of signal generation was about 10% that obtained with the latter. Only 2′,5′-dideoxyadenosylcobalamin generated a signal at a compar-

TABLE II: Extent of Deoxyadenosylcobalamin Conversion into Cob(II)alamin in the Presence of Various Nucleotides.[a]

Nucleotide	% Conversion into Cob(II)alamin
CTP, dCTP, GTP, dGTP, dGTP + dATP, UTP	70–90
ATP, AOPOPCP[b], AOPCPOP, dATP, ITP, dTTP, GDP	30–50
ADP, dGOPOPCP	1–10
CMP, GMP, AMP	0

[a] The composition of the reaction mixture was as in Figure 1A except that the nucleotide, present at a concentration of 5 mM, was as indicated. Mixtures were incubated anaerobically in the dark for 40 min at 37°. Electron spin resonance recording conditions were as in Figure 1. [b] The abbreviations are: AOPOPCP, the $\beta\gamma$-methylenephosphonate analog of ATP; AOPCPOP, the $\alpha\beta$-methylenephosphonate analog of ATP; dGOPOPCP; the $\beta\gamma$-methylenephosphonate analog of dGTP.

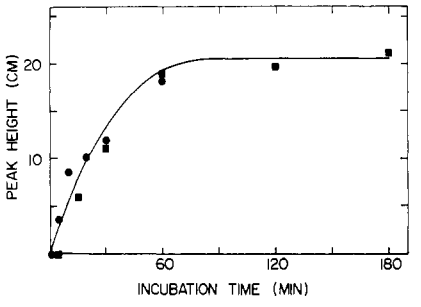

FIGURE 2: Rate of formation of cob(II) alamin from deoxyadenosylcobalamin in the presence of enzyme. A reaction mixture of the same composition as that used for Figure 1A was incubated anaerobically in the dark at 37°. Samples (0.22 ml) were withdrawn at intervals, transferred anaerobically to electron spin resonance tubes and frozen. Recording conditions were as follows. Experiment 1 (●): microwave frequency, 9.257 GHz; modulation amplitude, 40 gauss; microwave power, 50 mW; time constant, 1 sec; scanning rate, 100 gauss per min; temperature, 87°. Experiment 2 (■): microwave frequency, 9.112 GHz; modulation amplitude, 3.15 gauss; microwave power, 30 mW; time constant, 1 sec; scanning rate, 100 gauss per min; temperature, 77°K. The same gain settings were used in each case so that peak heights are directly comparable. Peak height was measured as indicated in Figure 1.

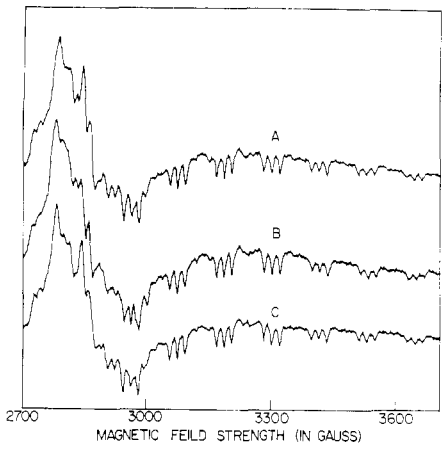

FIGURE 3: Highly resolved electron spin resonance spectra of cob(II)-alamin formed from hydroxocobalamin and dihydrolipoate. Reaction mixtures containing 0.2 mM hydroxocobalamin, 25 mM dihydrolipoate, 5 mM dGTP, 0.2 mM nucleoside, and approximately 0.3 mM ribonucleotide reductase (30 mg of protein per ml) were incubated 40 min at 37° in the dark under anaerobic conditions. Recording conditions were as follows: microwave frequency, 9.093 GHz; modulation amplitude, 3.15 gauss; microwave power, 30 mW; time constant, 3 sec; sanning rate, 20 gauss per min; temperature, 77° K. The nucleosides present were: A, 5′-deoxyadenosine; B, adenosine; C, 4′,5′-didehydro-5′-deoxyadenosine.

able rate to deoxyadenosylcobalamin, but the electron spin resonance spectrum generated in this case was significantly different as discussed later.

Identity of the Paramagnetic Species. The hyperfine structure of the high-field electron spin resonance spectrum (Figure 1, curve A) consists of eight lines (centered on $g = 2.003$) resulting from magnetic interaction of the unpaired electron with the ^{59}Co nucleus ($I = 7/2$). Seven of the lines are indicated by numbers in Figure 1A, the eighth being partly obscured by other peaks. The hyperfine coupling constant, $A_{\parallel Co}$, derived from the average spacing between lines 1 and 7 is $103 \pm 2 \times 10^{-4}$ cm^{-1} in all of the figures shown. In curve A each of the high-field hyperfine lines is further split into a triplet, in which the average superhyperfine spacing $A_{\parallel N}$ is $16.9 \pm 0.5 \times 10^{-4}$ cm^{-1}. The low-field part of the spectrum ($g = 2.25$ approximately) is not well enough resolved to permit estimation of the relevant constants except by computer simulation and analysis.

Spectrum A (Figure 1) is in general agreement with previously published electron spin resonance spectra for cob(II)-alamin. The spectrum for cob(II)alamin in the solid state shows much less hyperfine structure (Yamada *et al.*, 1966) and a frozen aqueous solution gives a spectrum showing little structure when other solutes are absent (Hogenkamp *et al.*, 1963). However, spectra with more structure have been reported for frozen solutions of cob(II)alamin containing other solutes or solvents by Schrauzer and Lee (1968), Cockle *et al.* (1969), and Bayston *et al.* (1970). Seven of the lines of the highfield part of the spectrum can be clearly distinguished and several, but not all, of these lines are resolved into triplets. Similar spectra were obtained from a frozen aqueous solution containing dihydrolipoate, dimethylglutarate, and (in one case) dGTP together with cob(II)alamin (curves B and C, Figure 1). The poorer resolution in B and C (as compared with A) does not appear to be due to differential saturation since spectra B and C were unchanged over the power range 5–30 mW and showed only a very slight change at 70 mW.

The resemblance between spectra A, B, and C (Figure 1) suggests that cob(II)alamin is the paramagnetic species giving rise to spectrum A. The differences between these spectra suggest that in the sample giving spectrum A cob(II)alamin is in an enviornment that enhances resolution so as to give the additional hyperfine structure in the low-field part of the spectrum and the superhyperfine splitting of *all* of the lines of the high-field part of the spectrum. The hyperfine coupling constant, $A_{\parallel Co}$, obtained from A agrees with published values for cob(II)alamin (103×10^{-4} cm^{-1} in the presence of cysteine and gluthathione). The splitting of the high-field lines into triplets is due to interaction of the electron with a nitrogen nucleus ($I = 1$) and the work of Schrauzer and Lee (1968), Cockle *et al.* (1969), and Bayston *et al.* (1970) indicate that the coupling is to the N^3-nitrogen of dimethylbenzimidazole. The $A_{\parallel N}$ constants obtained from A also agree well with those previously reported.

Conditions Required for "High-Resolution" Cob(II)alamin Spectra. No combination of solutes and solvents produces a spectrum showing either the resolution seen in the low-field portion of spectrum A or the resolution of triplets in all lines of the high-field part of the spectrum. This suggests that the high resolution of A is due to binding of the cob(II)alamin to the active center of the reductase. Supporting evidence was obtained from spectra of frozen solutions of cob(II)alamin that had been formed by reaction of hydroxocobalamin with dihydrolipoate. The spectra were like Figure 1C when enzyme was present in slight molar excess over cob(II)alamin, and even the additional presence of 5 mM dGTP did not enhance resolution significantly. However, in the presence of enzyme, dGTP, and 5′-deoxyadenosine the spectrum closely resembled Figure 1A (Figure 3A). Omission of any of the components of the system resulted in a poorly resolved spectrum like Figure 1C. The effect of 5′-deoxyadenosine in this system was highly specific. The following nucleosides were without effect on the spectrum of the cob(II)alamin: 2′,3′-isopropylideneadenosine, adenosine-5′-carbaldehyde, 5′,8-cyclic adenosine, 2′-deoxyadenosine, 3′-deoxyadenosine, 9β-D-arabinofuranosyladenine, 9β-D-xylofuranosyladenine, N^6-dimethyladenosine, 5′-thioethyladenosine, isoadenosine, guanosine, inosine, and cytidine. Adenine and 9-(4′-hydroxybutyl)adenine were also without effect. The only other nucleosides which enhanced resolution of the spectrum were adenosine and 4′,5′-didehydro-5′-deoxyadenosine. As seen in Figure 3 there were slight differences in the low-field region of the spectrum when either of these nucleosides replaced 5′-deoxyadenosine. Since cob(II)alamin generated from deoxyadenosylcobalamin by the enzyme system also has a highly resolved spectrum, the nucleoside derived from the deoxyadenosyl moiety of the cobamide must be 5′-deoxyadenosine or a closely related nucleoside.

The poorly resolved spectrum obtained in the presence of cob(II)alamin, enzyme, dGTP, and 2′-deoxyadenosine is consistent with the observation that after incubation of 2′,5′-dideoxyadenosylcobalamin with enzyme, dihydrolipoate, and dGTP the frozen solution exhibited a cob(II)alamin electron spin resonance spectrum, but the spectrum was of low resolution like Figure 1C. If the products fromed when 5′-deoxyadenosylcobalamin is incubated with the enzyme system are cob(II)alamin and 5′-deoxyadenosine, the products formed from 2′,5′-dideoxyadenosylcobalamin would be cob(II)alamin and 2′,5′-dideoxyadenosine, and since 2′-deoxyadenosine does not permit the high-resolution spectrum 2′,5′-dideoxyadenosine is unlikely to permit it either.

The enzyme–dGTP–5′-deoxyadenosine complex which

enhanced the resolution of the electron spin resonance spectrum of cob(II)alamin had no significant effect on the spectrum of cob(II)inamide. The peak height of the signal from the latter was small under all conditions examined, because of peak broadening. It is also possible that reaction of diaquocobinamide with dihydrolipoate gives a relatively low concentration of cob(II)inamide and a larger concentration of thiol adduct. However, the signal intensity was sufficient to obtain reasonable spectra and to determine the absence of signal resolution after incubation with the enzyme system.

Highly Resolved Spectra from Cob(II)alamin Produced by Photolysis. The highly resolved electron spin resonance spectrum could also be obtained, under appropriate conditions, from cob(II)alamin formed by anaerobic photolysis of deoxyadenosylcobalamin (Hogenkamp, 1964). When 0.2 mM deoxyadenosylcobalamin, 0.2 M sodium dimethylglutarate (pH 7.3), 5 mM dGTP, and 25 mM dihydrolipoate were mixed in an electron spin resonance tube under anaerobic conditions, and exposed to a 300-W tungsten lamp at a distance of 10 cm while maintained at 37° under a nitrogen stream, photolysis of the cobamide was complete in 3 min. If, after 3 min further anaerobic incubation at 37° in the dark, the solution was frozen, an electron spin resonance spectrum showing poor resolution was obtained (Figure 1B). However, if enzyme (final protein concentration 28 mg ml) was added prior to the incubation in the dark or prior to the photolysis period, a highly resolved spectrum was obtained resembling spectrum A in Figures 1 and 3. The highly resolved spectrum was obtained only if dGTP was added either before or after the photolysis period (Table III).

The presence of dihydrolipoate during photolysis was essential for generation of the highly resolved cob(II)alamin spectrum (Table III), although an equivalent concentration of mercaptoethanol was also effective. This requirement is believed due to the fact that during photolysis, the thiol reacts with the nucleoside radical derived from deoxyadenosylcobalamin to give a 5′-alkylthioadenosine (Johnson *et al.*, 1963), which presumably permits a highly resolved spectrum. Anaerobic photolysis of deoxyadenosylcobalamin in absence of a thiol gives 5′,8-cyclic adenosine or (in the presence of oxygen) adenosine-5′-carbaldehyde (Hogenkamp, 1964) which are unable to determine a resolved cob(II)alamin spectrum. This is seen in the results above on cob(II)alamin formed from hydroxocobalamin and dihydrolipoate. That it was the nucleoside product from photolysis of deoxyadenosylcobalamin in absence of thiol which caused the poor resolution was shown by the fact that when 5′-deoxyadenosine was added to the photolysis products together with dGTP, enzyme, and dihydrolipoate, the spectrum obtained was then highly resolved (Table III). When 2′,5′-dideoxyadenosylcobalamin or 2′,3′-isopropylidene-5′-deoxyadenosylcobalamin was photolyzed in the presence of dGTP and dihydrolipoate and the products subsequently allowed to react with enzyme, a poorly resolved cob(II)alamin spectrum resulted. The poor resolution of the spectrum obtained under these conditions was evidently due to the thioalkyl nucleoside formed under these conditions because the additional presence of 5′-deoxyadenosine resulted in a highly resolved cob(II)alamin spectrum (Table III).

Changes in the Active Center Indicated by Electron Spin Resonance Spectra. The above data suggest that when cob(II)alamin combines with the active center of ribonucleotide reductase or is formed *in situ* the hyperfine structure of the electron spin resonance spectrum of the bound cob(II)alamin is an index of the conformation of the active center. This is also suggested by the influence of nucleotides on the hyperfine structure of the spectrum. Such nucleotide-dependent differences are almost easily detected in the low-field spectral region (Figure 4). Each nucleotide determines a characteristic and highly reproducible cob(II)alamin spectrum, other parameters being constant. GTP, CTP, and ATP determine spectra identical with those shown with dGTP, dCTP, and dATP, respectively.

TABLE III: Conditions Required for the Resolution of the Spectrum of Cob(II)alamin Produced by Anaerobic Photolysis.

Present during Photolysis[a]	Added after Photolysis	Resolution of Spectrum
With Cob(II)alamin from Photolysis of 5′-Deoxyadenosylcobalamin		
Cobamide, dGTP, enzyme, dihydrolipoate		High
Cobamide, dGTP, dihydrolipoate	Enzyme	High
Cobamide, dihydrolipoate	dGTP, enzyme	High
Cobamide, dGTP, enzyme	Dihydrolipoate	Low
Cobamide, dGTP	Dihydrolipoate, enzyme	Low
Cobamide, dihydrolipoate	Enzyme	Low
Cobamide, dGTP, dihydrolipoate		Low
Cobamide, dGTP, 2-mercaptoethanol	Enzyme	High
Cobamide, dGTP	Dihydrolipoate, enzyme, 5′-deoxyadenosine	High
With Cob(II)alamin from Photolysis of 2′,5′-Dideoxyadenosylcobalamin		
Cobamide, dGTP, dihydrolipoate	Enzyme	Low
Cobamide, dGTP, dihydrolipoate, 5′-deoxyadenosine	Enzyme	High
With Cob(II)alamin from Photolysis of 2′,3′-Isopropylidene-5′-deoxyadenosylcobalamin		
Cobamide, dGTP, dihydrolipoate	Enzyme	Low
Cobamide, dGTP, dihydrolipoate, 5′-deoxyadenosine	Enzyme	High

[a] The concentration of components of reaction mixtures, when present, were: enzyme, approximately 0.3 mM (28 mg of protein per ml); sodium dimethylglutarate buffer (pH 7.3), 0.2 mM; cobamide, 0.2 mM; dGTP, 5 mM; dihydrolipoate, 25 mM; mercaptoethanol, 50 mM; and 5′-deoxyadenosine, 0.2 mM. Other details are given in the text. Recording conditions were as in Figure 1. A spectrum with high resolution is one similar to that shown in Figure 1A, and low resolution indicates a spectrum similar to those in Figure 1B or 1C.

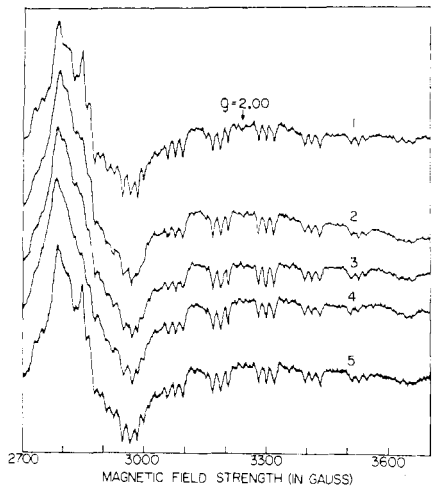

FIGURE 4: Dependence of the electron spin resonance spectrum of cob(II) alamin on the nucleoside triphosphate present. Reaction mixtures had the same composition as in Figure 1A except for the nucleotides present which were as follows (5 mM): 1, dGTP; 2, dCTP; 3, dATP; 4, UTP; 5, ITP. Incubation at 37° was carried out for 60 min. Recording conditions were as follows: microwave frequencies were 9.090 to 9.093 GHz; modulation amplitude, 3.15 gauss; microwave power, 30 mW; integration time constant, 3 sec; scanning rate, 20 gauss per minute; temperature, 77° K.

Effect of Recording Conditions on Spectra. The effect of variations in the recording conditions are shown in Figure 5. As the recording temperature is raised from 77° to 163° K, the expected reduction in intensity is observed (A is at half the amplifier gain used for B), but in addition, there is a broadening of the spectral lines and changes in the hyperfine structure in the low-field region. The two latter effects presumably arise from increased molecular motion within the frozen lattice.

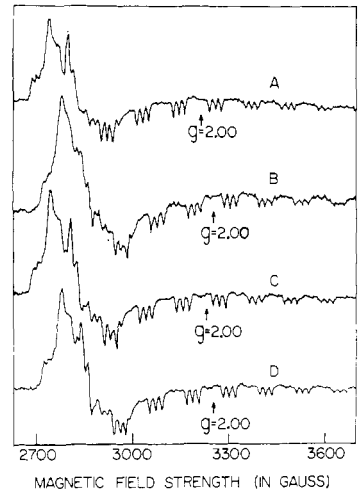

FIGURE 5: Effect of temperature and microwave frequency on the electron spin resonance spectrum of cob(II)alamin. Reaction mixtures contained 5 mM dGTP, 25 mM dihydrolipoate, 0.2 M sodium dimethylglutarate (pH 7.3), enzyme, and deoxyadenosylcobalamin. In A and B the concentration of enzyme was approximately 1 mM, and deoxyadenosylcobalamin 1.6 mM. In C and D the concentration of enzyme was approximately 0.3 mM and deoxyadenosylcobalamin 0.2 mM. Recording conditions were as in Figure 1 except for the following. Microwave frequencies were: A and B, 9.015 GHz; C, 9.049 GHz; D, 9.143 GHz. Temperatures were: A, C, and D, 77° K; B, 163° K. The gain setting in A was half that in B, C, and D.

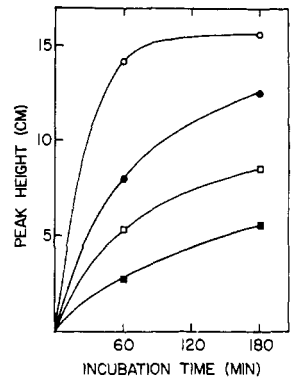

FIGURE 6: Formation of cob(II)alamin from deoxyadenosylcobalamin in the presence of limiting amounts of ribonucleotide reductase Except for the concentration of enzyme the experiment was carried out as in the case of Figure 2. Approximate concentration of ribonucleotide reductase were: ○, 0.3 mM; ●, 0.1 mM; □, 0.05 mM; ■, 0.03 mM. Recording conditions were as follows: microwave frequency, 9.093 GHz; modulation amplitude, 3.15 gauss; microwave power 30 mW; integration time constant, 1 sec; scanning rate, 100 gauss per min; temperature, 77° K. The same gain setting was used in each case so that peak heights are directly comparable, and peak height was measured as indicated in Figure 1.

Recording of spectra was performed at several slightly different microwave frequencies which were determined by the resonance conditions appropriate for various quartz dewar flasks which held liquid itrogen to cool the sample tube, or for the variable-temperature probe. It was observed that highly reproducible differences in the low-field spectra occurred with such small variations in the microwave frequency (curves B and C, Figure 4; curves C and D, Figure 5). These unexpected effects of such small changes in microwave frequency were not due to "Dewar signals" which were virtually absent from the low-field portion of the spectrum, and their cause and significance is not understood.

Relative Tightness of Binding of Cob(II)alamin and Deoxyadenosylcobalamin. In the experiments above enzyme was in excess over cobamide, so that probably almost all the cob(II)alamin was bound to the active center of the enzyme. When 3 mM deoxyadenosylcobalamin was incubated with approximately 0.3 mM enzyme in the presence of dGTP and dihydrolipoate, the cob(II)alamin formed gave a spectrum with poorer resolution than Figure 1A and with small differences in the low-field spectral region. This suggests that when present in excess, deoxyadenosylcobalamin can partially displace cob(II)alamin from the enzyme, leading to an observed spectrum that is the average of that for free and for bound cob(II)alamin.

Supporting evidence was obtained when 0.2 mM deoxyadenosylcobalamin was incubated with dGTP, dihydrolipoate and several limiting concentrations of enzyme (Figure 6). The amounts of cob(II)alamin formed at 180 min at enzyme concentrations of 0.1, 0.05, and 0.03 mM can be assessed from the peak heights to be approximately 0.14, 0.11, and 0.07 mM. The electron spin resonance spectra produced under these conditions showed a corresponding loss of resolution, but in no case was the decrease in resolution of the spectrum such as to indicate that all the cob(II)alamin had been displaced from the enzyme. However, the rate of cob(II)alamin formation was not greatly decreased when the amount formed was equimolar with enzyme (Figure 6), so that sufficient deoxyadenosylcobalamin was able to displace cob(II)alamin for the formation of the latter to continue.

Effect of pH on Cob(II)alamin Formation. Since formation of cob(II)alamin from deoxyadenosylcobalamin in the presence of the enzyme has been attributed to oxidative degration of a reactive intermediate containing monovalent cobalt, and since the stability of cob(I)alamin is decreased at lower pH (Das *et al.*, 1968), the rate of formation of cob(II)alamin should be greater at lower pH. It was found, however, that at pH 6.3, the rate of cob(II)alamin was the same as at pH 7.3 as judged by electron spin resonance spectra. The spectrum of cob(II)alamin formed under these conditions was similar to that formed at higher pH although not quite as well resolved.

Discussion

Cob(II)alamin as Degradation Product of a Reactive Cobamide. There can be little doubt that the paramagnetic species formed from deoxyadenosylcobalamin in the presence of ribonucleotide reductase, dihydrolipoate, and a nucleotide is cob(II)alamin. The electron spin resonance spectrum of the product closely resembles that of cob(II)alamin formed nonenzymically and hydroxocobalamin, the expected product of aerobic oxidation of cob(II)alamin, can be isolated from the enzyme system as the ultimate cobamide product (R. Yamada and R. L. Blakley, 1970, unpublished data). Furthermore, it seems probable that the cob(II)alamin is formed by the degradation of some reactive cobamide species that results from interaction of the dithiol and deoxyadenosylcobalamin at the active center of the enzyme. Formation of cob(II)alamin is considerably slower than the rate of ribonucleotide reduction and of hydrogen exchange between water and the Co-bound methylene group of deoxyadenosylcobalamin, so that cob(II)alamin cannot be an obligatory intermediate in these processes. On the other hand, cob(II)alamin is formed under those conditions in which hydrogen exchange occurs and this includes the conditions in which ribonucleotide reduction occurs. The data are, therefore, consistent with the view that cob(II)alamin formation results from oxidation of some reactive enzyme–cobamide complex closely associated with, if not actually an intermediate in, the hydrogen-exchange and ribonucleotide-reduction mechanisms. If, in this reactive cobamide complex, cobalt is monovalent, so that the cobamide is cob(I)alamin or its equivalent, then the observed rate of cob(II)alamin formation is in accord with expectation even if the steady-state concentration of complex is small, since cob(I)alamin is very readily oxidized to cob(II)alamin at neutral pH under anerobic conditions (Tackett *et al.*, 1963; Das *et al.*, 1968).

Significance of the "Highly Resolved" Electron Spin Resonance Spectrum of Cob(II)alamin. The increased resolution of the electron spin resonance spectrum of cob(II)alamin with changes in the solvent and solutes present has been discussed by Bayston *et al.* (1970), who have postulated that the principle effect of the solvent and solute is on the length and angle of the bond between cobalt and the nitrogen of dimethylbenzimidazole. It is suggested that in the frozen-state variability in the packing of solute and solvent molecules and ions between the corrinoid and the axial ligand causes inhomogeneity of the cob(II)alamin with respect to the bond between cobalt and the axial ligand. This inhomogeneity is considered a major cause of line boradening; particularly of the triplets in the high-field part of the spectrum. Such inhomogeneity must be supposed to be less in the presence of solvents, buffers, or other solutes that enhance resolution. Similar variability in the packing of solvent or solutes on the opposite side of the cobalt, that is in the vicinity of the orbital occupied by the unshared electron, may be considered a further potential cause of inhomogeneity and consequent line broadening.

The unusually sharp resolution of the electron spin resonance spectra of cob(II)alamin produced by the enzyme system can be attributed to the effect of binding of cob(II)alamin at the active center of the enzyme. This interpretation is strengthened by the observation that cob(II)alamin produced in other ways and treated with enzyme was found to give similar highly resolved electron spin resonance spectra provided appropriate additional components were present in the mixture. Binding of cob(II)alamin to the active center of the enzyme ensures the separation of the paramagnetic cobamide molecules from each other and thus prevents line broadening due to spin–spin interaction. Movement of propionamide and acetamide side chains and of the nucleotide moiety of cob(II)alamin is probably restricted in the complex so that variation in the magnetic environment of the cobalt atom due to such movement would be diminished. Finally, by restricting the access of solvent and solute molecules and ions to the corrin ring and by limiting the variability in packing of such molecules in the vicinity of the cobalt atom, hetereogeneity due to the effects of such molecules is possibly decreased.

The highly resolved spectrum of cob(II)alamin occurred only in the presence of a nucleoside triphosphate such as dGTP. Furthermore, the poorly resolved spectrum from cob(II)alamin obtained when 2′,5′-dideoxyadenosylcobalamin is incubated with the reductase system indicates that the nucleoside fragment derived from the cobamide has a marked effect on the spectrum. This was amply confirmed by experiments with cob(II)alamin formed by photolysis of cobalamins or by reaction of hydroxocobalamin with dihydrolipoate. In both cases observation of a highly resolved spectrum required the presence of a nucleoside like 5′-deoxyadenosine.

The interpretation of these requirements cannot be made with certainty, since it is possible that freezing of the solution and cooling to 77° K (for recording of spectra) causes conformational changes in the protein, expecially at the active center, or in molecules bound to the protein. However, the nucleoside and the nucleotide probably enhance the resolution of the spectrum of cob(II)alamin by promoting the formation of a complex of the latter with enzyme in which a special conformation of side chains and ligands at the active center severely limits variability in the magnetic enviornment of the unpaired electron. This is suggested by the specificity of the requirement for both nucleoside and nucleotide, by the allosteric properties of effective nucleotides (Vitols *et al.*, 1967), and by the small but significant spectral differences in the low-field region with different nucleotides.

The nucleoside requirement is characterized by very high specificity. The only nucleosides that are effective are 5′-deoxyadenosine, 4′,5′-didehydro-5′-deoxyadenosine, adenosine, and, possibly, certain 5′-alkythioadenosines. These active nucleosides may be presumed to resemble the deoxyadenosyl moiety of 5′-deoxyadenosylcobalamin so closely that they bind in the portion of the active center to which the deoxyadenosyl moiety normally attaches and, hence, presumably take up a position close to the cobalt atom of bound cob(II)alamin. Nucleosides related to cobalamins ineffective as coenzyme are ineffective in producing the highly resolved spectrum. Thus, 2′,5′-dideoxyadenosylcobalamin has little coenzyme activity and 2′,3′-isopropylidene-5′-deoxyadenosylcobalamin, 4-(adenin-9-yl)butylcobalamin, 5′-deoxycytidylcobalamin, and 5′-deoxy(adenine-β-D-arabinofuranosyl)cobalamin have none, though most of them do bind to the enzyme as indicated by

inhibitory properties (Vitols *et al.*, 1967; E. Vitols, R. L. Blakley and H. P. C. Hogenkamp, 1967, unpublished data). Corresponding nucleosides, 2′-deoxyadenosine, 2′,3′-isopropylidene adenosine, 9-(4′-hydroxybutyl)adenine, cytidine, and 9β-D-arabinofuranosyladenine, produce no effect on the spectrum of cob(II)alamin. These nucleosides, as well as other ineffective ones probably bind weakly to the active center of the reductase, or alternatively, bind but assume a different orientation within the active center to that assumed by active nucleosides.

Deoxyribonucleoside triphosphates are as effective as ribonucleoside triphosphates in promoting spectral resolution (Table II). Since the deoxyribonucleotides are very weak inhibitors of the enzyme and therefore bind poorly to the active center, but are activators and therefore bind to an allosteric site (Vitols *et al.*, 1967), it seems likely that their spectral effects result from their combination at the allosteric site and consequent conformational changes in the protein. A similar explanation has been postulated for their effect on hydrogen exchange between water and the 5′-methylene of deoxyadenosylcobalamin (Hogenkamp *et al.*, 1968). Ribonucleotides possibly produce spectral effects by combination at the allosteric site also (Vitols *et al.*, 1967), but combination at the active center may also be involved.

The proposal that 5′-deoxyadenosine and dGTP produce their spectral effects by combining, respectively, at the active center and the allosteric site may be understood in two ways. The enzyme may be unable to bind cob(II)alamin to a significant extent unless 5′-deoxyadenosine and dGTP (or their equivalents) are complexed with it, that is the enzyme conformation induced by the nucleoside and nucleotide may specify binding of cob(II)alamin to the enzyme. An alternative explanation is that although cob(II)alamin binds to the enzyme in absence of nucleotide and nucleosides, combination of the latter with the enzyme induces a unique configuration at the active center and thus alters the immediate environment of the bound cob(II)alamin. It has previously been suggested that each deoxyribonucleoside triphosphate determines a specific but different configuration at the active center and that this accounts for the specific activation effects exhibited by the deoxynucleotides (Hogenkamp *et al.*, 1968). These postulated different specific configurations could also account for the differences in the cob(II)alamin electron spin resonance spectra produced when different nucleotides are present in the enzyme system (Figure 4). The effect of 5′-deoxyadenosine on the magnetic environment of the unpaired electron of cob(II)alamin may be explained by assuming that binding of 5′-deoxyadenosine at the active center alters the configuration of amino acid side chains and bound ligands there. An additional consideration is that if the cob(II)alamin and 5′-deoxyadenosine bind to the respective parts of the active center where the cobalamin and deoxyadenosyl moieties of deoxyadenosylcobalamin bind, the nucleoside and cob(II)alamin would be held in very close juxtaposition in the complex—so close that the nuclei of the protons in the 5′-methyl group of the nucleoside might interact with the unpaired electron. Although this would not explain the greater resolution of triplets in the high-field spectrum, it might account for some of the additional lines that become visible in the low-field part of the electron spin resonance spectrum when deoxyadenosine is present.

If the foregoing explanation of the effect of enzyme, 5′-deoxyadenosine, and nucleotide on the spectrum of cob(II)alamin is correct in principle, then the electron spin resonance spectrum provides a sensitive method for signaling changes at the active center. One immediate conclusion is that, in the complex, N^3 of the dimethylbenzimidazole moiety remains coordinated to the cobalt atom. The superhyperfine splitting into triplets in the high-field region indicates coupling of the electron spin with nitrogen nucleis, and values of the coupling constants ($A_{\perp Co}$ and $A_{\parallel N}$) suggest that the coupled nitrogen is in the benzimidazole moiety rather than in a protein side chain (Bayston *et al.*, 1970; Cockle *et al.*, 1969). This is in contrast to the complex of methylcobalamin with 5-methyltetrahydrofolate homocysteine transmethylase in which it appears likely that nitrogen of a side chain histidine becomes the axial ligand to cobalt (Pailes and Hogenkamp, 1968). The electron spin resonance results do not, of course, preclude the possibility that dimethylbenzimidazole is displaced from cobalt in the reactive complex formed from deoxyadenoxylcobalamin on the enzyme.

Acknowledgments

The authors are indebted to Misses Barbara Craker, Barbara Humphrey, and Kathleen Kelly, and to Mrs. Mary Schrock for assistance in the preparation of enzyme, to Dr. R. Bramley, Dr. J. R. Pilbrow, and Dr. R. E. Coffman for helpful advice and discussion, and the the generous donors whole gifts of materials are acknowledged above.

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In Vitro Dechlorination of a 4,6-Dichloro-$\Delta^{4,6}$-keto Steroid*

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ABSTRACT: Investigation of the *in vitro* metabolism of 4,6-dichloro-17α-hydroxypregna-4,6-diene-3,20-dione acetate (I) showed that in addition to the expected 4,6-dichloro-3-hydroxy derivatives IIIa and IIIb, the 6-monochloro compound II and the corresponding 6-monochloro-3-hydroxy compounds IVa and IVb were formed. Although proportions varied, the qualitative distribution of the metabolites was the same whether a 9000*g* rat liver microsomal preparation or the 105,000*g* supernatant fraction was used. After the metabolites had been characterized, attempts were made to identify the factor or factors responsible for the unique 4-dechlorination of I.

The effects of various substituents on the *in vitro* and *in vivo* metabolism of compounds possessing the basic progesterone nucleus have been widely studied. It is well known that the usual metabolic fate of progesterone in mammalian systems follows the path of Δ^4-3-keto, and 20-keto reduction as well as several ring hydroxylations, depending on species and tissue (Dorfman and Ungar, 1965).

There have been exceptions to this pattern, such as that reported by Ungar *et al.* (1957) where rat liver tissue was shown to be capable of reducing Δ^4-3-ketones to Δ^4-3-ols. Particularly germane to the present work was the report by Ringold *et al.* (1964) that substitution by halogen at C-2, C-4, or C-6 enhanced both rate and yield of Δ^4-3-ol formation from the corresponding Δ^4-3-ones. Conversely, these investigators found methyl substitution at these positions to severely retard reduction, and they offered cogent arguments for the electronic destabilization of the Δ^4-3-keto group by electronegative substitution, with consequent stabilization of the proposed transition state for reduction. It is of interest that the metabolism of 4-chlorotestosterone by human liver slices also produced the Δ^4-3-hydroxy derivative (Starka *et al.*, 1969).

An investigation by Cooke and Vallance (1965) of the metabolism of megestrol acetate[1] by rat and rabbit liver preparations clearly demonstrated the retardation of metabolism by modification of the progesterone skeleton. It can be inferred from this study that introduction of a 6,7-double bond may be partially responsible for the absence of 6-methyl hydroxylation, a conversion reported earlier (Castegnaro and Sala, 1962; Helmreich and Huseby, 1962) to be the major pathway of medroxyprogesterone acetate metabolism in humans.

The only chloro-substituted C_{21}-progestational steroid to have been widely used is chlormadinone acetate (II) (Ringold *et al.*, 1959; Brückner *et al.*, 1961). Its metabolism in women has been investigated (Bermudez *et al.*, 1968), but the identity of its metabolites has not been reported.

The 4-dechlorination undergone by the 4,6-dichloro steroid I in our experiments is without precedent in biological systems. It should be mentioned, however, that a similar reaction, the reductive dechlorination of DDT to DDD, has been observed in several systems (Klein *et al.*, 1964; Miskus *et al.*, 1965; Bunyan *et al.*, 1966). It was shown by Bunyan *et al.* (1966) that this dechlorination was enhanced under anerobic conditions by the presence of GSH. The present work devotes considerable attention to this tripeptide as a possible participant in the unique 4-dechlorination of I.

Experimental Section

Synthesis of 4,6-Dichloro-3β,17α-dihydroxypregna-4,6-dien-20-one 17-Acetate (IIIa). A solution of 2.000 g (4.5 mmoles) of I (Kierstead *et al.*, 1970) in 20 ml of anhydrous tetrahydrofuran was added dropwise over a 15-min period to 3.280 g (13.68 mmoles) of lithium aluminum tri-*tert*-butoxyhydride in 20 ml of anhydrous tetrahydrofuran under a nitrogen atmosphere. After stirring at room temperature for 2 hr, 20 ml of acetone was added followed by 150 ml of 10% acetic acid in water. The mixture was extracted with two 75-ml portions of chloroform; the organic phases were

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[1] Trivial names and abbreviations used are: megestrol acetate, 17α-hydroxy-6-methylpregna-4,6-diene-3,20-dione acetate; medroxyprogesterone acetate, 17α-hydroxy-6α-methylpregn-4-ene-3,20-dione acetate; chlormadinone acetate, 6-chloro-17α-hydroxypregna-4,6-diene-3,20-dione acetate; DDT, 1,1-bis-(*p*-chlorophenyl)-2,2,2,-trichloroethane; DDD, 1,1-bis(p-chlorophenyl)-2,2-dichloroethane; GSH, reduced glutathione; GSSG, oxidized glutathione; NEM, *N*-ethylmaleimide; SKF-525A, 2-diethylamino-2,2-diphenylvalerate hydrochloride; Glc-6-P, glucose 6-phosphate.