Sheppard, E. P., Hogan, K., and Roberts, K. B. (1970), *Biochim. Biophys. Acta 217*, 159.
Sox, H. C., Jr., and Hoagland, M. B. (1966), *J. Mol. Biol. 20*, 113.
Takagi, M., Tanaka, T., and Ogata, K. (1970), *Biochim. Biophys. Acta 217*, 148.
Tanaka, T., Takagi, M., and Ogata, K. (1970), *Biochim. Biophys. Acta 224*, 507.
Wilson, S. H., and Hoagland, M. B. (1967), *Biochem. J. 103*, 556.
Wittman, J. S., II, Lee, K. L., and Miller, O. N. (1969), *Biochim. Biophys. Acta 174*, 536.

A Monomeric, Allosteric Enzyme with a Single Polypeptide Chain. Ribonucleotide Reductase of *Lactobacillus leichmannii*†

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ABSTRACT: Ribonucleoside triphosphate reductase has been purified from *Lactobacillus leichmannii* on a large scale as well as on the conventional scale. The final preparation has been shown homogeneous by behavior in plateau gel filtration, velocity sedimentation as examined by several criteria, equilibrium sedimentation, and polyacrylamide gel electrophoresis. The mean value of the molecular weight determined by all methods is 76,000. Equilibrium sedimentation under various denaturing conditions and polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate indicated that the protein cannot be disaggregated into subunits. The amino acid analysis is consistent with a molecular weight of 76,000 and indicates the presence of eight methionine residues. Cleavage with cyanogen bromide gave the expected nine peptides as shown by polyacrylamide gel electrophoresis and by gel filtration. The only detectable N-terminal residue is serine and the only C-terminus lysine. No aggregation of the enzyme occurred in the absence or presence of allosteric modifiers under the experimental conditions employed, except under denaturing conditions.

The ribonucleotide reductase of *Lactobacillus leichmannii* catalyzes reduction of GTP, ATP, CTP, ITP, and to a much smaller extent, UTP by dithiols such as dihydrolipoate or by reduced thioredoxin, a low molecular weight protein with two sulfhydryls (Blakley and Vitols, 1968). This reduction is absolutely dependent on the presence of deoxyadenosylcobalamin (coenzyme B_{12}). Several of the deoxynucleoside triphosphate products specifically activate reduction of particular ribonucleotides. For example, dGTP specifically activates ATP reduction and dATP specifically activates CTP reduction. On the basis of kinetic studies it has been suggested (Goulian and Beck, 1966; Beck, 1967; Vitols *et al.*, 1967a) that the deoxynucleotides produce such activation by binding at a site other than the catalytic site, that is at an allosteric or regulatory site. Kinetic evidence has also been obtained (Vitols *et al.*, 1967a) which suggests that ribonucleoside triphosphates also bind at such a site.

Binding of deoxynucleotide modifiers at the regulatory site has a profound effect on the interaction of cobamides with the enzyme. Thus, binding of cob(II)alamin (B_{12r}) to the active site is greatly enhanced by such modifiers, each of which probably also determines a specific conformation of the enzyme–cobamide complex (Hamilton *et al.*, 1971; Yamada *et al.*, 1971). Exchange of hydrogen between water and the cobalt-bound methylene group of 5′-deoxyadenosylcobalamin is dependent on a modifier nucleotide (Hogenkamp *et al.*, 1968), and degradation of deoxyadenosylcobalamin in presence of enzyme and dithiol to 5′-deoxyadenosine and cob(II)alamin, presumably *via* an active intermediate, occurs only in presence of a modifier (Hamilton *et al.*, 1971; Yamada *et al.*, 1971).

Many enzymes subject to regulatory control, including most of those studied intensively, have proven to be oligomeric proteins. However, estimates of the molecular weight of ribonucleotide reductase of *L. leichmannii* (Goulian and Beck, 1966; Vitols *et al.*, 1967b) indicate that this enzyme is unlikely to contain many subunits, and indeed suggest that it might be monomeric. It was therefore of interest to redetermine the molecular weight of the lactobacillus reductase, to determine whether any aggregation or gross conformational changes occur in the presence of modifiers and to establish the number of polypeptide chains constituting the catalytically active enzyme.

Materials

Materials were obtained commercially as follows: sodium dodecyl sulfate and urea, Fisher (both chemicals were recrystallized from 95% ethanol); guanidine hydrochloride (enzyme grade), ammonium sulfate, imidazole, and diisopropyl fluorophosphate (DFP) treated bovine pancreatic carboxypeptidase A, Mann Research Laboratories; 3,3-dimethylglutaric acid, Tris, serononin creatinine sulfate, didimethylaminonaphthalenesulfonyl chloride (dansyl chlo-

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ride), danyslamino acids, ribonuclease A, cytochrome *c*, 1,4-dithiothreitol, and DFP-treated hog pancreatic carboxypeptidase B, Sigma Chemical Co.; *N*-ethylmorpholine, Eastman Organic Chemicals (this chemical was redistilled before use); cyanogen bromide, Aldrich Chemical Co.; tritiated water, New England Nuclear Corp.; nucleotides, P-L Biochemicals; human serum albumin, Pentex Inc.; human γ-globulin, Commonwealth Serum Laboratories, Melbourne, Australia; protamine sulfate, Sigma and Krishell Laboratories; crystalline rabbit muscle creatine kinase, Worthington; ovalbumin, Sigma and Worthington.

Methods

Purification of Ribonucleotide Reductase. The enzyme was prepared essentially according to the method previously described (Vitols *et al.*, 1967a) but with several modifications which increased the purification while permitting adaption to a large scale procedure. The method has been used extensively on the small scale (120 g of cell paste) and several times on the large scale (4–5 kg). In the following, quantities applicable to both scales are given. The early stages of the first two large-scale preparations were carried out at the New England Enzyme Center, Boston.

The organism and its growth and harvesting were as previously described (Blakley, 1965; Vitols *et al.*, 1967a), except as follows. The organism was maintained by monthly transfers in stab cultures prepared by addition of agar (1.5 g/100 ml) to the liquid medium used for growth. The original medium (Blakley, 1965) was modified by decreasing the casein hydrolysate to 1.5 g/l. and adding L-tyrosine (50 mg/l.), the amino acid which analyses suggested most likely to be present in limiting amounts. Casamino Acids low in cyanocobalamin must be used in order to avoid repression of reductase synthesis (Ghambeer and Blakley, 1966). The minimum concentration of added cyanocobalamin necessary for optimum growth was determined with each batch of casamino acids and varied from 50 to 0 ng per l. When the absorbance at 660 nm reached 1.1, the culture was chilled rapidly (below 20° within 15 min) and was kept below 20° during harvesting which was completed within 1.5 hr. Since experiments showed that the reductase content of cells in suspension slowly declined even at 5°, the cell paste was not washed but immediately frozen in liquid nitrogen or Dry Ice. Cells were grown in the laboratory in volumes of medium up to 60 l. Large batches (two 860-l. portions) were grown by the Grain Processing Co., Muscatine, Iowa. In both cases the yield was about 2 g of cell paste/l.

Frozen cells were thawed in the cold room (5°) overnight or in a few hours by standing the containers in water at room temperature. The thawed cells were suspended in five to eight volumes of 0.1 M potassium phosphate buffer (pH 7.3) and disrupted either in a Ribi cell fractionator at 20,000 psi or, on the large scale, by two passages through a Manton-Gaulin mill. The insoluble material was removed by centrifugation (2 hr at 25,000*g*) at 5° and all further steps were carried out at 5°.

The soluble extract was dialyzed overnight against 0.01 M potassium phosphate buffer (pH 7.3; at least four volumes), and diluted with the same buffer to a protein concentration of 10 mg/ml. A protamine sulfate solution was prepared by stirring 2 g of the soild/100 ml of water and adjusting to pH 5.6 at room temperature with 5 N KOH. It is not necessary to remove undissolved material. The dialyzed enzyme solution in batches that could be centrifuged at one time (1–4 l.) was adjusted to pH 5.6 with 5 N acetic acid. The freshly prepared protamine sulfate solution (at 25°) was then run into the vigorously stirred enzyme solution (at 0–5°) during a 15- to 20-min period. Stirring was continued for 15 min after all the protamine sulfate was added and the mixture then centrifuged for 10 min (25,000*g*). The optimum volume of protamine sulfate solution to be added must be determined in a preliminary trial with a series of 10-ml portions of enzyme solution, and varies from 0.05 to 0.16 volume depending on the batch of cells and the batch of protamine sulfate. The supernatant from the centrifugation was readjusted to pH 7.3 with 5 N NaOH as soon as possible and treated with 0.1 volume of 0.1 M Na EDTA (pH 7.3).

Enzyme was next precipitated from the combined solutions obtained in the previous step by addition of solid ammonium sulfate at 1°. A total of 360 g/l. was added slowly with constant stirring, and stirring was continued at 0° for 1 hr after all the crystals had dissolved. After recovering the precipitate by centrifugation (15 min at 25,000*g*), a minimum volume of 0.01 M Tris-acetate buffer (pH 7.3), containing 1 mM EDTA, was used to partially dissolve and suspend the precipitate. The mixture was dialyzed overnight against the same buffer, with the use of dialysis tubing previously soaked for at least 2 hr in 0.1 M EDTA (pH 7).

The deep yellow dialyzed solution was next subject to gel filtration on Sephadex G-100 equilibrated with 0.01 M Tris-acetate buffer (pH 7.3). In small-scale preparations a 50- to 100-ml sample was passed through a 4 × 140 cm column with collection of 15-ml fractions. On the large scale a 500-ml sample was applied to a 16 × 130 cm column with collection of 250-ml fractions. The latter column was constructed from Plexiglass tubing and the gel was packed over a disk of porous polypropylene sheet ($^3/_{16}$ in. thick, Labpor sheet, Bel Art Products). Fines must be thoroughly removed from the Sephadex so that a flow rate of about 500 ml/hr is obtained with the large column or 30 ml/hr with the small column. At this flow rate the enzyme is eluted from the column within 2 days and is associated with the second of two large, yellow protein peaks. A third fainter yellow band and sometimes an orange band elute after the enzyme.

The active fractions from the column were combined and treated with 0.1 volume of 0.1 M EDTA (pH 7.0) and the enzyme precipitated from the solution by solid ammonium sulfate as previously, except that this time 430 g of solid was added per l. After suspension in, and dialysis against, Tris-acetate buffer as before the solution was combined with other batches brought to the same stage of treatment.

The enzyme was next passed through another G-100 Sephadex column of the same dimensions as in the earlier step, but this time equilibrated with 0.01 M imidazole acetate buffer (pH 5.0), containing 1 mM EDTA. Before the enzyme was passed through this column it was dialyzed against 0.01 M imidazole acetate buffer (pH 5.0), containing 1 mM EDTA for about 16 hr. A considerable pale yellow precipitate that formed during dialysis was removed by centrifugation and discarded, before the dialyzed solution was placed without delay on the column. Other conditions were as for the first Sephadex step. Some inactive protein precedes the enzyme from the column and is incompletely separated from the enzyme.

As soon as possible, active fractions were combined and treated with 0.1 volume of 0.1 M EDTA, and the pH adjusted to 7.0 with 1 N NaOH. Care must be taken not to exceed this pH since at higher pH the enzyme denatures in the presence of imidazole (Orr *et al.*, 1972). The proteins were then frac-

TABLE I: Purification of Ribonucleotide Reductase from *L. leichmannii*.[a]

Purification Step	Vol (ml)	Total Protein (g)	Total Act. (Units)	Sp Act. (Units/mg)
Dialyzed extract	34,500	480	1,900,000	4.0
Protamine sulfate treated extract[b]	44,900	259	1,670,000	6.6
First ammonium sulfate precipitation[c]	2,540	85.7	1,430,000	17.5
First gel filtration[c]	17,270	34.5	1,602,000[e]	46
Second ammonium sulfate precipitation[c]	806	19.0	1,050,000	55
Second gel filtration[d]	7,300	6.6	572,000	87
Third ammonium sulfate precipitation[d] A	173	3.12	378,000	121
B	175	1.78	154,000	86

[a] From two 860 liter cultures. Approximately 4 kg of cell paste. [b] Carried out in 14 batches. Results refer to combined product of all batches. [c] Carried out in 5 batches. Results refer to combined product of all batches. [d] Carried out in 2 batches. A refers to the combined second fractions from both batches. B refers to the combined third fractions from both batches. [e] The activity at this step frequently seemed high compared with the preceding step. The explanation of this is presently unknown.

tionally precipitated with ammonium sulfate, the first fraction being precipitated by addition of $290 \times V$ g of solid ammonium sulfate in the manner described previously, where V l. is the volume of the enzyme solution after pH adjustment. After recovery of the precipitate by recentrifugation the second fraction was precipitated from the supernatant by addition of $50 \times V$ g of ammonium sulfate, and from the supernatant from the second fraction the third was precipitated by $90 \times V$ g of ammonium sulfate. The precipitates were each dissolved without delay in a minimum volume of 0.1 M sodium 3,3-dimethylglutarate buffer (pH 7.3) and were dialyzed overnight against either the same buffer (for keeping at 0°) or against 0.01 M dimethylglutarate buffer (pH 7.3) preparatory to freeze-drying.

The enzyme solution lost activity slowly at 0°, the activity declining to 50% over a period of 2–3 weeks. About 20% loss in activity occurred on freeze-drying, but there was no further loss in activity from the frozen-dried powder over several months at 0°.

Results for a large-scale preparation are shown in Table I. The fraction containing the majority of the activity in the final step (usually the second fraction) had a specific activity of 100–190 units/mg. These fractions were judged to be 70–80% pure on the basis of analytical polyacrylamide gel electrophoresis (Orr *et al.*, 1972). Of many attempts to remove the residual contaminating proteins none was successful except preparative electrophoresis, which was used to purify 40- to 50-mg samples (Orr *et al.*, 1972). The resulting preparation contained no impurities detectable by polyacrylamide gel electrophoresis, although it may have contained some denatured reductase (Orr *et al.*, 1972) and was used in all the experiments reported in this paper. Preparative electrophoresis of the material referred to in A of the final step of Table I gave a preparation with specific activity 160 units/mg.

Assay of Ribonucleotide Reductase. The colorimetric method was used to estimate dATP production under conditions previously defined (Orr *et al.*, 1972). A unit of activity corresponds to formation of 1 μmole of dATP/hr.

Radioactivity Determination. This was carried out in a Tri-Carb scintillation counter in vials containing 10 ml of scintillation fluid prepared as described by Bray (1960).

Analytical Gel Filtration. The zonal methods of Andrews (1964) and Determann (1966) were used at 4° for molecular weight estimations on Sephadex G-100 equilibrated with 0.05 M potassium phosphate (pH 7.3), containing 1 mM EDTA ($\Gamma/2 = 0.13$) or 0.1 M sodium dimethylglutarate buffer, pH 7.3 ($\Gamma/2 = 0.27$). Samples of 10 mg of each protein in a volume of 1 ml of the appropriate buffer were applied to a column (1 × 100 cm). Collection of the first fraction was commenced immediately after the sample was applied. Protein was eluted into weighed tubes at flow rates between 10 and 21 ml per cm^2 per hr and the volume of each fraction (approximately 0.4 ml) determined from its mass. Elution profiles were determined by measurement of absorbance at 280 nm and in some cases by activity determinations. The molecular weight values for the reference compounds were obtained from the literature as follows: horse cytochrome *c*, 12,400 (Margoliash, 1962); ribonuclease A, 13,700 (Hirs *et al.*, 1956); trypsin inhibitor, 21,500 (Wu and Sheraga, 1962); ovalbumin, 45,000 (Warner, 1954); human serum albumin monomer, 69,000 (Oncley *et al.*, 1947); human γ-globulin, 160,000 (Phelps and Putnam, 1960).

Homogeneity of the enzyme was examined by gel filtration experiments that produced a plateau region in the elution profile (Winzor and Scheraga, 1963). A column (0.6 × 30 cm) of Sephadex G-100 was used. Collection of fractions and other details were as in the zonal experiments but the sample volume was 10 ml.

Sedimentation Velocity Measurements. Except where stated otherwise, sedimentation velocity measurements were made in a Spinco Model E analytical untracentrifuge with schlieren optics at 50,740 rpm. Solutions were buffered with 0.1 M sodium dimethylglutarate (pH 7.3) and temperature was controlled with the RTIC unit. The weight-average sedimentation coefficients, $\bar{s}$, were calculated from the rates of movement of the square roots of the second moments of the schlieren patterns (Goldberg, 1953). When comparisons between experiments at different temperatures were required, the $\bar{s}$ values were corrected to 20° in water giving the values $\bar{s}_{20,w}$. Concentrations were determined refractometrically using peak areas (obtained by trapezoidal integration) and assuming a specific refractive increment of 0.0018 dl g^{-1}. In the determination of the concentration dependence of the sedimentation coefficient mean plateau region concentrations were used.

As a test of homogeneity an analysis of the schlieren patterns was carried out (Baldwin, 1954). A sedimentation co-

efficient distribution (a plot of $g^*(S)$ *vs.* S) was obtained at different times during an experiment. The terms $g^*(S)$, an apparent differential distribution function, and S, a reduced coordinate with the same dimensions as the sedimentation coefficient, have been clearly defined (Baldwin, 1954; Signer and Gross, 1934). The curves of $g^*(S)$ *vs.* S were very nearly symmetrical. The range of S decreased progressively with time indicating the significant contribution of diffusion to the spreading of the boundary. These effects were eliminated by extrapolating to infinite time (Baldwin, 1959). The quantity $(S - \bar{s})^2$, where $\bar{s}$ is the weighted mean of the distribution of S, was plotted against $1/te^{\bar{s}\omega^2 t}$ at fixed values of $g^*(S)_{max}$ (where ω is the angular velocity). Since the weighted means of the distributions of S did not vary significantly from the corresponding weight-average sedimentation coefficients the single symbol $\bar{s}$ has been used throughout. While the method accounts for the diffusional spreading of the boundary it does not account for the concentration dependence of the sedimentation coefficient. This should not markedly affect the analysis because of the relative closeness of the measured sedimentation coefficient to that at infinite dilution (Baldwin, 1959).

A boundary analysis procedure (Creeth and Pain, 1967) based on a simplified form (Van Holde, 1960) of an expression for the height and area of a gradient curve as a function of time (Fujita, 1959) accounts for small linear concentration-dependence effects and provides a further test of homogeneity and a method for determining apparent diffusion coefficients. This method has been applied to the schlieren patterns from the sedimentation velocity experiments. The apparent diffusion coefficients, D^*, so obtained have been corrected to 20° in water giving the values $D^*_{20,w}$.

Equilibrium Sedimentation. Experiments in the absence of denaturants and in urea were performed at 14,210 rpm; Kel-F polymer oil was used as an inert base fluid; volumes of polymer oil, equilibrium diffusate and solution were taken so that the diffusate column just overlapped the solution column (1.5 mm) at both ends; most experiments were carried out in the 0.1 M sodium dimethylglutarate buffer (pH 7.3); the An-J equilibrium rotor and schlieren optics were used. Separate experiments with solutions of different initial concentrations were performed. Equilibrium was usually reached in 15 hr (33 hr in 7 M urea). Measurements (20–30) were made of the refractive index gradient at equal intervals throughout the solution columns using a Gaertner toolmakers' microscope, type M2001, As-P.

The molecular weight at any point in the cell can be calculated from the slope, at that point, of a graph of ln c *vs.* $r^2/2$ or of ln $[(1/r)(dc/dr)]$ *vs.* $r^2/2$ by multiplying by the constant factor $RT/(1 - \bar{v}\rho)\omega^2$ where c is the protein concentration at distance r from the center of rotation, R is the gas constant, T is the absolute temperature, $\bar{v}$ is the partial specific volume of the protein, ρ is the density of the solvent, and ω the angular velocity of the rotor. The former method gives point weight-average molecular weights and the latter point z-average molecular weights. In practice these graphs were almost linear, so apparent weight-average ($\overline{M}_w$) and z-average ($\overline{M}_z$) molecular weights were obtained from the slopes of the best straight lines through the data, the slopes being calculated by the method of least squares. Point concentrations were computed (Schachman, 1957) using initial concentrations obtained by measurements with a differential refractometer (Cecil and Ogston, 1951).

Sedimentation equilibrium experiments in the presence of guanidine hydrochloride were performed by the method of Yphantis (1964) at 30,000 rpm in an AN-H rotor and the use of Rayleigh optics. The sample was 0.1 ml of a solution containing 0.2–0.5 mg of protein/ml and equilibrium diffusate was placed in the second sector of the cell. Fluorocarbon was not used in these experiments, and equilibrium was reached after 20 hr. Photographs were analyzed with a Nikon microcomparator by measuring vertical fringe displacement, D, in microns, as a function of r at small intervals throughout the solution. The positions of five fringes, three black and two white, were measured at each value of r, and the average displacement of these five fringes was used in a plot of ln D *vs.* r^2 after correction for the average displacement of five fringes in a base-line photograph. The slope of this plot, *i.e.*, $d(\ln D)/dr^2$, was obtained by computing the least-squares fit to the data on a Linc 8 computer and inserted into the usual equation to obtain the weight-average molecular weight.

Partial Specific Volume of the Reductase. The partial specific volume of the reductase, required for calculation of molecular weight from the sedimentation data, was deduced from the amino acid composition by the method outlined by Schachman (1957). A value of 0.73 was obtained. Since there is considerable evidence that for many proteins the value of $\bar{v}$ in denaturants is the same or very slightly lower than the value in dilute salt solutions (Castellino and Barker, 1968; Kielley and Harrington, 1960; Woods *et al.*, 1963; Marler *et al.*, 1964), the same value for $\bar{v}$ was used for calculation of molecular weight in 7 M urea and in 6 M guanidine hydrochloride in the presence of 0.01 M 2-mercaptoethanol.

Amino Acid Analysis. Since ribonucleotide reductase is insoluble in water, samples were dialyzed against 0.01 M sodium phosphate buffer (pH 7.3). Samples of approximately 1 mg in 1 ml of 6 N HCl were hydrolyzed in evacuated Pyrex tubes for periods of 24, 48, 96, and 140 hr at 110°. Two to six samples were hydrolyzed at each time interval. Analyses were performed by the method of Spackman *et al.* (1958) on a Beckman Model 120B amino acid analyzer. An 8-cm column of Aminex-A5 and a 50-cm column of Aminex-A4 were used. Results for serine, threonine, methionine and tyrosine were plotted *vs.* time of hydrolysis and extrapolated to zero time (Lindberg, 1967). Values at the longest period of hydrolysis were used for leucine, valine, and isoleucine. Results for other amino acids were the mean for all determinations. Alkaline hydrolysis for determination of tryptophan was carried out in 4.2 N NaOH in the presence of 25 mg of acid-washed soluble starch according to the method of Hugli and Moore (1971). Analysis was carried out on a 12.5-cm Aminex-A5 column with 0.25 M sodium citrate (pH 5.28) as eluting buffer. For more accurate determination of cystine, these residues in the protein were oxidized by performic acid and the cysteic acid determined on the analyzer after hydrolysis. The procedure of Hirs (1967a) was followed except that after treatment of the protein with reagent the solution was diluted with 200 ml of cold water and freeze-dried. The dried protein was hydrolyzed in 6 N HCl for analysis on the analyzer.

Spectroscopic Determination of Tyrosine and Tryptophan. The procedure of Edelhoch (1967) was used. The tryptophan content was determined from the absorbance of the protein (1.0 mg/ml) in 6.0 M guanidine hydrochloride at 288 and 280 nm after correction for absorbance due to tyrosine, cystine, and reagents. The tyrosine content was calculated from the change in absorbance at 295 or 300 nm after adjustment to pH 11.0 with 5 N NaOH, and extrapolation to zero time.

Determination of Carboxyl-Terminal Amino Acids. Two methods were used for identification of the amino acid residue at the carboxyl terminus of the polypeptide chain. (1) The

first was by means of selective tritiation carried out by a slight modification of the procedure of Holcomb *et al.* (1968). An electrophoretically pure sample of ribonucleotide reductase (8.0 mg) was dialyzed against 0.01 M ammonium acetate, the dialyzed solution freeze-dried and the residue dissolved in 0.5 ml of tritiated water (0.5 Ci). Pyridine (1 ml) and acetic anhydride (0.5 ml) were added and the mixture incubated at 37° for 4 hr. The reagents were removed by dialysis with stirring against 2 l. of water in a stoppered vessel overnight, with several changes of water. The suspension of precipitated protein was transferred to a hydrolysis tube in which it was freeze-dried. Hydrolysis of the protein was performed with 1.0 ml of 6 N HCl at 110° for 24 hr. After evaporation of solvent under reduced pressure the residue was dissolved in 3.0 ml of 0.2 M citrate buffer (pH 2.2). After removal of samples of hydrolysate for determination of total radioactivity, 1.5 ml of the solution was transferred to a 135-cm column (type B Chromobeads) of a Technicon NC1 amino acid analyzer. The effluent line from the column was first passed through a 1-ml anthracene-packed flow cell in a Nuclear-Chicago Unilux-IIA scintillation counter before entering the mixer assembly of the analyzer. The apparatus had been calibrated so that the delay time between recording of counts (over 4-min periods) in the scintillation counter and recording of ninhydrin color on the analyzer chart for the same material was known to be 18–20 min, thus permitting determination of specific radioactivity for each amino acid. (2) The carboxy-terminal amino acid was also determined by identifying amino acids released by carboxypeptidase according to the procedures outlined by Ambler (1967). Ribonucleotide reductase was dialyzed against 0.2 M *N*-ethylmorpholine buffer (pH 8.5) and treated with a solution of carboxypeptidase A (carboxypeptidase to reductase ratio, 1:30), or carboxypeptidase B (carboxypeptidase to reductase, 1:30 or 1:90), or a mixture of carboxypeptidases A and B (A–B–reductase, 1:2:40). In some experiments urea was also present as a denaturant in the reaction mixture. During incubation of the reaction mixture at 37° samples were removed at intervals and adjusted to pH 2.0–3.0 (indicator paper) by addition of Dowex 50 H^+ resin (100–200 mesh). The suspension was stirred mechanically for 15 min and then poured into a glass column (0.9 × 20 cm) where the resin was allowed to drain and was then twice washed with two bed volumes of water. The adsorbed amino acids were eluted with 5 M ammonia, the solution was evaporated to dryness and the residue dissolved in citrate buffer (pH 2.2) and applied to the column of the amino acid analyzer. The analysis for each amino acid was corrected for the amount found in a control experiment performed under identical conditions except that the reductase was omitted. Reductase concentration in the complete reaction mixture was estimated from amino acid analysis of a sample of the stock reductase solution after acid hydrolysis, and from the amino acid composition of the reductase given in the Results section.

Determination of Amino-Terminal Amino Acid. The amino-terminal amino acids were identified by two methods. (i) Carbamylation was carried out as described by Stark (1967) on approximately 40 mg of reductase in 4 M guanidine hydrochloride. In control experiments the entire procedure was carried out except for addition of potassium cyanate and protein, respectively; amino acid analyses obtained in this control were deducted from values found in the complete experiment. Protein concentration in the reaction mixture was determined as in the preceding section. (ii) Dansylation was carried out according to Gray (1967) and the dansylamino acids were identified by thin-layer chromatography (Hartley, 1970) on Cheng Chin polyamide plates (Gallard-Schlesinger Chemical Manufacturing Corp., New York).

Cyanogen Bromide Cleavage and Gel Electrophoresis of Peptides. The reductase (6.5 mg) in 6 M guanidine hydrochloride, about 0.2 M 2-mercaptoethanol and 4 mM EDTA (total volume 3 ml) was adjusted to pH 8.1 by addition of 1 N NaOH. Solid dithiothreitol was added to a concentration of 0.25 M and reduction of cystine residues was allowed to proceed for 4.5 hr under nitrogen. Aminoethylation of cysteine residues was accomplished by adding ethylenimine (cautiously adjusted to pH 11 with HCl) in 13-fold excess over all thiol groups present. After a reaction time of 1 hr at room temperature the reaction mixture was dialyzed against distilled water and freeze-dried (Schroeder *et al.*, 1967). The residue was dissolved in 70% formic acid and solid cyanogen bromide was added (40 moles/mole of protein). After the reaction mixture had stood at room temperature for 24 hr it was diluted ten times with water and the mixture freeze-dried. The number of cyanogen bromide peptides was determined by electrophoresis on polyacrylamide gel in the presence of sodium dodecyl sulfate.

Electrophoresis in the presence of sodium dodecyl sulfate was carried out according to Weber and Osborn (1969), except that the gel was prepared from 15% acrylamide and 0.5% bisacrylamide. After destaining, the gels were scanned at 600 nm in a Gilford microdensitometer, Model 2000, and the curves analyzed on a DuPont curve resolver. When the reductase was electrophoresed the reference proteins were creatine kinase which has subunits of mol wt 40,000 (Dawson *et al.*, 1967) and serum albumin, molecular weight of monomer 68,000 (Tanford *et al.*, 1967). Polyacrylamide gel electrophoresis in triethanolamine–*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (buffer system I) was carried out as previously described (Orr *et al.*, 1972).

Cyanogen Bromide Cleavage and Gel Filtration of Peptides. Approximately 70 mg of the reductase was reduced by approximately 40 mM dihydrolipoate in a solution containing 0.2 M Tris·HCl buffer (pH 8.6), 0.135 mM EDTA, and 6 M guanidine hydrochloride in a total volume of 12.0 ml. The mixture was flushed with nitrogen and the reaction was allowed to proceed for 60 min.

A 15-fold excess of ethylenimine over the total sulfhydryl groups was added to the mixture. The pH of the reagent had previously been adjusted cautiously to pH 11.0 with 6 N HCl. After a period of 1 hr, the solution was dialyzed against water, and freeze-dried. Hydrolysis and amino acid analysis of a portion of the product indicated the presence of 8.6 moles of *S*-aminoethylcysteine/mole of enzyme (*cf.* 9 moles of half-cystine/mole of unmodified enzyme, Table IV). The residue was dissolved in 70% trifluoroacetic acid and 500 mg of CNBr was added. After 24 hr the solution was freeze-dried, and amino acid analysis showed a very small amount of residual methionine and no methionine sulfone present, corresponding to 98% cleavage. The residue was dissolved in 6 ml of 5% formic acid and the solution applied to a column of Sephadex G-50 (fine, 200 × 2.5 cm) which had been equilibrated with 5% formic acid. The elution rate was 15 ml/hr and 3-ml fractions were collected. They were analyzed by measuring the absorbance at 280 nm and by reaction with ninhydrin after alkaline hydrolysis (Hirs, 1967b).

The number of peptides under each peak was determined by high-voltage paper electrophoresis and by thin-layer chromatography on cellulose sheets. For the latter, two solvents were used: (A) isopropyl alcohol–5% formic acid (6:4.5,

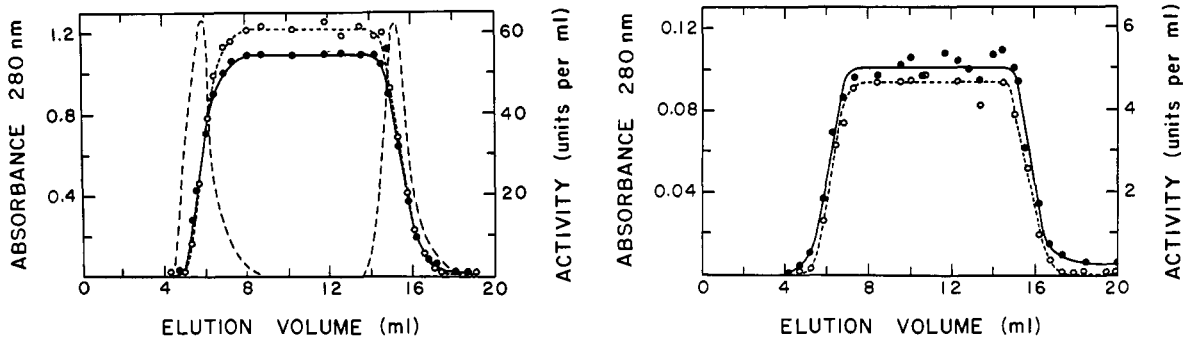

FIGURE 1: Homogeneity of ribonucleotide reductase by analytical gel filtration. The protein concentration was 1.2 mg/ml in A (left) and 0.09 mg/ml in B (right). (●) Absorbance at 280 nm; (○) activity (units/ml). The superimposed broken curves in A show the first differential of the absorbance curve.

v/v) and (B) *sec*-butyl alcohol–ammonia–water (6:3:1, v/v). Electrophoresis was carried out in a Gilson Model D-2 electrophorator in pyridine–acetic acid (pH 6.5) (Bennett, 1967) or formic acid–acetic acid (pH 2.0) (Smith, 1960). The chromatograms were stained for arginine with the phenanthrenequinone reagent (Yamada and Itano, 1966), for histidine with the Pauly reagent (Easley, 1965) or with *p*-bromoaniline (Elliott, 1959), and with the ninhydrin–cadmium reagent (Yamada and Itano, 1966).

Results

Homogeneity of the Ribonucleotide Reductase Preparation. Reductase purified by preparative electrophoresis has previously been shown to give a single band in analytical electrophoresis on polyacrylamide gel in buffer systems operating over the pH range 6–8.5 (Orr *et al.*, 1972). However, the total purification achieved in the preparation was only 30- to 40-fold and since it was important to demonstrate that this was a single enzyme capable of catalyzing the reduction of several ribonucleotides rather than a collection of isoenzymes of differing specificity, the homogeneity was further examined by several other methods.

In studies on the reductase by zonal gel filtration, elution patterns of protein and enzyme activity were both symmetrical and had identical elution volumes. In plateau experiments, leading and trailing edges provided first differential curves that departed only slightly from the enantiographic pattern expected for a pure protein (Figure 1A). The elution volume and pattern did not change significantly at lower protein concentration (Figure 1B).

In sedimentation velocity experiments a single, apparently symmetrical schlieren peak was observed (Figure 2), the area under which remained constant (after correction for radial dilution) for the duration of the experiment (3–4 hr), a result indicating the absence of gross heterogeneity. An apparent content of 3–4% of slower sedimenting material was seen in some preparations, but as no corrections were made for the Johnston–Ogston effect (1946) the true proportion of these components is probably smaller.

The sedimentation coefficient of the reductase varied linearly with concentration over the range 0.03–0.3 g dl^{-1}, and from the least-squares treatment of the experimental data the following expression for the concentration dependence of $s_{20,w}$ was derived. $s_{20,w} = 5.2(1 - 0.09C) \times 10^{-13}$, where C is the concentration of protein in g dl^{-1}. This relationship was used in the boundary analysis of Creeth and Pain (1967) where the apparent diffusion coefficient was calculated at various times. Since the apparent diffusion coefficient was constant with time, the analysis provided additional evidence for homogeneity.

The apparent diffusion coefficient varied linearly with concentration in the range 0.03–0.3 g dl^{-1} and least-squares treatment of the experimental data gives the following expression for the concentration dependence of $D^*_{20,w}$. $D^*_{20,w} = 6.4(1 + 0.4C) \times 10^{-7}$, where C equals concentration in g dl^{-1}.

The results of the boundary analysis of Baldwin (1954) are shown in Figure 3 where $(S - s)^2$ is plotted against $1/te^{s\omega^2 t}$ for various values of $g^*(S)/g^*(S)_{max}$ and the extrapolations to infinite time are shown by the dashed lines. The values of $(S - s)^2$ at infinite time are close to zero, indicating the absence of measurable heterogeneity in terms of sedimentation coefficient. The plots of $g^*(S)_{max}$ *vs.* S were symmetrical which gives a further indication of the homogeneity.

Examination of the reductase by equilibrium sedimentation permitted the calculation of weight-average and z-average molecular weights from the slopes of the plots log (c *vs.* r^2)/2 and log [(1/r)(dc/dr)] *vs.* $r^2/2$, respectively. The linearity of the analytical plots and the similarity of the slopes from which the $\bar{M}_w$ and $\bar{M}_z$ were calculated also indicated the homogeneity of the preparations.

Molecular Weight of Ribonucleotide Reductase. Comparison of the elution volume of the reductase (initial concentrations of 7, 0.7, and 0.07 mg per ml) from Sephadex G-100 with those experimentally determined with proteins of known

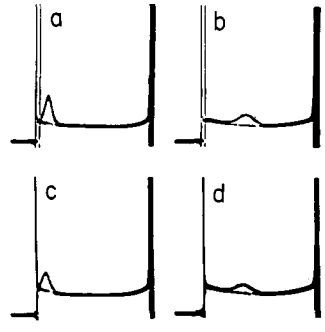

FIGURE 2: Schlieren patterns obtained during sedimentation velocity determinations on ribonucleotide reductase. For a and b: concentration, 0.2 g dl^{-1}; schlieren phase-plate angle 70°, temperature 4.1°. Time after reaching speed in a, 20 min; in b, 108 min. In c and d: concentration 0.13 g dl^{-1}; schlieren phase-plate angle 65°, temperature 5°. Time after reaching speed in c, 19 min; in d, 99 min.

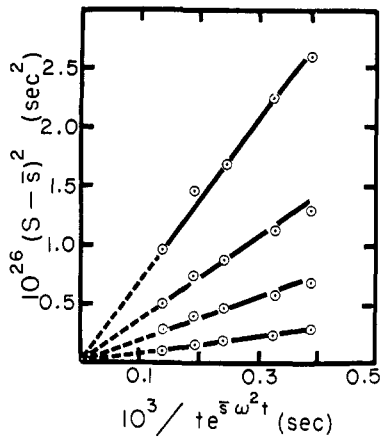

FIGURE 3: Boundary analysis of Baldwin (1954) applied to data for sedimentation of ribonucleotide reductase. For details, see the Methods section.

TABLE III: Molecular Weight of Ribonucleotide Reductase under Various Conditions as Determined by Sedimentation Equilibrium.

Conditions	Mol Wt
0.1 M Dimethyl glutarate (pH 7.3)	76,000
6 M Guanidine·HCl–0.01 M 2-mercaptoethanol–0.1 M dimethyl glutarate (pH 7.3)	74,300
0.1 M Glycine·HCl–0.01 M 2-mercaptoethanol (pH 2.0)	79,950
0.1 M Sodium carbonate buffer–0.01 M 2-mercaptoethanol (pH 11.0)	74,300
7 M Urea–0.01 M 2-mercaptoethanol–0.1 M dimethyl glutarate (pH 7.3)	76,000
0.1% Sodium dodecyl sulfate–0.1% mercaptoethanol–0.01 M sodium phosphate buffer (pH 7.0)	70,000

molecular weight (Andrews, 1964) indicated a molecular weight for the reductase of about 70,000.

The relationships given in the preceding section were used to calculate the $s_{20,w}$ and the $D^*_{20,w}$ at infinite dilution. The partial specific volume ($\bar{v}$) calculated as described in Methods was 0.73. Substitution of these values into the Svedberg equation gave an apparent molecular weight of 73,000. Molecular weights calculated from equilibrium sedimentation at four different initial concentrations (0.03, 0.08, 0.13, and 0.20 g dl^{-1}) are shown in Table II. The weight-average and the z-average molecular weights are identical within the expected experimental variation of the method. The average value of molecular weight of the reductase from all estimations by gel filtration, sedimentation diffusion, and equilibrium sedimentation is 76,000.

Number of Polypeptide Chains in Ribonucleotide Reductase. When the reductase was examined by sedimentation equilibrium in the presence of 6 M guanidine hydrochloride and 0.01 M 2-mercaptoethanol, the plot of ln D *vs.* r^2 was linear indicating homogeneity under these conditions. From determinations of the slope of this plot in three experiments, values calculated for the weight-average molecular weight were 70,940, 78,880, and 73,140 giving a mean value of 74,300.

Equilibrium sedimentation was also carried out under the following conditions: in 0.1% sodium dodecyl sulfate, in glycine·HCl buffer (pH 2.0), in carbonate buffer (pH 11.0), and in 7 M urea. Mercaptoethanol (0.01 M) was present in all denaturing conditions since, except at pH 2.0, it prevented the formation of aggregated material which otherwise occurred. At pH 2.0 the plot indicated the presence of a minor component of molecular weight about 118,000 even with mercaptoethanol present. In all other cases the plots showed that a single major species was present with molecular weight as shown in Table III. Clearly this evidence indicates that the protein does not disaggregate into subunits when the polypeptide chain is unfolded.

When the reductase was treated with 0.1% sodium dodecyl sulfate and 0.1% mercaptoethanol and subsequently electrophoresed on polyacrylamide gel in the presence of dodecyl sulfate, only one band, with mobility less than that of serum albumin and of creatine kinase, was observed. Calculation of the molecular weight from the mobility gave a value of approximately 80,000. This result provides confirmation that the reductase contains no subunits.

Amino Acid Analysis and Cyanogen Bromide Cleavage. Table IV shows the number of residues of each amino acid found per molecule of enzyme assuming 9.0 half-cystine residues/mole. These values represent the best estimate obtained from a large number of determinations, as indicated in the Methods section. The integral numbers of residues shown in column 2 correspond to a molecular weight of 75,966 which must be increased on account of an unknown number of amide groups. It should be noted in particular that there are 8 methionine residues/76,000 so that if these are in a single polypeptide chain, 9 different peptide fragments should be produced by cyanogen bromide treatment.

Peptides Formed by Cyanogen Bromide Treatment. When the peptides produced by cyanogen bromide cleavage were subjected to electrophoresis on polyacrylamide gel in the presence of sodium dodecyl sulfate and mercaptoethanol ten bands were detected after staining with Coomassie Blue, that is, one in excess of the expected number for a single chain. This is seen clearly in the densitometer scan and the corresponding resolution into individual curves (Figure 4). Amino acid analysis of the material after cyanogen bromide treatment indicated that 0.3 mole of methionine residue remained per 76,000 g of protein, so that it is likely that peptide I and perhaps peptide II contained a methionine residue at which cleavage did not occur. If both peptides I and II, which together constituted about 10% of the total protein, contained unreacted methionine, another band (presumably X) may have contained two peptides.

The elution pattern from the gel filtration of the peptides

TABLE II: Molecular Weight of Ribonucleotide Reductase by Equilibrium Sedimentation.

Initial Concn (g dl^{-1})	Concn Range (g dl^{-1})	App Mol Wt $\bar{M}_w$	App Mol Wt $\bar{M}_z$
0.2	0.06–0.5	73,000	72,000
0.13	0.05–0.3	80,000	73,000
0.08	0.02–0.17	82,000	77,000
0.03	0.01–0.09	79,000	79,000

TABLE IV: Amino Acid Composition of Ribonucleotide Reductase.

Amino Acid	Column 1[a]	Column 2[b]
Lysine	43.4	43
Histidine	8.1	8
Arginine	33.4	33
Aspartic acid	78.1	78
Threonine	31.6[c]	32
Serine	54.4[c]	54
Glutamic acid	78.2	78
Proline	30.6	31
Glycine	55.6	56
Alanine	54.7	55
Cystine (half)	9.0	9
Valine	47.5[d]	48
Methionine	7.7	8
Isoleucine	39.4[d]	39
Leucine	59.3[d]	59
Tyrosine	23.8	24
Phenylalanine	24.7	25
Tryptophan	9.0	9

[a] Mean values (except as indicated). Results calculated on the basis of 9 half-cystines. [b] Nearest integral values. [c] Obtained by extrapolation to zero hydrolysis time. [d] Obtained by extrapolation to maximum value.

produced by cyanogen bromide action contained nine peaks (Figure 5). Peak I was eluted in the void volume as determined by gel filtration of Blue Dextran and was probably uncleaved protein. Peak II was probably some incompletely cleaved material, possibly with intact disulfide bridges since aminoethylation was only 95.5% complete. This peak remained at the origin both in high-voltage paper electrophoresis at pH 2.0 or pH 6.5 and in thin-layer chromatography in a number of solvents.

On high-voltage electrophoresis the material from peaks

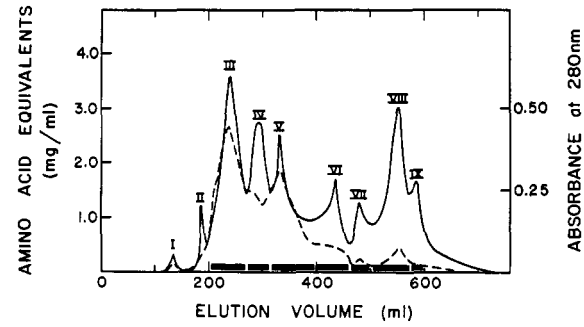

FIGURE 5: Gel filtration of peptides formed by cyanogen bromide treatment of ribonucleotide reductase. The continuous curve indicates amino acid equivalents calculated from ninhydrin color after alkaline hydrolysis: broken curve, absorbance at 280 nm. Horizontal bars indicate pooled fractions. Other details are given in Methods.

VIII and IX moved toward the cathode with identical mobilities, both at pH 2.0 and 6.5. The material from both was arginine and ninhydrin positive but histidine negative. Since the remaining peptides stayed at the origin during electrophoresis, the peptides in peaks VIII and IX were different from that in the other peaks. Peak VIII was shown by thin-layer chromatography on cellulose in solvent B to contain two peptides and peak IX one which had different mobility from either peptide of peak VIII (Figure 10). Since some material remained at the origin, especially from the peaks containing large peptides, two-dimensional thin-layer chromatography, first in solvent A and then in B, was employed to further resolve and distinguish the peptides in peaks III to VII. As seen in Figure 6 these peaks contained a total of six major peptides. Of these, all were arginine positive and all except peptide 4 histidine positive. These data indicate that peaks III to IX contained a total of nine different peptides in major amounts.

End-Group Determinations. Determinations of the carboxyl terminus by the selective tritiation method clearly indicated that lysine was the only C terminus present. The specific activities (counts per minute per micromole) of various amino

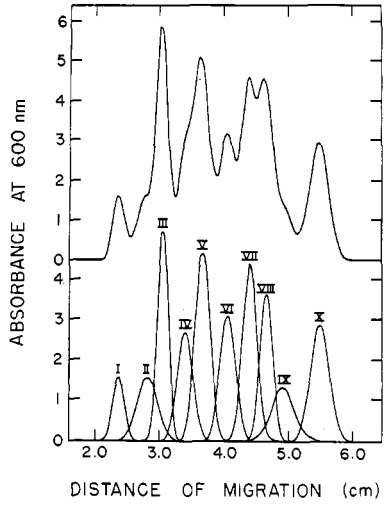

FIGURE 4: Polyacrylamide gel electrophoresis of peptides formed by cyanogen bromide treatment of ribonucleotide reductase. The upper curve is the densitometer scan of the gel and the lower curves are the component curves obtained by the curve resolver.

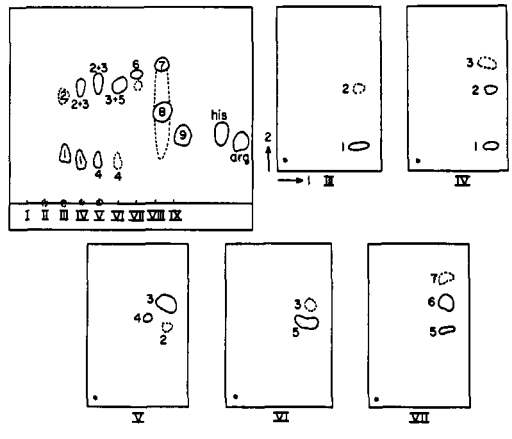

FIGURE 6: Chromatographic separation of peptides formed by cyanogen bromide treatment of ribonucleotide reductase. Samples in the one-dimension chromatogram at upper left were the fractions from the gel filtration described in Figure 5 and the solvent was solvent B. The other diagrams represent two-dimensional chromatograms of individual fractions developed with solvent A in the first direction and solvent B in the second. Peptides were detected with phenanthrene quinone.

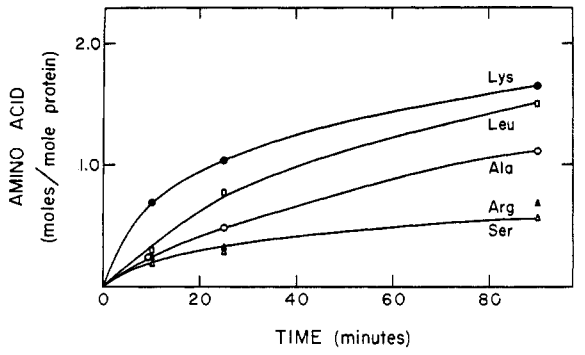

FIGURE 7: Release of amino acids from ribonucleotide reductase by carboxypeptidase B. The reductase (40) mg) was treated with 0.45 mg of carboxypeptidase under the conditions described in Methods.

TABLE V: Amino Acids Released from the N Terminus of Ribonucleotide Reductase by the Carbamylation Method.

Amino Acid	Mole of Amino Acid/ Mole of Protein[a]
Arginine	0.041
Aspartic acid	0.020
Threonine	0.015
Serine	0.740
Glutamic acid	0.079
Glycine	0.021
Alanine	0.015

[a] Values corrected according to the recovery factors of Stark (1967).

acids released after selective tritiation were as follows: Lys, 13,900; Asp, 3950; Glu, 1,900. All other values were less than 650. The much smaller amount of radioactivity introduced into glutamic and aspartic acids is probably due to mixed anhydride formation at nonterminal residues (Holcomb *et al.*, 1968) and has been observed in several other proteins which do not have glutamic or aspartic acid at the C terminus (Holcomb *et al.*, 1968; Hsieh *et al.*, 1971). In order to confirm terminal lysine and eliminate the possibility of terminal glutamic or aspartic acid the reductase was treated with carboxypeptidases A and B. Lysine was the amino acid released at the fastest rate in the presence of both carboxypeptidases, and glutamic and aspartic acids were not released at a significant rate with carboxypeptidase A alone or with A and B. Treatment with carboxypeptidase A alone resulted in release of rather small amounts of amino acids. After 15-min incubation the amounts of amino acids released (moles per mole of reductase) were: Lys, 0.111; Ser, 0.047; Leu, 0.061. After 60 min they were: Lys, 0.29; Ser, 0.178; Leu, 0.135. However, carboxypeptidase B rapidly released not only lysine but several other amino acids although in smaller amounts (Figure 7). Liberation of leucine and alanine in this experiment was favored by carboxypeptidase A present in commercial preparations of carboxypeptidase B, but the negligible rate of their release by carboxypeptidase A alone indicates that they could not have been present at the C terminus. To ensure that all C termini were accessible, treatment with carboxypeptidase B was also carried out in urea but this did not result in change in the pattern of amino acids released. The results shown in Figure 7 indicate that the C terminus is Ala-Leu-Lys.

Determination of the amino terminus by carbamylation of the protein, cyclization, isolation of the resulting hydantoins, and their subsequent hydrolysis to amino acids yielded serine as the only N-terminal residue (Table V). Recovery of lysine from the carbamylated protein after hydrolysis with 6 N HCl at 10° for 24 hr was 20%, which is within the range expected to be formed from homocitrulline during hydrolysis of a fully carbamylated protein (Stark and Smyth, 1963), so that carbamylation was probably complete.

Thin-layer chromatography of the hydrolysis products from the dansylated protein revealed the presence of five fluorescent products which were identified by comparison to authentic materials as dimethylaminonaphthalenesulfonamide (dansylamine), dimethylaminonaphthalenesulfonic acid (dansyl-OH), N^{ϵ}-dansyllysine, *O*-dansyltyrosine, and N^{α}-dansylserine. The first two products result from reaction of the reagent with ammonia and water, respectively, and the N^{ϵ}-dansyllysine and *O*-dansyltyrosine result from reaction of reagent with nonterminal lysine and tyrosine residues. The only amino-terminal residue detected was therefore serine. When authentic dansylserine was added to the hydrolytic products prior to chromatography, the authentic material reinforced the fluorescence of the product in this part of the chromatogram, whereas when authentic dansylthreonine was added it was clearly resolved from this product. In the absence of the added authentic compounds only one product was found in this area of the chromatogram.

Sedimentation and Electrophoresis of the Reductase in the Presence of Modifiers. Although the evidence presented above indicates that the native reductase consists of a single polypeptide chain, formation of an oligomeric protein might occur in the presence of modifiers of the enzyme activity, that is deoxyribonucleoside triphosphates. It has been shown that polymerization of an enzyme in the presence of modifier can provide, at least in theory, a mechanism for allosteric behavior (Nichol *et al.*, 1967; Frieden, 1967). Moreover, the ribonucleoside diphosphate reductase complex of *Escherichia coli* undergoes aggregation in presence of the negative modifier dATP (Brown and Reichard, 1969a).

Gel filtration data reported above for the native enzyme in absence of modifiers indicated that no detectable polymer was present under the conditions employed. Thus the elution volume was unaltered over a 100-fold concentration range in the zonal experiments and over a 10-fold range in the plateau experiments and the data showed no evidence of more than one species of protein or enzyme activity. The weight-average sedimentation coefficient of the reductase determined by equilibrium sedimentation did not decrease on dilution and in fact the concentration dependence was negative down to a concentration of 0.03 g dl^{-1} and there was a small negative (or no) concentration dependence of molecular weight down to 0.01 g dl^{-1}. These data also indicate the absence of aggregated forms.

In the presence of 1–5 mM dGTP, which produces maximum activation of ATP reduction, the sedimentation coefficient of the reductase did not differ significantly from the value obtained in absence of dGTP, and plots of $g^*(S)_{max}$ *vs.* S for the treated reductase were identical with the distribution for the enzyme in absence of nucleotide. The z-average molecular weight from sedimentation equilibrium experiments was

78,000, a value within the range for the reductase in absence of nucleotides. Similarly, the substrate ATP (10 mM) did not change the sedimentation coefficient in velocity experiments. When reductase was electrophoresed on polyacrylamide gel in the presence of 1 mM dGTP or GTP and buffer system I, only a single band of protein was revealed by staining. The mobility of this band was increased slightly (18%) with respect to Bromophenol Blue, probably due to the the higher charge density of the enzyme–nucleotide complex.

Discussion

The homogeneity of the reductase is important since the molecular weight and amino acid analysis are in conflict with previously reported values (Blakley *et al.*, 1965; Goulian and Beck, 1966). By every method used, including polyacrylamide gel electrophoresis (Orr *et al.*, 1972), the maximum amount of contaminants or aggregates was a few per cent. Also the specific activity of the enzyme used in this work was 2.5 to 4 times higher than that used in the work of Goulian and Beck (1966) even after allowance for the suboptimal assay conditions used by the latter, and about 10–20 times higher than that used in the work of Blakley *et al.* (1965). As previously noted (Orr *et al.*, 1972), the enzyme after preparative electrophoresis often did not have the maximum specific activity, but this was probably due to the presence of variable amounts of denaturated reductase rather than to contamination with other protein species. There seems little doubt, therefore, that the higher purity of the preparation used in the present work has permitted more reliable assessment of the properties of the enzyme.

Estimates of the molecular weight in the present study ranged from 70,000 (zonal gel filtration) through 72,000–82,000 (sedimentation) with a mean from all determination of 76,000. The lower value from gel filtration may have been due to enzyme–Sephadex interaction or to a lower frictional ratio for the reductase compared with reference proteins (Siegel and Monty, 1966). Clearly, both values reported previously are erroneous, and the discrepancy between them can not be explained by aggregation–disaggregation phenomena as had been suggested (Larsson and Reichard, 1967) since no evidence for aggregation was found.

Sedimentation equilibrium studies in 6 M guanidine hydrochloride, in 7 M urea, in buffer at pH 2 or 11 or in 0.1% sodium dodecyl sulfate indicated that these denaturants cause no change in molecular weight, and electrophoretic mobility in polyacrylamide gel in the presence of dodecyl sulfate confirmed this. These data therefore strongly suggest that the enzyme consists of a single polypeptide chain.

Since no amino acid is present in an amount less than 8 residues/76,000 molecular weight, determination of a minimum chemical molecular weight from the amino acid analysis was not very accurate, but the value obtained on the assumption of nine half-cystine residues is remarkably close to 76,000. Molecular weight determination from the number of tryptic peptides was unsuccessful due to incomplete hydrolysis of the polypeptide chain. The acid-insoluble core has a molecular weight of about 20,000 (gel filtration, unpublished results) and contained about nine basic residues. Why tryptic digestion did not proceed further is uncertain, but may be related to the many acidic residues present (about 60). However, cleavage of the reductase by cyanogen bromide appeared from polyacrylamide gel electrophoresis in the presence of dodecyl sulfate and from gel filtration to yield the nine peptides expected for a single polypeptide chain of mol wt 76,000. The finding of a single N terminus (Ser) and one type of C terminus (Lys) is also consistent with the absence of subunits.

Several large proteins with a single polypeptide chain are known: DNA polymerase (109,000) (Jovin *et al.*, 1969), leucyl tRNA synthetase (110,000) (Hayashi *et al.*, 1970), phosphorylase subunit (90,000) (Hedrick *et al.*, 1969), myosin subunit (212,000) (Gersham *et al.*, 1969), and β-galactosidase subunit (135,000) (Steers *et al.*, 1965). The molecular weight of 76,000 for ribonucleotide reductase is therefore not exceptionally high for a single polypeptide chain.

Most of the enzymes showing allosteric behavior (that is altered activity in the presence of ligands that bind at a site other than the catalytic center) are oligomeric, or have such high molecular weights that it is probable that they are oligomeric. Because of this association of oligomeric structure with allosteric behavior, allosteric mechanisms are usually discussed in terms of interaction between protomers. Such mechanisms are inappropriate for the ribonucleotide reductase of *L. leichmannii*, which, according to the evidence presented above, has no quaternary structure. Yet the enzyme shows specific activation of the reduction of its various ribonucleotide substrates by appropriate deoxyribonucleoside triphosphates, and nonlinear kinetics under appropriate conditions. Equilibrium dialysis studies indicated that the modifiers bind tightly to the enzyme (Morley, 1968), yet have negligible inhibitory effect (Vitols *et al.*, 1967a) so that binding probably occurs at a modifier site. Frieden (1964) has shown that a monomeric enzyme that catalyzes even a one substrate reaction and has a single modifier site can give nonlinear kinetics of several types and can therefore show all aspects of allosteric behavior except the multisite cooperative interactions characteristic of the oligomeric enzymes. It seems very probable that the reductase is such an enzyme except that it catalyzes a multireactant process. To our knowledge it is the first well-documented example of a monomeric enzyme subject to regulatory control.

Ribonucleotide reductase of *E. coli* is in a category between *L. leichmannii* reductase and the classical type of oligomeric protein that shows cooperative allosteric behavior. Thus the *E. coli* reductase consists of two different subunits but these are loosely associated, separate during purification, and reassociate only in the presence of a relatively high concentration of Mg^{2+} (Brown and Reichard, 1969a). One subunit appears to carry the catalytic site (Brown *et al.*, 1969) and the other is regulatory in function (Brown and Reichard, 1969b) but only the associated subunits are catalytically active. The functions that are performed by a single polypeptide chain in the case of the *L. leichmannii* enzyme are therefore carried out by two larger chains in the case of the *E. coli* enzyme.

Acknowledgments

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References

Ambler, R. P. (1967), *Methods Enzymol. 11*, 155.
Andrews, P. (1964), *Biochem. J. 91*, 222.
Baldwin, R. L. (1954), *J. Amer. Chem. Soc. 76*, 402.
Baldwin, R. L. (1959), *J. Phys. Chem. 63*, 1570.
Beck, W. S. (1967), *J. Biol. Chem. 242*, 3148.

Bennett, J. C. (1967), *Methods Enzymol. 11*, 330.
Blakley, R. L. (1965), *J. Biol. Chem. 240*, 2173.
Blakley, R. L., Ghambeer, R. K., Nixon, P. F., and Vitols, E. (1965), *Biochem. Biophys. Res. Commun. 20*, 439.
Blakley, R. L., and Vitols, E. (1968), *Annu. Rev. Biochem. 37*, 201.
Bray, G. A. (1960), *Anal. Biochem. 1*, 279.
Brown, N. C., Eliasson, R., Reichard, P., and Thelander, L. (1969), *Eur. J. Biochem. 9*, 512.
Brown, N. C., and Reichard, P. (1969a), *J. Mol. Biol. 46*, 25.
Brown, N. C., and Reichard, P. (1969b), *J. Mol. Biol. 46*, 39.
Castellino, F. J., and Barker, R. (1968), *Biochemistry 7*, 2207.
Cecil, R., and Ogston, A. G. (1951), *J. Sci. Instrum. 28*, 253.
Creeth, J. M., and Pain, R. H. (1967), *Progr. Biophys. 17*, 219.
Dawson, D. M., Eppenberger, H. M., and Kaplan, N. D. (1967), *J. Biol. Chem. 242*, 210.
Determann, H. (1966), *Protides Biol. Fluids Proc. Colloq. 14*, 563.
Easley, C. W. (1965), *Biochim. Biophys. Acta 107*, 386.
Edelhoch, H. (1967), *Biochemistry 6*, 1948.
Elliott, D. C. (1959), *in* Data for Biochemical Research, Dawson, R. C., Elliott, D. C., Elliott, W. H., and Jones, K. M., Ed., London, Oxford University Press, p 230.
Frieden, C. (1964), *J. Biol. Chem. 239*, 3522.
Frieden, C. (1967), *J. Biol. Chem. 242*, 4045.
Fujita, H. (1959), *J. Phys. Chem. 63*, 1092.
Gersham, L. C., Stracher, A., and Dreizen, P. (1969), *J. Biol. Chem. 244*, 2726.
Ghambeer, R. K., and Blakley, R. L. (1966), *J. Biol. Chem. 241*, 4710.
Goldberg, R. J. (1953), *J. Phys. Chem. 57*, 194.
Goulian, M., and Beck, W. S. (1966), *J. Biol. Chem. 241*, 4233.
Gray, W. R. (1967), *Methods Enzymol. 11*, 139.
Hamilton, J. A., Yamada, R., Blakley, R. L., Hogenkamp, H. P. C., Looney, F. D., and Winfield, M. E. (1971), *Biochemistry 10*, 347.
Hartley, B. S. (1970), *Biochem. J. 119*, 805.
Hayashi, H., Knowles, J. R., Katze, J. R., Lapointe, J., and Soll, D. (1970), *J. Biol. Chem. 254*, 1401.
Hedrick, J. L., Smith, A. J., and Bruening, G. E. (1969), *Biochemistry 8*, 4012.
Hirs, C. H. W., Moore, S., and Stein, W. H. (1956), *J. Biol. Chem. 219*, 623.
Hirs, C. H. W. (1967a), *Methods Enzymol. 11*, 59.
Hirs, C. H. W. (1967b), *Methods Enzymol. 11*, 325.
Hogenkamp, H. P. C., Ghambeer, R. K., Brownson, C., Blakley, R. L., and Vitols, E. (1968), *J. Biol. Chem. 243*, 799.
Holcomb, G. N., James, S. A., and Ward, D. N. (1968), *Biochemistry 7*, 1291.
Hsieh, W. T., Gundersen, L. E., and Vestling, C. S. (1971), *Biochem. Biophys. Res. Commun. 43*, 69.
Hugli, T. E., and Moore, S. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 1181.
Johnston, J. P., and Ogston, A. G. (1946), *Trans. Faraday Soc. 42*, 789.
Jovin, T. M., Englund, P. T., and Bertsch, L. L. (1969), *J. Biol. Chem. 244*, 2996.
Kielley, W. W., and Harrington, W. F. (1960), *Biochim. Biophys. Acta 41*, 401.
Larsson, A., and Reichard, P. (1967), *Progr. Nucl. Acid Res. 7*, 303.
Lindberg, U. (1967), *Biochemistry 6*, 323.
Margoliash, E. (1962), *J. Biol. Chem. 237*, 2161.
Marler, E., Nelson, C. A., and Tanford, C. (1964), *Biochemistry 3*, 279.
Morley, C. G. D. (1968), Ph.D. Thesis, Australian National University, Canberra, A. C. T., Australia.
Nichol, L. W., Jackson, W. J. H., and Winzor, D. J. (1967), *Biochemistry 6*, 2449.
Oncley, J. L., Scatchard, G., and Brown, A. (1947), *J. Phys. Colloid Chem. 51*, 184.
Orr, M. D., Blakley, R. L., and Panagou, D. (1972), *Anal. Biochem. 45*, 68.
Phelps, R. A., and Putnam, F. W. (1960), *in* The Plasma Proteins, Vol. 1, Putnam, F. W., Ed., New York, N. Y., Academic, p 171.
Schachman, H. K. (1957), *Methods Enzymol. 4*, 32.
Schroeder, W. A., Shelton, J. R., and Robberson, B. (1967), *Biochim. Biophys. Acta 147*, 590.
Siegel, L. M., and Monty, K. J. (1966), *Biochim. Biophys. Acta 112*, 346.
Signer, R., and Gross, H. (1934), *Helv. Chim. Acta 17*, 726.
Smith, I. (1960), Chromatographic and Electrophoretic Techniques, Vol. 2, New York, N. Y., Interscience, p 170.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Stark, G. R. (1967), *Methods Enzymol. 11*, 125.
Stark, G. R., and Smyth, D. G. (1963), *J. Biol. Chem. 238*, 214.
Steers, E., Craven, G. R., Anfinsen, C. B. (1965), *J. Biol. Chem. 240*, 2478.
Tanford, C., Kawahara, K., and Lapanje, S. (1967), *J. Amer. Chem. Soc. 89*, 729.
Van Holde, K. E. (1960), *J. Phys. Chem. 64*, 1582.
Vitols, E., Brownson, C., Gardiner, W., and Blakley, R. L. (1967a), *J. Biol. Chem. 242*, 3035.
Vitols, E., Hogenkamp, H. P. C., Brownson, C., Blakley, R. L., and Connellan, J. (1967b), *Biochem. J. 104*, 58c.
Warner, R. C. (1954), *Proteins 2*, 435.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.
Winzor, D. J., and Scheraga, H. A. (1963), *Biochemistry 2*, 1263.
Woods, E. F., Himmelfarb, S., and Harrington, W. F. (1963), *J. Biol. Chem. 238*, 2374.
Wu, Y. U., and Scheraga, H. A. (1962), *Biochemistry 1*, 698.
Yamada, S., and Itano, H. A. (1966), *Biochim. Biophys. Acta 130*, 538.
Yamada, R., Tamao, Y., and Blakley, R. L. (1971), *Biochemistry 10*, 3959.
Yphantis, D. A. (1964), *Biochemistry 3*, 297.