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Ribonucleotide Reductase from Calf Thymus. Purification and Properties†

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ABSTRACT: Ribonucleotide reductase from calf thymus was purified 3400-fold in good yield by using a rapid and highly reproducible procedure which included ammonium sulfate fractionation, chromatography on DEAE-cellulose and hydroxylapatite and affinity chromatography on dATP–Sepharose. Nonheme iron is an essential component of the enzyme since EDTA causes inactivation which can be reversed by readdition of iron. Data from polyacrylamide gel electrophoresis, glycerol gradient centrifugation, iron analysis, and kinetic experiments indicated that the enzyme preparation consists of two kinds of polypeptide, both necessary for activity. One polypeptide has a molecular weight of about 84 000 and constitutes the bulk of the protein in the final enzyme preparation, while the other iron-binding polypeptide is present in low, nonstoichiometric amounts. The active enzyme complex has a sedimentation coefficient of 10 S but, on addition of the inhibitory effector dATP, most of the protein sediments more rapidly at 16 S. In this respect, the thymus reductase resembles the *Escherichia coli* ribonucleotide reductase which also forms dATP-induced aggregates. Furthermore, the proposed subunit structure of the thymus enzyme is very similar to the one of the bacterial enzyme, which consists of two kinds of polypeptide, molecular weight 80 000 and 39 000, where the 39 000 polypeptide contains nonheme iron and a free radical. However, the thymus enzyme is inhibited reversibly by hydroxyurea or 2′-deoxy-2′-azidocytidine diphosphate in contrast to the *E. coli* reductase which is inactivated by these reagents. These results suggest the possibility of a different structure or environment for the free radical in the mammalian enzyme.

Deoxyribonucleotides are synthesized by direct reduction of the corresponding ribonucleotides in a reaction catalyzed by ribonucleotide reductase. The level of enzyme activity is correlated to the growth rate of the tissue involved. This observation plus the finding that the pools of deoxyribonucleoside triphosphates in cells are very low indicates that the reaction catalyzed by ribonucleotide reductase plays a critical role in DNA synthesis and cell division (Thelander & Reichard, 1979).

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Ribonucleotide reductase has been isolated from *Escherichia coli* and *Lactobacillus leichmannii* and carefully studied (Thelander & Reichard, 1979). However, no eucaryotic reductase has been obtained as a homogeneous preparation and most studies have been done with crude extracts (Moore & Hurlbert, 1966; Larsson, 1969; Hopper, 1972). The best studied enzyme is the reductase from Novikoff hepatoma and in this case a high degree of purification of the enzyme has been reported (Moore, 1977). Also, Hopper (1978) has recently reported extensive purification of ribonucleotide reductase from rabbit bone marrow. The bacterial reductases are controlled by allosteric effectors in a complicated well-defined manner, but the lack of pure preparations of the mammalian enzyme has made detailed studies of allosteric control, subunit structure and reaction mechanism impossible. A major problem is the low level of reductase present in most eucaryotic cells. We have chosen calf thymus as a source for the purification of this enzyme since it contains a high proportion of proliferating cells and it is available in large quantities.

In this paper, a 3400-fold purification of ribonucleotide reductase from calf thymus is described and the preparation is characterized with respect to homogeneity, stability, sedimentation properties, involvement of iron in the reaction, and reaction mechanism. In an accompanying paper, the allosteric regulation of the enzyme is reported (Eriksson et al., 1979).

Experimental Procedure

Materials

Thymuses from 2–4 month old calves were obtained from local slaughter houses. DEAE (DE 52) was purchased from Whatman and hydroxylapatite (Hypatite C) from Clarkson Chemical Co. dATP–Sepharose was synthesized according to Berglund and Eckstein as modified by Knorre et al. (1976). Streptomycin sulfate was obtained from Sigma Chemical Co. and [^{3}H]CDP, UltraPure Tris base, and NaCl from Schwarz/Mann. Radioactive iron, $^{59}FeCl_3$, specific activity 8.5 μCi/μg of Fe, was purchased from the Radiochemical Centre, Amersham, England.

Methods

Assay. The following reagents were incubated at 37 °C for 30 min in a final volume of 50 μL: 25 nmol of [^{3}H]CDP (specific activity, 20 000 cpm/nmol), 2 μmol of 4-(2-hydroxyethyl)-1-piperazinesulfonic acid buffer, pH 7.6, 0.15 μmol of ATP, 0.32 μmol of $MgCl_2$, 0.5 μmol of dithiothreitol, 5 μmol of KCl, 1 nmol of $FeCl_3$, and enzyme as indicated. The reaction was stopped by the addition of 0.5 mL of 1 M perchloric acid, 0.5 μmol of dCMP was added, and precipitated protein was removed by centrifugation. This is an important step since the presence of protein sometimes results in high blank values. After hydrolysis for 10 min at 100 °C, the deoxycytidine monophosphate formed was isolated by chromatography on Dowex-50 W-X8, 200–400 mesh. To gain time, the original procedure (Reichard, 1958) was scaled down in the following way: Dowex-50 columns, 1 × 3.5 cm (Bio-Rad Econo columns, 1 × 10 cm with polypropylene funnels), were used, and CMP was eluted in 55 mL of 0.2 M acetic acid and dCMP in a second wash of 25 mL of 0.2 M acetic acid. The whole chromatographic procedure was finished within 2 h, and the recovery of the dCMP carrier was about 70%.

Before assaying fractions from the DEAE or hydroxylapatite chromatograms, they had to be concentrated. Solid ammonium sulfate was added to 70% saturation and the protein precipitates were collected by centrifugation and dissolved in 50 mM Tris–Cl, pH 7.6, to give a protein concentration of about 10 mg/mL. The presence of ammonium sulfate inhibited in the assay and, therefore, aliquots (200 μL) of the solutions were freed from this salt prior to assaying by centrifugation through 1-mL Sephadex G-25 columns equilibrated with 50 mM Tris-Cl, pH 7.6, in polyallomer tubes (7/16 × 2 3/8 in., Beckman) (Neal & Florini, 1973).

One unit of ribonucleotide reductase activity is defined as the amount of enzyme which catalyzes the formation of 1 nmol of deoxycytidine diphosphate (isolated as dCMP) per min under standard conditions.

Protein Determinations. In the fractions preceding the hydroxylapatite step, protein was determined according to Lowry as modified by Jovin by using bovine serum albumin as a standard (Jovin et al., 1969).

In the analytical glycerol gradient centrifugations, protein was determined by using Coomassie Brilliant Blue G-250 as described by Bradford (1976). The presence of effectors and dithiothreitol absorbing at 280 nm did not interfere with this assay.

In all other cases, the absorbance at 280 nm in 1.0-cm cuvettes was used by assuming that an absorbance of 1.0 corresponded to a protein concentration of 1 mg/mL.

Conductivity Measurements. All measurements were made at about 0 °C with a Radiometer conductivity meter CDM 3 by using a cell type CDC 314.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. To one volume of protein solution containing 6–30 μg of protein in a centrifuge tube was added 1 volume of 10% (w/v) trichloroacetic acid and the sample was incubated on ice for 5 min. After centrifugation, the supernatant solution was removed and the precipitate was dissolved and incubated for 10 min at 25 °C in 50 μL of a solution containing 26% glycerol, 0.4 M Tris base, 1% (v/v) 2-mercaptoethanol, 1% (w/v) sodium dodecyl sulfate, and 0.01% (w/v) bromphenol blue. Then 5 μL of 1 M HCl was added followed by heating to 100 °C for 2 min. Ten or twenty microliters of the resulting solution was analyzed on a 7.5% linear polyacrylamide slab gel by using a stacking gel and a discontinuous buffer system (O'Farell, 1975). After staining, the gels were scanned in a Joyce-Loebl microdensitometer.

Inactivation with EDTA. About 5 mg of enzyme in 250 μL of 50 mM Tris-Cl (pH 7.6)–0.1 M KCl was dialyzed overnight at 4 °C in a collodion bag (Sartorius-Membranfilter GmbH) against 0.1 M EDTA–1 mM dithiothreitol. The protein solution was then chromatographed on a 0.7 × 6.4 cm column of Sephadex G-25 washed with EDTA and then equilibrated with 50 mM Tris-Cl, pH 7.6, 0.1 M NaCl (UltraPure).

Reactivation with ^{59}Fe. About 3 mg of EDTA-inactivated enzyme in 380 μL of 50 mM Tris-Cl (pH 7.6)–0.1 M NaCl was mixed with 60 μL of 30 mM sodium ascorbate–0.2 M Tris-Cl, pH 7.6, containing 120 nmol of $^{59}FeCl_3$ (specific activity, 75 000 cpm/nmol) and incubated for 5 min at 37 °C. Before mixing, the iron ascorbate solution was kept for 5 min at 25 °C to allow reduction of the Fe^{3+} and formation of the violet iron(II) ascorbate complex (Atkin et al., 1973). Solid ammonium sulfate was added to 80% saturation; the protein precipitate was collected by centrifugation at 0 °C, washed twice in 80% saturated ammonium sulfate–50 mM Tris-Cl (pH 7.6)–10 mM sodium ascorbate, and dissolved in 200 μL of 50 mM Tris-Cl, pH 7.6. The solution was immediately passed through a 0.36 × 21 cm column of Sephadex G-25 equilibrated with 50 mM Tris-Cl (pH 7.6)–0.1 M KCl, and

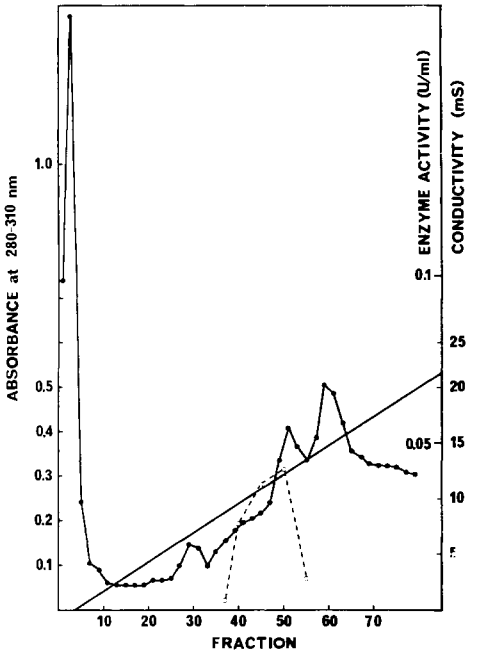

FIGURE 1: DEAE-cellulose chromatography of calf thymus ribonucleotide reductase. This represents a scaled down version of the standard DEAE chromatography described in the text. Dialysate after ammonium sulfate precipitation (4100 mg corresponding to about 0.4 kg of thymus) was applied to a 6 × 23 cm column of DEAE-cellulose. Protein was eluted with a linear potassium chloride gradient, 4.5 L of 10 mM potassium phosphate buffer, pH 7.0, and 4.5 L of the same buffer plus 0.25 M KCl. Fractions of 90 mL were collected. (●—●) $A_{280-310}$; (O---O) reductase activity (units/mL); and (—) conductivity (mS).

the protein eluate was analyzed by glycerol gradient centrifugation.

Purification of Ribonucleotide Reductase. All steps in the procedure were performed at about 4 °C except for the dATP–Sepharose chromatography which was done at 25 °C.

Preparation of Crude Extract. Frozen thymuses (2700 g) were split into pieces which were thawed in 8 L of 50 mM Tris-Cl, pH 7.6, for 60 min at 4 °C. This half-thawed tissue was minced and extracted in a homogenizer (CUT-O-MAT, type H 10, Kneubühler & Co., Luzern) for 5 min, and the slurry was centrifuged for 30 min at 20000*g* to remove insoluble debris. The supernatant solution was filtered through glass wool to remove flotating lipids, giving 7 L of opalescent crude extract.

Precipitation with *Streptomycin.* A 6.5% (w/v) solution of streptomycin sulfate (700 mL) was added during 30 min to 7 L of the crude extract with vigorous stirring. The solution was centrifuged for 20 min at 20000*g*, the precipitate was discarded, and the supernatant (7.5 L) was used in the next step.

Precipitation with Ammonium Sulfate. Solid ammonium sulfate (0.243 g/mL, corresponding to 40% saturation) was slowly added to the streptomycin supernatant with stirring and, after 30 min of additional stirring, the suspension was centrifuged 20 min at 20000*g*. The supernatant solution was discarded or used for preparing thioredoxin, and the precipitate was dissolved in 10 mM Tris-Cl, pH 7.6, and dialyzed against the same buffer overnight. The precipitate was removed by centrifugation. The resulting solution had a volume of 1500 mL and a conductivity of 2–3 mS.

Chromatography on DEAE-cellulose. The dialysate after ammonium sulfate precipitation was diluted with 10 mM Tris-Cl, pH 7.6, to give a conductivity of less than 1.8 mS and then adsorbed to a column of DEAE-cellulose (11 × 32 cm)

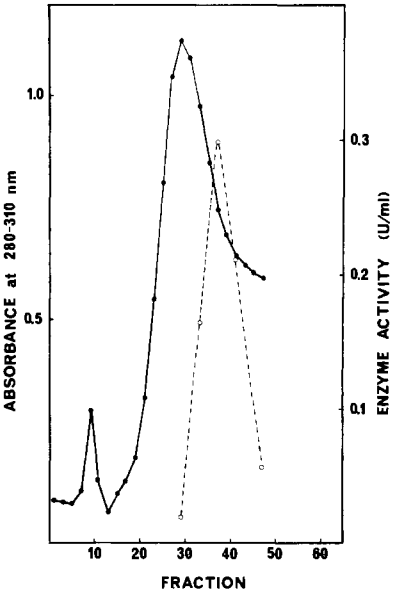

FIGURE 2: Hydroxylapatite chromatography of calf thymus ribonucleotide reductase. The pooled fractions, containing the enzyme activity from a DEAE-cellulose chromatogram (8000 mg corresponding to 2.7 kg of thymus), were adsorbed to a column of hydroxylapatite (see text). Fractions of 90 mL were collected every 20 min and analyzed for absorbance at 280 nm (●—●) and enzyme activity (O---O).

equilibrated with 10 mM potassium phosphate buffer, pH 7.0. The protein was eluted at 18 mL/min with a linear gradient composed of 13.5 L of 10 mM potassium phosphate, pH 7.0, and 13.5 L of the same buffer, containing 0.25 M potassium chloride. The active fractions (Figure 1) were combined and adsorbed directly to hydroxylapatite. The elution pattern from the DEAE columns was very reproducible, and the fractions containing ribonucleotide reductase were pooled routinely only according to conductivity (between 7.6 and 14.3 mS).

Chromatography on Hydroxylapatite. The pooled DEAE chromatography fractions (about 9 L) were adsorbed directly to a hydroxylapatite column (8.4 × 16 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.0, and elution was performed with a linear gradient of potassium phosphate, pH 7.0, from 10 to 200 mM with a total volume of 6 L (Figure 2). The active fractions (about 1.5 L containing 1100 mg of protein) were pooled, the protein was precipitated by dialysis overnight against saturated ammonium sulfate solution, pH 7.0, and the precipitate was collected by centrifugation and suspended in 80% saturated ammonium sulfate–50 mM Tris-Cl, pH 7.6.

Chromatography on dATP–Sepharose. The ammonium sulfate suspension from one hydroxylapatite chromatogram was centrifuged, and the precipitate was dissolved in 50 mM Tris-Cl, pH 7.6, to give a protein concentration of about 25 mg/mL. After removal of insoluble precipitates by centrifugation, the ammonium sulfate was removed from the solution by chromatography on a column of Bio-Gel P-10 (10 column volumes per volume of solution) equilibrated with 50 mM Tris-Cl, pH 7.6. The eluate from the Bio-Gel column (100 mL containing 860 mg of protein) was warmed in a 37 °C water bath to 25 °C and adsorbed slowly to a 1.5 × 2 cm column of dATP–Sepharose equilibrated with the same buffer at room temperature. Chromatography was then performed at this temperature (Hoffman & Blakley, 1975). The column was first washed with buffer until the A_{280} of the eluate was less than 0.1 (usually about 30 mL of buffer) and then with 36 mL of buffer containing 0.5 mM ATP. Ribonucleotide reductase activity was eluted during 60 min with 12 mL of

Table I: Purification of Calf Thymus Ribonucleotide Reductase from 2.7 kg of Thymus

	protein (mg)	total act. (units)	sp act. (units/mg)
crude extract	115 500	800[a]	0.007[a]
streptomycin supernatant	90 000	900[a]	0.01[a]
ammonium sulfate	26 550	800	0.03
DEAE-cellulose	8 010	640	0.08
hydroxylapatite	1 100	429	0.39
dATP-Sepharose	10	237	23.7

[a] Approximate figures since the extracts at this level of purity contain inhibitory factors. The assays were performed at 25 °C, and the values obtained were doubled.

buffer containing 50 mM ATP (compare Thelander, 1973), and the eluate was collected in a cylinder immersed in ice. The dATP–Sepharose chromatography usually took about 3.5 h. The protein in the ATP eluate was precipitated by dialysis overnight against saturated ammonium sulfate solution as described for the hydroxylapatite pool and the protein suspension was stored at −70 °C. Before use, ATP and ammonium sulfate were removed from the protein by chromatography on Sephadex G-25, and this is the material used in the following studies.

Results

Purification of Calf Thymus Ribonucleotide Reductase. The purification is summarized in Table I. One kilogram of thymus usually yields 3–4 mg of protein with a specific activity of about 20 units/mg, although values as low as 13 or as high as 33 were occasionally observed. The variation probably reflects both the difficulties in assaying ribonucleotide reductase (see below) and differences in the purity of the enzyme preparations. The recovery in the early fractions was difficult to estimate but in all later steps the recoveries were 50–70%, giving a total recovery of approximately 25% (Table I).

Stability and Storage. The enzyme activity was quite stable during the preparation as long as no separation method based on differences in molecular size such as gel filtration or density gradient centrifugations was applied (see below). In contrast to *E. coli* ribonucleotide reductase, the presence of dithiothreitol and Mg^{2+} during the purification was not required (Thelander, 1973). The ammonium sulfate suspension of the enzyme was routinely dissolved in 50 mM Tris-Cl, pH 7.6, and equilibrated with the same buffer containing 0.1 M KCl on columns of Sephadex G-25 before use without any loss of activity. The presence of KCl was necessary to prevent the enzyme from precipitating. The activity of the enzyme was stable for several months at −70 °C either as an ammonium sulfate suspension or as a protein solution in 50 mM Tris-Cl (pH 7.6)–0.1 M KCl.

Analytical Gel Electrophoresis under Nondenaturing Conditions. To test the homogeneity of the ribonucleotide reductase preparation after chromatography on dATP–Sepharose, samples were analyzed by electrophoresis on polyacrylamide gels containing glycerol by using a discontinuous buffer system as described (Fisher & Korn, 1977). A single, slightly asymmetric band was observed (Figure 3A).

Electrophoresis under Denaturing Conditions. In Figure 3B, the result of analysis of the enzyme by sodium dodecyl sulfate–polyacrylamide gel electrophoresis is shown. There is one major peak (molecular weight 84 000) and in addition some minor components of lower molecular weight. From these experiments, the enzyme appeared nearly homogeneous, but there were no data showing that the activity corresponded to the observed protein.

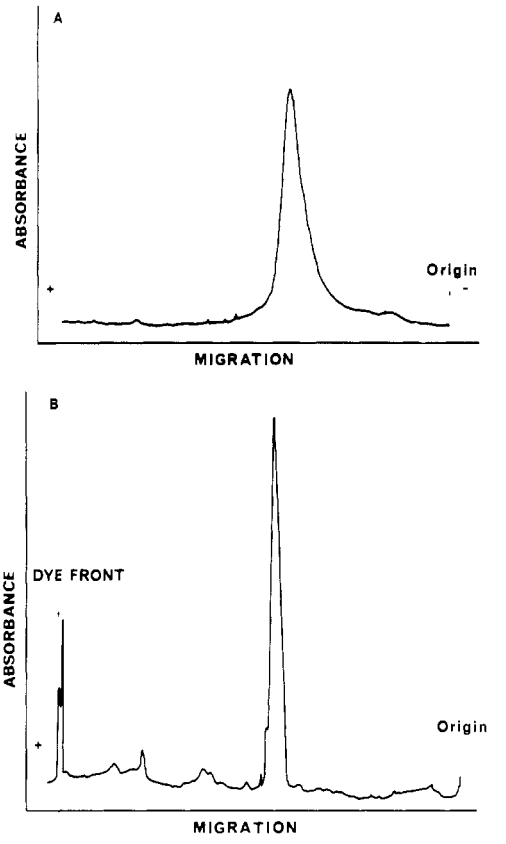

FIGURE 3: Polyacrylamide gel electrophoresis of the thymus ribonucleotide reductase. (A) Nondenaturing conditions: enzyme (18 μg) with a specific activity of 20.0 U/mg was analyzed on a 0.5 × 10 cm cylindrical 5% polyacrylamide gel as described by Fisher & Korn (1977). Bromphenol blue was used as a tracking dye, and the run was stopped when the dye was 0.5 cm from the bottom of the tube. Migration was toward the anode. The gel was scanned in a Joyce-Loebl microdensitometer. (B) Denaturing conditions: enzyme (11 μg) with a specific activity of 15.0 U/mg was analyzed on a 7.5% linear polyacrylamide slab gel containing 0.1% sodium dodecyl sulfate as described under Experimental Procedure.

Analytical Glycerol Gradient Centrifugations. The enzyme was analyzed further by glycerol gradient centrifugations in an attempt to show correspondence between activity and protein. When analyzed by centrifugation through a glycerol gradient containing 10^{-4} M dTTP, the protein sedimented as one major peak (9 S) followed by two minor ones (Figure 4A). However, the activity did not coincide with the protein profile but instead moved with a sedimentation coefficient of 10 S, corresponding to the leading half of the major protein peak. This pattern was repeated in numerous gradients. Only about 15% of the applied enzyme activity was recovered in the gradient, and no significant stimulation of the activity was observed when various fractions were recombined. The same pattern as in Figure 4A was obtained in the absence of effector but the peaks were less distinct. The 84 000 molecular weight polypeptide was the principal component in analyses by sodium dodecyl sulfate–polyacrylamide gel electrophoresis of fractions from various parts of the gradient (Figure 4B). Another constant feature was the presence of a 55 000 molecular weight polypeptide in gels from fractions in the leading part of the major protein peak, i.e., corresponding to the enzyme activity. The results fit the hypothesis that the active ribonucleotide reductase complex consists of two loosely associated subunits, with one present in excess over the other.

Influence of Allosteric Effectors on the Sedimentation Profile of the Enzyme. Ribonucleotide reductases from calf

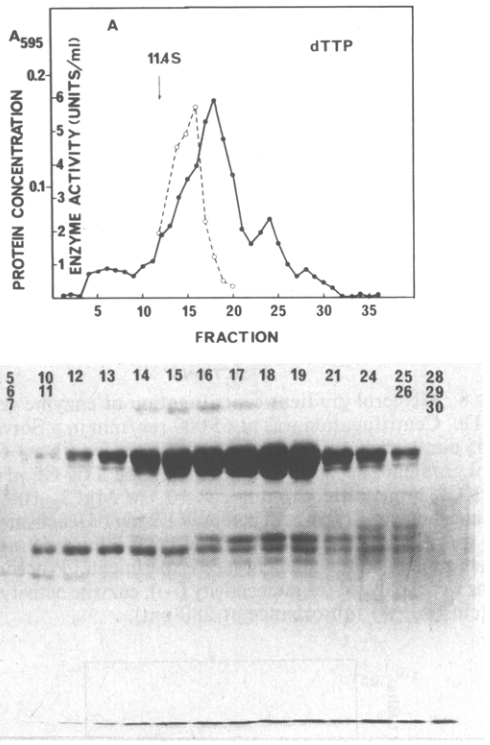

FIGURE 4: Analytical glycerol gradient centrifugation of the thymus ribonucleotide reductase and electrophoretic analysis of the fractions. (A) Enzyme (1.3 mg) with a specific activity of 13 U/mg was applied to a glycerol gradient (25–40%) containing 50 mM Tris-Cl (pH 7.6)–0.1 M KCl–2 mM dithiothreitol–10 mM $MgCl_2$–10^{-4} M dTTP in a volume of 3.8 mL. Catalase ($s_{20,w}$ = 11.4 S) was added as a reference. Sedimentation (from the right to the left) was performed at 60 000 rev/min in a Spinco Model L2-65B centrifuge in an SW 60 rotor for 16 h at −5 °C. Aliquots of the fractions indicated were tested directly for activity (O---O). Protein was determined on 10-μL aliquots of each fraction, by reading the A_{595} (●—●) after addition of 1.0 mL of Coomassie Brilliant Blue protein reagent (see Experimental Procedure). (B) Before sodium dodecyl sulfate gel electrophoresis, 50 μL of the fractions indicated was precipitated with trichloroacetic acid as described under Experimental Procedure, and 10 μL of the sample solutions was analyzed on the slab gel. Fractions are numbered as in A. The upper arrow shows the position of the 84 000 molecular weight polypeptide and the lower arrow the position of the 55 000 molecular weight polypeptide.

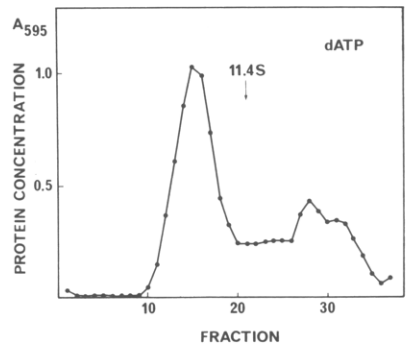

FIGURE 5: Influence of dATP on the sedimentation behavior of thymus ribonucleotide reductase. Enzyme (0.4 mg) with a specific activity of 23.3 U/mg was centrifuged as described in the legend to Figure 4 but the gradient contained 5×10^{-5} M dATP instead of dTTP. To each fraction of the gradient 1.0 mL of Coomassie Brilliant Blue protein reagent was added after removal of 5 μL for catalase assay and then the absorbance at 595 nm was recorded (see Experimental Procedure).

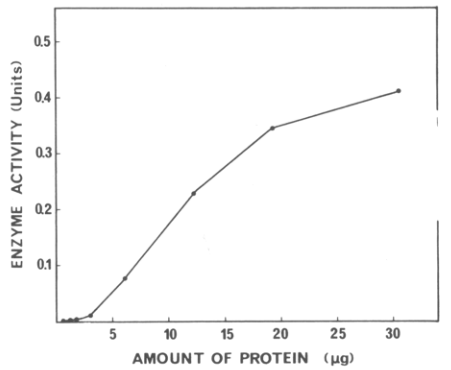

FIGURE 6: Relationship between reaction rate and enzyme concentration. The enzyme had a specific activity of 18.9 U/mg.

thymus or *E. coli* are controlled by allosteric effectors in a similar way (Eriksson et al., 1979). Since the sedimentation properties of the bacterial enzyme are influenced strongly by effectors (Brown & Reichard, 1969a), it is important to see if the calf thymus enzyme behaves in the same way. Glycerol gradient centrifugation of ribonucleotide reductase in the presence of 10^{-4} M dGTP showed the same basic pattern as in Figure 4A. In contrast, 5×10^{-5} M dATP shifts the position of the major protein peak completely to about 16 S (Figure 5). When fractions from this heavy peak were tested in the presence of high concentrations of ATP, ribonucleotide reductase activity was detected, although in very low yields (compare Eriksson et al., 1979). Finally, in the presence of ATP, the protein sediments in a complex manner, with at least four components (16–4.7 S). The same pattern as with ATP alone was observed with 2×10^{-5} M dTTP + 1 mM ATP, which is the best combination of effectors for GDP reduction (Eriksson et al., 1979).

Requirements for the Enzyme Reaction. Nonlinear Enzyme Concentration Curve. The calf thymus ribonucleotide reductase resembled reductases from other mammalian tissues in not giving a strict linear relationship between activity and amount of protein (Figure 6) (Larsson, 1969; Hopper, 1972, 1978; Moore, 1977). An initial lag at low protein concentrations was always observed and the extent of this lag varied between different preparations. Above the lag, the activity was proportional to the amount of enzyme up to about 50% conversion of the substrate CDP.

Time and Temperature Dependence. The lag period was not due to any time-dependent reactivation process at low protein concentrations since the initial rate of ribonucleotide reduction was linear with time both at high (0.5 mg/mL) and at low (0.1 mg/mL) protein concentrations in the assay. Instead, the data suggest the presence of a dissociating system in the enzyme preparations.

The reaction rate was about twice as fast at 37 °C as the rate at 25 °C (data not shown).

Salt Dependence. Optimal activity was observed in 40 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, pH 7.6, in the presence of 0.08–0.12 M KCl. In lower salt the enzyme started to precipitate, and in higher salt there was a pronounced inhibition.

Magnesium Ion Dependence. Unlike the *E. coli* ribonucleotide reductase (Larsson & Reichard, 1966), the calf thymus enzyme showed about 50% of maximal activity in the absence of added magnesium ions. Optimal magnesium ion concentration varied with the concentration of nucleotide effector, with maximal activity at a concentration of magnesium about twice the concentration of the effector (data not shown).

Involvement of Iron. Ribonucleotide reductase preparations from mammalian tissues were reported to be stimulated by iron (Larsson, 1969; Hopper, 1972, 1978; Moore, 1977) and, therefore, we routinely added $FeCl_3$ to our assay mixtures.

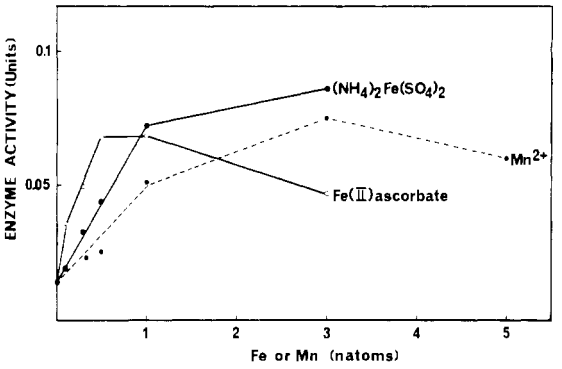

FIGURE 7: Reactivation of EDTA-treated enzyme by iron or manganese. To a series of tubes, each containing 11.6 μg of EDTA-treated enzyme (see text) in 30 μL of 50 mM Tris-Cl, pH 7.6, 0.1–5 ng-atoms of ferrous ammonium sulfate, Fe(II) ascorbate, or $MnCl_2$ was added. After mixing, the standard assay components except for $FeCl_3$ were added and incubation was made at 37 °C for 30 min in the usual way.

However, we never observed any significant stimulation of our reductase preparations by iron. Still, repeated analyses for bound nonheme iron by the bathophenanthrolinesulfonate method (Atkin et al., 1973) gave no indication of the presence of stoichiometric amounts of iron in the enzyme. (The lower limit of the assay is 0.6 ng-atom of iron per mg of protein.)

We then evaluated the effects of some potent metal chelators on ribonucleotide reductase activity. Dialysis against 8-hydroxyquinoline-5-sulfonate, 1 M imidazole hydrochloride, pH 7.0, as described for removing iron from *E. coli* ribonucleotide reductase (Atkin et al., 1973) did not influence the activity of the mammalian enzyme, nor did dialysis against 1 mM 8-hydroxyquinoline-5-sulfonate–50 mM Tris-Cl (pH 7.6)–0.1 M KCl for 1 week at 4 °C. However, incubation in 1.5 mM bathophenanthrolinesulfonate for 30 min at 25 °C resulted in complete inactivation, and addition of $FeCl_3$ to the assay mixture gave complete reactivation. However, complete reactivation was also obtained in the *absence* of added iron after passage of the inactivated protein through a Sephadex G-25 column to remove the bathophenanthrolinesulfonate. Therefore, inhibition by this chelator could not be taken as evidence for the presence of iron in the reductase.

Finally, we tried incubation with EDTA. Dialysis against 0.1 M EDTA–50 mM Tris-Cl, pH 7.6, for 72 h at 4 °C followed by removal of the EDTA by passage through a Sephadex G-25 column equilibrated with 50 mM Tris-Cl (pH 7.6)–0.1 M NaCl resulted in an enzyme preparation retaining only 10–20% of the activity as compared with a control, both assayed in the absence of iron. Addition of iron as $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ or as an Fe(II) ascorbate complex gave a maximal reactivation of 88%, the latter being more efficient (Figure 7). Surprisingly, $MnCl_2$ reactivated the enzyme almost as well as iron, while $CuCl_2$, zinc(II) acetate, $CoCl_2$, and $NiCl_2$ had no effect. The iron-containing ribonucleotide reductase of *E. coli* was not reactivated by $MnCl_2$ (Brown et al., 1969).

In Figure 8 is shown an analysis by glycerol gradient centrifugation of an enzyme preparation inactivated with EDTA and reactivated with radioactive ^{59}Fe (see Experimental Procedure). The starting enzyme had a specific activity of 13.3 U/mg, the EDTA-inactivated enzyme a specific activity of 2.8 U/mg, and the ^{59}Fe-reactivated enzyme a specific activity of 23.3 U/mg. There is a fair correlation between enzyme activity and radioactivity sedimenting in the leading half of the main protein peak. The active fractions (18–26) were pooled and concentrated by dialysis against saturated ammonium sulfate overnight, and the precipitated protein was

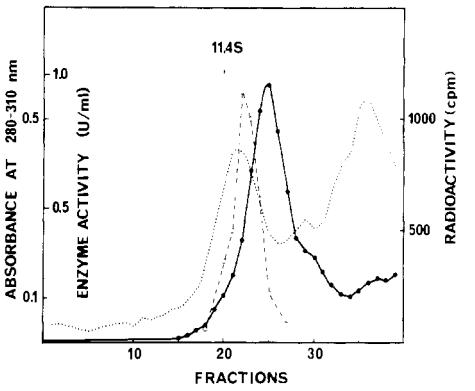

FIGURE 8: Glycerol gradient centrifugation of enzyme reactivated with ^{59}Fe. Centrifugation was at 65 000 rev/min in a Sorvall Model OTD-65 ultracentrifuge with a TV-865 B rotor for 5 h at −5 °C. The glycerol gradient (25–40%) contained 50 mM Tris-Cl, pH 7.6, and 0.1 M KCl–2 mM dithiothreitol–2×10^{-4} M $MgCl_2$–10^{-4} M dTTP in a final volume of 17.0 mL. There was 1.9 mg of reactivated enzyme with a specific activity of 23.3 U/mg containing 0.12 ng-atom of Fe/mg of protein on the gradient (see Experimental Procedure). The fractions were analyzed for radioactivity (···), enzyme activity (O---O), or protein (●—●) (absorbance at 280 nm).

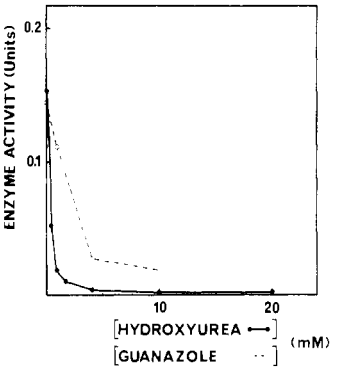

FIGURE 9: Inhibition of the thymus reductase by hydroxyurea or guanazole. Increasing amounts of each drug were added to a series of standard assay mixtures containing 9.6 μg of enzyme with a specific activity of 17 U/mg and incubation was performed for 30 min at 37 °C.

dissolved in 50 mM Tris-Cl, pH 7.6. About 15% of the applied enzyme activity was recovered in this concentrated pool after correction for losses of protein. The recovery of protein in the pool after concentration was 52%, while only 35% of the radioactivity was recovered, indicating a preferential loss of ^{59}Fe during the concentration step.

When the pooled and concentrated material was reanalyzed by glycerol gradient centrifugation only about 50% of the radioactivity sedimented with the leading part of the protein peak, while the rest sedimented near the top of the gradient, again indicating loss of iron from the protein (data not shown). Taken together, these results strongly argue for the presence of iron as a necessary component of the mammalian ribonucleotide reductase.

Effects of the Radical Scavengers Hydroxyurea and 2′-Deoxy-2′-azidocytidine Diphosphate on Thymus Ribonucleotide Reductase. Hydroxyurea inhibits DNA synthesis in eucaryotic cells and also in *E. coli*, and this effect is a result of interference with ribonucleotide reduction (Castellot et al., 1978). In *E. coli*, the drug acts as a radical scavenger and destroys the free radical of the B2 subunit (Atkin et al., 1973). Figure 9 shows the results of adding increasing amounts of hydroxyurea to the incubation mixture; 1 mM gave about 90% inhibition. The same degree of inhibition was observed at different concentrations of substrate or dithiothreitol, or when

the thioredoxin system (Luthman et al., 1979) replaced dithiothreitol. As a comparison, the inhibition caused by the drug guanazole is shown (Figure 9). Guanazole also inhibits DNA synthesis and is supposed to act by the same mechanism as hydroxyurea (Wright & Lewis, 1974).

The degree of inhibition of the thymus reductase by hydroxyurea is very similar to that observed with *E. coli* ribonucleotide reductase (Brown et al., 1969). However, incubation of the thymus enzyme in 10 mM hydroxyurea for 30 min at 37 °C followed by removal of the drug by passing the enzyme solution through a column of Sephadex G-25 did not result in inactivation, in strong contrast to what happens with the *E. coli* enzyme (Atkin et al., 1973). This picture was not changed by addition of effectors, magnesium ions, dithiothreitol, or substrate in various combinations together with hydroxyurea and enzyme. We conclude that the effect of hydroxyurea on the thymus enzyme is reversible.

2′-Deoxy-2′-azidocytidine diphosphate was shown earlier to inactivate the *E. coli* ribonucleotide reductase specifically as a k_{cat} inhibitor by destroying the free radical of protein B2 only during reduction (Thelander et al., 1976). However, when this analogue was tested in assays of the thymus reductase, it acted as a reversible inhibitor competitive with the normal substrate CDP, with a K_I of 2.3×10^{-5} M as compared with the K_m for CDP of 1.4×10^{-5} M, determined in the same experiment. Incubation of enzyme, ATP–Mg^{2+}, dithiothreitol, and nucleotide analogue (5×10^{-4} M) for 30 min at 37 °C followed by passage through a column of Sephadex G-25 to remove the analogue gave no inactivation of the enzyme, again in strong contrast to what happens with the *E. coli* reductase (Thelander et al., 1976). These results indicate that the catalytic site and probably also the catalytic mechanisms are different for the *E. coli* and mammalian enzymes even though both contain iron.

Discussion

This paper describes the first extensive purification of ribonucleotide reductase from calf thymus. One great advantage of this tissue compared with those used earlier (Larsson, 1969; Hopper, 1972; Moore, 1977) is that it is available in large amounts. However, only thymus from calves 2–4 months old contained high levels of ribonucleotide reductase. The specific activity in extracts from younger or older calves was much lower and gave quite irreproducible results (Abrams et al., 1960).

Several kinds of data indicate that the mammalian ribonucleotide reductases consist of at least two subunits, each one inactive when assayed alone (Hopper, 1972; Moore, 1977; Cory et al., 1978). However, attempts in our laboratory to purify those subunits separately gave very low yields and similar experiences were reported from another laboratory (Eriksson et al., 1977; Moore, 1977). Therefore, we aimed at a purification procedure which minimized separation of the subunits. The procedure is rapid and highly reproducible and results in a good yield of enzyme with the highest specific activity reported so far for a eucaryotic reductase.

The final preparation showed one major protein peak during gel electrophoresis (Figure 3). Under denaturing conditions, the molecular weight of this peak corresponded to 84 000. In equilibrium dialysis experiments, the protein bound up to 0.3 mol of dTTP or 0.8 mol of dATP per 84 000 g of protein with dissociation constants at 0 °C of 1.5×10^{-6} and 0.4×10^{-6} M, respectively (Eriksson, 1979, unpublished experiments). The B1 subunit of the *E. coli* ribonucleotide reductase consisting of two 80 000 molecular weight polypeptide chains binds 1 mol of dTTP or 2 mol of dATP per 80 000 g of protein (Brown & Reichard, 1969b). By implication, our results therefore suggest that the protein having a polypeptide molecular weight of 84 000 corresponds to the B1 subunit of the bacterial reductase and that about half of the final preparation is made up of this protein.

The second subunit of the *E. coli* reductase (B2 protein) contains iron and an organic free radical which can be inactivated by hydroxyurea (Atkin et al., 1973). Earlier work reported stimulation of mammalian reductases by iron. We did not find such an effect with the thymus enzyme but our results with the EDTA inactivated enzyme as well as the reactivation experiments with radioactive iron clearly demonstrate that iron is an essential component of the enzyme. However, the amount of iron in the enzyme was very low. Thus direct iron analyses showed less than 0.6 ng-atom of iron per mg of enzyme (limit of the assay) and a ^{59}Fe-reactivated enzyme with full activity contained only 0.12 ng-atom of iron per mg of protein (Figure 8). By assuming a molecular weight of the enzyme in the range of 200 000–300 000, this corresponds to about 0.03 atom of iron per mol of enzyme.

These data, as well as the inhibition of the enzyme by hydroxyurea, suggest that the thymus reductase also contains an iron-containing subunit, corresponding to the B2 protein of *E. coli*, but that this subunit in our final preparation is present in substoichiometric amounts. We propose that the iron-containing subunit is loosely associated with the protein having a polypeptide molecular weight of 84 000 and that the two proteins together make up the active enzyme. It is not clear why the final preparation contains so little of the iron subunit. It may be lost during an early purification step or, alternatively, be present in nonstoichiometric amounts already in the cell.

Our interpretation of the subunit structure is supported by the centrifugation experiment in glycerol gradient where activity did not coincide with the protein profile but instead sedimented in a position expected for a complex heavier than the major protein component. Gel electrophoresis of individual fractions showed the presence of the 84 000 polypeptide both in the position of enzyme activity and in the major protein peak. A polypeptide of molecular weight 55 000 was seen in those fractions from the glycerol gradient containing the activity and might correspond to the iron-containing subunit (Figure 4B). Further evidence for the involvement of the protein having a polypeptide molecular weight of 84 000 as a subunit of the reductase derives from the finding that addition of the negative effector dATP causes most of the protein to sediment more rapidly in the glycerol gradient. Such a dATP-induced aggregation was first observed for the *E. coli* ribonucleotide reductase and it has also been reported for a partially purified reductase from Ehrlich tumor cells (Brown & Reichard, 1969a; Klippenstein & Cory, 1978).

The plot of activity vs. enzyme concentration was nonlinear, with a distinct lag at low protein concentrations, also suggesting a dissociating system with at least two components (Larsson, 1969; Hopper, 1972, 1978; Moore, 1977). If one of the components is present in excess, most of it must be modified in a way that prevents it from forming an active complex with the other subunit readily. Such a modification could also explain the difficulties encountered in recombining the subunits of mammalian ribonucleotide reductase (Moore, 1977).

The thymus reductase has maximal activity with dithiothreitol or with a thioredoxin system as reducing substrate (Luthman et al., 1979). The reductase preparations were free of endogenous thioredoxin as analyzed by a radioimmune assay (data not shown) and, therefore, the electrons from dithio-

threitol were not transferred via thioredoxin.

The inhibition studies with hydroxyurea and 2′-deoxy-2′-azidocytidine diphosphate suggested that the thymus enzyme might not permanently contain a free radical species but instead formed such a species only during the reaction. However, conclusive results regarding the reaction mechanism and catalytic site of mammalian reductases will require homogeneous preparations of the iron-binding subunit and an understanding of the conditions needed for recombination of the two proteins into an active enzyme complex.

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Allosteric Regulation of Calf Thymus Ribonucleoside Diphosphate Reductase†

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ABSTRACT: A highly purified ribonucleotide reductase from calf thymus catalyzed the reduction of all four ribonucleoside diphosphates at almost identical rates. Substrate specificity is regulated by allosteric effects. The activities toward CDP and GDP were purified in parallel and the two nucleotides competed for the same catalytic site. Taken together the data show that the same enzyme also in mammalian cells can reduce all four ribonucleotides. In the absence of positive effectors, the enzyme was inactive with any ribonucleoside diphosphate. Reduction of CDP and UDP was stimulated by ATP, reduction of GDP by dTTP, and reduction of ADP by dGTP. Reduction of the purine ribonucleotides was further stimulated by ATP combined with dTTP or dGTP. dATP served as a general inhibitor whose negative effects could be reversed by ATP. Inhibition was also caused by dTTP or dGTP which, in stimulating reduction of a single substrate, inhibited reduction of the other three substrates at the same time. The general pattern of regulation is similar to that observed for the *Escherichia coli* enzyme, but the effector requirements were more distinct with the mammalian enzyme. Our results fully explain the variations in pools of deoxyribonucleoside triphosphates observed earlier for cells in tissue cultre upon exposure to certain inhibitors of DNA synthesis. They may also explain the mechanism behind two immunodeficiency diseases associated with an inherited deficiency in the enzymes adenosine deaminase or purine nucleoside phosphorylase.

Two types of ribonucleotide reductases have been studied in detail: the cobamide-dependent ribonucleoside *tri*phosphate reductase from *Lactobacillus leichmannii* and the ribonucleoside *di*phosphate reductase from *Escherichia coli* which contains nonheme iron (Thelander & Reichard, 1979). In both cases the same enzyme reduces all four ribonucleotides and the substrate specificity and overall activity of the enzyme are regulated by nucleoside triphosphates which act as allosteric effectors (Thelander & Reichard, 1979).

Studies on chick embryo homogenates (Reichard et al., 1961) first demonstrated a regulatory mechanism for the synthesis of deoxyribonucleotides from ribonucleotides.

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