chem. 20, 1285.
Margolis, R. U. (1967), *Biochim. Biophys. Acta 141,* 91.
Margolis, R. U., and Margolis, R. K. (1972a), *Biochim. Biophys. Acta 264,* 426.
Margolis, R. U., and Margolis, R. K. (1972b), in Research Methods in Neurochemistry, Vol. 1, Marks, N., and Rodnight, R., Ed., New York, N.Y., Plenum Press, p 249.
Margolis, R. U., and Margolis, R. K. (1973c), *Biochem. Pharmacol. 22,* 2195.
Margolis, R. U., and Margolis, R. K. (1974), *Biochemistry 13,* 2849.
Margolis, R. U., Margolis, R. K., and Atherton, D. M. (1972), *J. Neurochem. 19,* 2317.
Margolis, R. U., Margolis, R. K., Chang, L. B., and Preti, C. (1975), *Biochemistry 14,* 85.
Matthieu, J.-M., Quarles, R. H., Webster, H. de F., Hogan, E. L., and Brady, R. O. (1974), *J. Neurochem. 23,* 517.
Morgan, I. G., Zanetta, J.-P., Breckenridge, W. C., Vincendon, G., and Gombos, G. (1973), *Brain Res. 62,* 405.
Quarles, R. H., Everly, J. L., and Brady, R. O. (1973), *J. Neurochem. 21,* 1177.
Raghupathy, E., Ko, G. K. W., and Peterson, N. A. (1972), *Biochim. Biophys. Acta 286,* 339.
Truding, R., Shelanski, M. L., Daniels, M. P., and Morell, P. (1974), *J. Biol. Chem. 249,* 3973.
Van Nieuw Amerongen, A., Roukema, P. A., and Van Rossum, A. L. (1974), *Brain Res. 81,* 1.
Wood, J. G., and McLaughlin, B. J. (1975), *J. Neurochem. 24,* 233.
Zanetta, J. P., Morgan, I. G., and Gombos, G. (1975), *Brain Res. 83,* 337.

An Affinity Adsorbent Containing Deoxyguanosine 5′-Triphosphate Linked to Sepharose and Its Use for Large Scale Preparation of Ribonucleotide Reductase of *Lactobacillus leichmannii*†

Peter J. Hoffmann and Raymond L. Blakley*

ABSTRACT: P^3-(6-(*N*-Trifluoroacetyl)aminohex-1-yl) deoxyguanosine triphosphate has been prepared by the reaction of *N*-trifluoroacetyl-6-aminohexanol 1-pyrophosphate with the imidazolide of dGMP and has been characterized. This compound and the corresponding free amine, obtained by removal of the protective trifluoroacetyl group, are activators of ribonucleotide reductase of *Lactobacillus leichmannii.* An affinity adsorbent for the reductase, prepared by reaction of the amine derivative with CNBr-activated Sepharose, contains dGTP covalently attached through the γ-phosphate via a six-carbon chain to the matrix. The method of synthesis of the dGTP derivative is generally applicable to the synthesis of P^3-(ω-aminoalk-1-yl)nucleoside triphosphate esters for the preparation of analogous affinity adsorbents. Ribonucleotide reductase can be rapidly purified to homogeneity, on a large scale, by use of dGTP–Sepharose and conditions for optimum recovery of the enzyme have been determined. The affinity of ribonucleotide reductase and other proteins for dGTP–Sepharose is increased by either raising the ionic strength or lowering the temperature of the eluent. Elution of the enzyme from the adsorbent can be achieved between pH 5.8 and 7.3, whereas at pH 5.3 the reductase is bound extremely tightly and cannot be recovered. Ribonucleotide reductase can be eluted from the adsorbent with dGTP or urea. Elution with urea is carried out at pH 6.3, where the enzyme is stable and maximum recovery is obtained. Affinity chromatography consistently produces ribonucleotide reductase of high specific activity (170–180 units/mg). In the presence of 0.1 to 1.2 *M* urea or hydroxyurea, the enzyme is inhibited, but allosteric activation is unchanged. No alteration in the structure or function of the reductase was detected when the enzyme was exposed to 2.0 *M* urea during elution from the affinity adsorbent, but exposure for longer periods causes some inactivation.

The allosteric ribonucleotide reductase of *Lactobacillus leichmannii* has been shown to be a monomeric enzyme with a single polypeptide chain (Panagou et al., 1972). The allosteric effectors, which are the deoxyribonucleotide products of the reaction catalyzed by the enzyme, show a pattern of specific activation. For example, dGTP specifically activates ATP reduction and dATP specifically activates CTP reduction (Vitols et al., 1967).

Although the enzyme has previously been purified to homogeneity by conventional procedures (Panagou et al., 1972), different preparations were not of the same high specific activity. Instead, the final purification step, preparative polyacrylamide gel electrophoresis, raised the specific activity of all preparations 20–30% irrespective of the specific activity of the sample electrophoresed (Orr et al., 1972). Furthermore, preparative gel electrophoresis is tedious and unsuitable for large scale purification.

It was considered that an affinity adsorbent of one of the

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nucleotide activators might be successful in the rapid purification of the reductase and conveniently produce, on a large scale, an enzyme of consistently high specific activity.

For the synthesis of the affinity adsorbent, it was not considered feasible to immobilize the activator by covalent attachment via the nucleotide base. Since the base moiety confers the specificity of activation, it is likely that this part of the nucleotide is tightly bound to the allosteric site, and substitution on the base would probably result in greatly decreased affinity of the resulting ligand for the allosteric site. Linkage of the deoxynucleoside trisphosphate activator to Sepharose gel through the terminal phosphate seemed more promising. This paper describes the synthesis of such an affinity adsorbent and its application to the purification of ribonucleotide reductase.

Materials and Methods

Tri-*N*-octylamine (Aldrich Chemical Co.), tributylamine (Eastman Chemical Co.), and *N,N*-dimethylformamide (Fisher) were redistilled before use. Ethyl trifluorothiol acetate and cyanogen bromide were purchased from Pierce Chemical Co., 1,1′-carbonyldiimidazole and 6-aminohexanol were from Aldrich Chemical Co., and crystalline phosphoric acid was from K&K Laboratories. Thin-layer cellulose sheets were obtained from Eastman Chemical Co. (Eastman Chromagram Sheet 6064) and PEI-cellulose sheets from J. T. Baker Chemical Co. Elon (*p*-methylaminophenol) was purchased from Kodak. *N*-Trifluoroacetyl-6-aminohexanol 1-phosphate and 6-aminohexanol 1-phosphate were prepared as described by Barker et al. (1972). Methyltri-*N*-octylammonium hydroxide was prepared according to Letters and Michelson (1963). Urea solutions were deionized by passage through a column of mixed anion and cation exchange resin (Rexyn 300, Fisher) just prior to use. Bio-Gel P-2 was obtained from Bio-Rad Laboratories and Dowex-1, Dowex-50, Sepharose 4B-200, and DEAE-cellulose were from Sigma Chemical Co.

Ribonucleotide reductase was isolated from *L. leichmannii* and purified on a large scale to the second ammonium sulfate precipitation step, as described previously (Panagou et al., 1972) or by a modification of this procedure involving the use of lower pH.[1] Preparations were not freeze dried but stored at 0–4°C. Sodium azide (0.02%) was added to all buffers as an antibacterial agent. Snake venom (*Crotalus adamanteus*) was purchased from Sigma. Other materials used were obtained as previously described (Orme-Johnson et al., 1974; Panagou et al., 1972; Vitols et al., 1967).

Assay of Ribonucleotide Reductase. The enzyme was assayed either colorimetrically (Orr et al., 1972) using dihydrolipoate as the reductant or spectrophotometrically (Vitols et al., 1967) using the thioredoxin system. The final concentrations of reactants in the spectrophotometric assay were: 2 m*M* ATP, 6 μM thioredoxin, 0.3 unit of thioredoxin reductase, 0.2 m*M* NADPH, and 4 μM AdoCbl in a total volume of 0.5 ml. A unit of activity corresponds to the formation of 1 μmol of dATP/hr under the conditions of the colorimetric assay.

Analytical Procedures. Analytical ultracentrifugation (Panagou et al., 1972) and analytical polyacrylamide gel electrophoresis (Orr et al., 1972) were carried out as described previously. In the latter, the triethanolamine, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) buffer system, pH 6.8, was used. Protein concentrations were determined by the biuret method (Gornall et al., 1949) or by a modification of the method of Lowry et al. (Hartree, 1972) with bovine serum albumin as standard. As discussed later the values obtained were high because pure ribonucleotide reductase was found to give higher color values than serum albumin. Circular dichroic measurements were made on a Cary 60 recording spectropolarimeter. Ellipticities are expressed as mean residue ellipticities, $[\theta]$. The mean residue weight of 110 g/mol of amino acid for ribonucleotide reductase was calculated from the amino acid composition (Panagou et al., 1972).

Thin-layer chromatography of synthesized compounds was carried out with isobutyric acid–concentrated ammonium hydroxide–water (66:1:33, v/v) as solvent (system 1) and on PEI-cellulose using 1.0 *M* KCl as solvent (system 2). Spots were located under ultraviolet light or developed with a molybdate spray for phosphates (Brandursky and Axelrod, 1951) or a ninhydrin spray for amines (Levy and Chung, 1953).

Inorganic phosphate was determined by a modified method of Fiske and Subbarow using 1% *p*-methylaminophenol–3% sodium bisulfite as the reducing agent (Jaenicke, 1974). Acid hydrolyzable phosphate plus inorganic phosphate was determined as inorganic phosphate after samples were hydrolyzed in 1 *N* HCl at 100°C for 7 min. Total phosphate was determined as inorganic phosphate after samples were ashed with $Mg(NO_3)_2$ (Ames and Dubin, 1960). Quantitative ninhydrin assays were performed by the method of Rosen (1957), with 6-aminohexanol 1-phosphate as standard. Contributions to ninhydrin color by triethylammonium bicarbonate buffer and the amino group of guanine were subtracted. The concentration of ultraviolet absorbing compounds was determined using a molar extinction coefficient of $\epsilon = 13.7 \times 10^3$ at 252 nm. Concentration of nonabsorbing compounds was determined by dry weight measurements.

Alkaline hydrolysis of *N*-trifluoroacetyl-containing compounds was carried out by adjusting a 2–5 m*M* solution of the compound to pH 11.5 with NaOH and incubating at room temperature overnight. The pH was then readjusted to neutrality with acetic acid.

Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn.

Sequence Determination. Purified enzyme (40 mg) was reduced and carboxymethylated as follows. The enzyme in a volume of 8 ml was precipitated by treatment with 16 ml of acetone overnight at 0°C. The precipitate was removed by centrifugation, washed with acetone, and air dried. The residue was dissolved by the addition of 2.29 g of solid guanidine hydrochloride (Eastman), 0.4 ml of 1 *M* Tris-phosphate buffer (pH 8.1), and 3 ml of water. Solid disodium EDTA was added to a final concentration of 0.2% and the pH then adjusted to 8.1 with 10 *N* NaOH. A few drops of octanol were added to prevent frothing and humidified nitrogen was then bubbled through the solution for 30 min. Reduction of the protein was commenced by the addition of 0.4 ml of 0.5 *M* disodium dihydrolipoate (pH 7.0) and the stream of nitrogen continued for 2.5 hr, with adjustment of the pH to 8.1–8.5 with 10 *N* NaOH. The temperature was then raised to 38°C and a further 0.4 ml of 0.5 *M* dihydrolipoate and 0.6 g of guanidine hydrochloride were added. The nitrogen stream was continued, the pH kept in the range 8.1 to 8.5, and the temperature maintained at 38°C for a further 2 hr. The solution was then cooled to room temperature and while the nitrogen stream continued, 148

[1] D. Singh and R. L. Blakley, unpublished results.

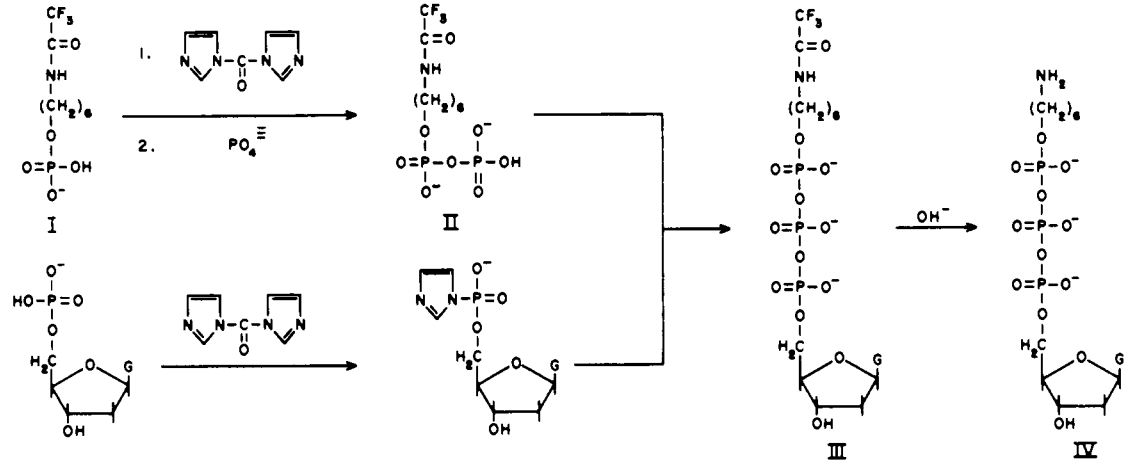

FIGURE 1: The synthesis of *N*-trifluoroacetyl-6-aminohexanol 1-pyrophosphate (II) and P^3-(6-aminohex-1-yl)-dGTP (IV) by the imidazolide method; G = guanine.

mg of recrystallized iodoacetic acid dissolved in 0.55 ml of 1 *N* NaOH was slowly added, with the addition of 10 *N* NaOH to maintain the pH in the range 8.1–8.5. When no further change in pH occurred (about 10 min) two drops of 2-mercaptoethanol were added. After a further 10 min the solution was transferred to a dialysis bag and exhaustively dialyzed against water. The protein suspension was freeze-dried and the residue dissolved in 2 ml of 70% formic acid for transfer to the sequenator cup. Amino acid analysis showed complete carboxymethylation of cystine and cysteine residues. Sequencing was carried out by methods previously described (Gleisner et al., 1975).

Organic Salts of Phosphate Esters. The sodium salt of dGMP and the lithium salt of *N*-trifluoroacetyl-6-aminohexanol 1-pyrophosphate were converted to the pyridinium salt by passing an aqueous solution of the compound through a 4.4 × 30 cm column of Dowex-50 (X2, pyridinium form, 200–400 mesh). The fractions containing the compound were determined by spotting a 5-μl sample of each fraction on a cellulose thin-layer chromatogram sheet containing fluorescent indicator in the case of dGMP, or by determining acid-labile phosphate on a 0.05-ml sample in the case of the pyrophosphate compound. One molar equivalent of methyltri-*N*-octylammonium hydroxide or of tributylamine was added to the combined fractions and ethanol added, if necessary, to give a homogenous solution.

Tributylammonium phosphate was prepared by adding a one molar equivalent of tributylamine to an approximately 1 *M* solution of phosphoric acid.

Solutions were concentrated on the rotary evaporator and the syrupy residues dried by repeated addition of anhydrous dimethylformamide (5 × 70 ml) and evaporation (bath at 45°C). Dimethylformamide was dried over molecular sieves (type 4A) for at least 1 week prior to use. The compounds were further dried and stored in a desiccator under high vacuum over P_2O_5 for at least 24 hr.

Synthesis of N-Trifluoroacetyl-6-aminohexanol 1-Pyrophosphate and P^3-(6-Aminohex-1-yl)-dGTP. Both compounds were prepared via the imidazolide of the respective monophosphate (Figure 1). The imidazolides were formed by reaction with carbonyldiimidazole (Hoard and Ott, 1965), and it was necessary to first protect the primary amino group of 6-aminohexanol 1-phosphate to prevent its reaction with carbonyldiimidazole. The protective trifluoroacetyl group is readily removed later by hydrolysis at pH 11.

Synthesis of N-Trifluoroacetyl-6-aminohexanol 1-Pyrophosphate (II). To a solution of the tributylammonium salt of *N*-trifluoroacetyl-6-aminohexanol 1-phosphate (I, 200 mmol) in 300 ml of dry dimethylformamide was added 50 g (312 mmol) of 1,1′-carbonyldiimidazole. The reagent dissolved rapidly and some gas was evolved. The solution was stirred under a drying tube, over P_2O_5 in a desiccator at room temperature for a further 6 hr. Methanol (6.2 ml, 150 mmol) was added to decompose excess diimidazole (Hoard and Ott, 1965). After 30 min, tributylammonium phosphate (400 mmol) in 500 ml of dry dimethylformamide was added with stirring. A precipitate of imidazolium phosphate formed immediately and the mixture was stirred in a desiccator over P_2O_5 at room temperature for a further 24 hr. Most of the precipitate was removed by centrifugation (3400*g* for 10 min) and washed with dimethylformamide (3 × 50 ml). The slightly turbid supernatant and washings were combined and clarified by filtration (Whatman No. 1 filter paper). The filtrate was treated with an equal volume of methanol and the solution concentrated to a thick syrup on the rotary evaporator. The syrup was dissolved in 1 l. of water and chromatographed at 4°C on a 6 × 100 cm column of Dowex-1 (X2, chloride form, 100–200 mesh) with a gradient formed with 0.01 *N* HCl (15 l.) in the mixing chamber and 0.55 *M* LiCl in 0.01 *N* HCl (15 l.) in the reservoir (Figure 2A). The flow rate was about 300 ml/hr. Each fraction (~250 ml) was neutralized with 5 *M* NH_4OH soon after collection and a sample (0.05 ml) assayed for acid-labile + inorganic phosphate. Fractions 96 to 110 containing the major peak of acid hydrolyzable phosphate (peak D, Figure 2A) were combined and concentrated to dryness on a rotary evaporator (bath at 45°C). Lithium chloride was extracted from the solid with 6 × 300 ml of ice-cold methanol and discarded. The remaining solid was collected on a coarse sintered glass funnel and dried under high vacuum, at room temperature overnight; yield 42.8 g (56%). The compound was recrystallized from concentrated aqueous solution by the addition of methanol or ethanol until the solution was slightly turbid and by then keeping at 4°C overnight. This product (II) was ninhydrin negative but gave a strong positive ninhydrin reaction after incubation at pH 11 to 12 for several minutes at 25°C. Chromatography of II in system 1 (R_f 0.69) or system 2 (R_f 0.34) showed the presence of only one phosphate compound. When 6-aminohexanol 1-pyrophosphate, obtained by hydrolysis of II at pH 11.5, was chromatographed, a sin-

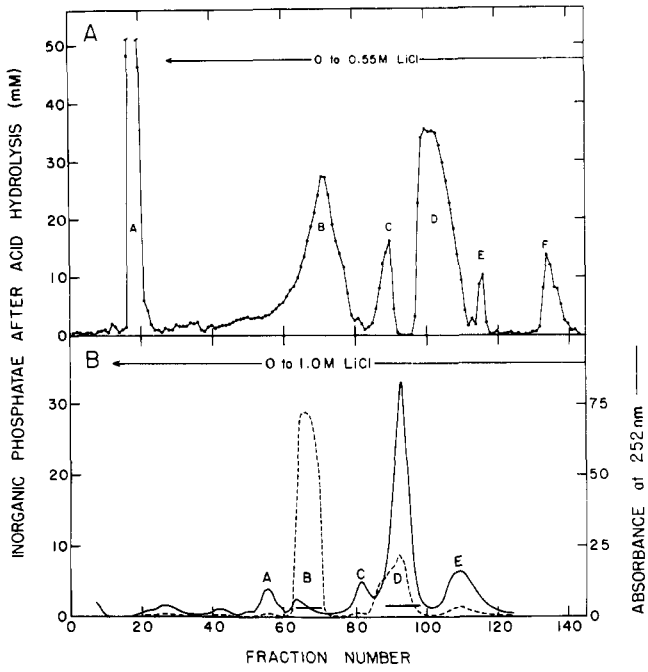

FIGURE 2: Purification of (A) *N*-trifluoroacetyl-6-aminohexanol 1-pyrophosphate (II) and (B) P^3-(6-(*N*-trifluoroacetyl)aminohex-1-yl)-dGTP by chromatography on Dowex-1. Fractions which were combined are indicated by the bars.

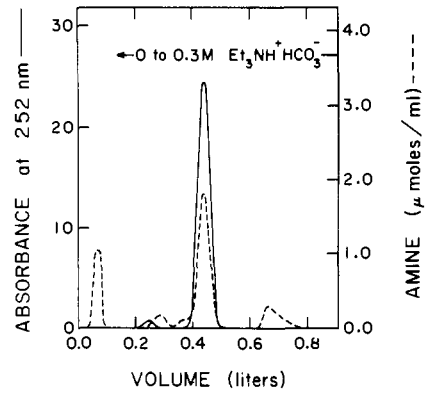

FIGURE 3: Purification of P^3-(6-aminohex-1-yl)-dGTP (IV) by chromatography on DEAE-cellulose. Compound IV (0.1 mmol) obtained after hydrolysis (pH 11.5) of compound III was chromatographed on DEAE-cellulose (bicarbonate form, 1.4 × 28 cm). The column was eluted with a gradient prepared from 500 ml of water in the mixing chamber and 500 ml of 0.5 *M* triethylammonium bicarbonate in the reservoir. Each fraction (7 ml) was assayed for free amino (0.5 ml sample, - - -) and guanine absorbance at 252 nm (—). Alternate fractions across the main ultraviolet absorbing peak were assayed for total phosphate (0.1-ml sample).

gle ninhydrin positive phosphate compound was observed in system 1 (R_f 0.58) and system 2 (R_f 0.45). Elemental analysis performed on crystalline II was as follows. Anal. Calcd for $C_8H_{14}NO_8P_2F_3Li_2$ (mol wt 385.02): C, 24.96; H, 3.67; N, 3.64; P, 16.09. Found: C, 25.10; H, 3.56; N, 3.70; P, 16.07.

Synthesis of P^3-(6-Aminohex-1-yl)-dGTP (IV). To a solution of the methyltri-*N*-octylammonium salt of dGMP (13.0 mmol) in 140 ml of dimethylformamide was added 12 g (75 mmol) of 1,1′-carbonyldiimidazole. After the solution had been stirred for 8 hr, in a desiccator over P_2O_5, at room temperature, 2.88 ml (70 mmol) of methanol was added and the solution stirred for a further 40 min. To this solution was added 43 mmol of II (tributylammonium salt) in 500 ml of dimethylformamide. The reaction mixture was stirred in a desiccator, over P_2O_5 at room temperature for 24 hr. The dimethylformamide was then removed on the rotary evaporator at 45°C and 1 l. of 67% methanol added to the syrupy residue. This solution was then applied to a 6 × 100 cm column of Dowex-1 (X2, chloride form, 100–200 mesh). The column was washed with 500 ml of 50% methanol and then eluted with a gradient generated by 0.01 *M* HCl (15 l.) in the mixing chamber and 1.0 *M* LiCl in 0.01 *M* HCl (15 l.) in the reservoir (Figure 2B). Elution was at 4°C at an average flow rate of 280 ml/hr. Fractions of about 200 ml were collected and neutralized with 5 *N* NH_4OH soon after collection. The absorbance at 252 nm and the acid-labile phosphate (0.1-ml samples) were determined on each fraction. The desired product [P^3-(6-(*N*-trifluoroacetyl)aminohex-1-yl)-dGTP, III] was the major ultraviolet absorbing peak and fractions 86–98 (peak D, Figure 2B) containing this material were pooled and concentrated on the rotary evaporator. The sample was divided into portions (2 × 90 ml) and the lithium chloride was removed from each portion by passage through a 4.4 × 110 cm column of Bio-Gel P-2 (200–400 mesh) equilibrated in 0.05 *M* triethylammonium bicarbonate (pH 7.0). Fractions (200 ml) containing ultraviolet absorbing material were combined.

Analysis for total phosphate and for amine after hydrolysis at pH 11.5 gave significantly higher values per mole (based on ultraviolet absorbance) than that expected. Chromatography in system 2 revealed a phosphate-containing contaminant (R_f 0.10) in addition to the major product (R_f 0.53).

The desalted material (780 ml) was incubated at pH 11.5 at room temperature overnight to hydrolyze the trifluoroacetyl derivative (III) to the free amine (IV) and then rechromatographed at 4°C on DEAE-cellulose (4.4 × 45 cm column, bicarbonate form) with a gradient generated by water (3.5 l.) in the mixing chamber and 0.2 *M* triethylammonium bicarbonate (pH 7.5) (3.5 l.) in the reservoir. Purification on a small scale is shown in Figure 3. The sample was adjusted to pH 7.5 and diluted to 1800 ml (conductance, 2.8 mmho) before application to the column. The flow rate was 200 ml/hr and fractions (20 ml) containing the ultraviolet absorbing peak were combined and concentrated to a syrup. Triethylammonium bicarbonate was removed by addition and evaporation of ethanol (5 × 200 ml). Water (100 ml) was added to the syrup and the solution adjusted to pH 7.5 with NaOH before storage at −15°C.

Compound IV has an identical spectrum to dGTP at pH 7.0, with an absorbance maximum at 252 nm and a minimum at 223 nm. On analysis it gave 1 mol of free amine and 3 mol of phosphate per mol of IV (calculated from absorbance at 252 nm).

Chromatography showed only one ultraviolet-absorbing, phosphate-containing, and ninhydrin-positive compound in both system 1 (R_f 0.52) and system 2 (R_f 0.53). The trifluoroacetyl derivative (III) has R_f 0.67 in system 1 and R_f 0.38 in system 2.

Compound IV was further characterized by incubation with phosphodiesterase and 5′-nucleotidase from snake venom. Compound IV (20 μmol) was incubated at 37°C in Tris-acetate (50 m*M*), magnesium acetate (30 m*M*) (pH 8.8) (Koerner and Sinsheimer, 1957), and 1 mg of snake venom in a total volume of 1 ml. A sample taken after 60 min and chromatographed in system 1 showed an ultraviolet spot corresponding to deoxyguanosine (R_f 0.65), and

Table I: Specificity of Elution of Ribonucleotide Reductase from dGTP–Sepharose.[a]

Column Size	Eluent	Vol of Pooled Fractions (Bed Vol)	Recovery of Act. Applied (%)
1 × 13 cm (10 ml)	5 m*M* ATP in 0.2 *M* potassium phosphate	2.0	0
1 × 13 cm (10 ml)	2 m*M* GTP in 0.2 *M* potassium phosphate	2.0	11
1 × 13 cm (10 ml)	0.5 m*M* dGTP in 0.2 *M* potassium phosphate	2.0	56
1 × 65 cm (50 ml)	0.5 m*M* dGTP in 0.1 *M* sodium citrate	2.0	53
1 × 65 cm (50 ml)	2.0 *M* urea in 0.02 *M* sodium citrate	0.4	73

[a] All buffers were at pH 6.3 and columns were operated at 24°C. Elution with nucleotides was limited to 2 bed volumes.

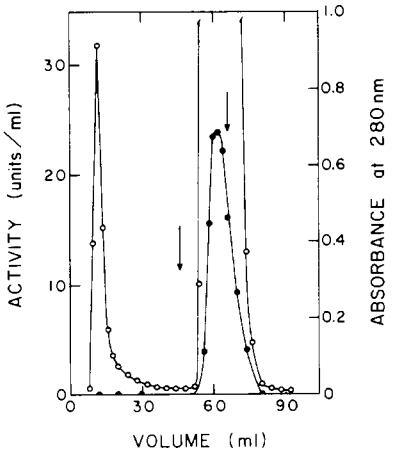

FIGURE 4: Affinity chromatography of ribonucleotide reductase on dGTP-Sepharose. The enzyme (200 units) in 2 ml of 0.1 *M* potassium phosphate buffer (pH 7.3) was applied to a column of dGTP-Sepharose (1 × 13 cm) which was equilibrated and eluted with the same buffer. Ribonucleotide reductase was eluted with two column volumes (20 ml) of 0.5 m*M* dGTP in the eluting buffer (between the arrows). The column was operated at 24°C and enzyme activity was determined by the spectrophotometric method: (●) enzyme activity; (○) absorbance at 280 nm.

phosphate-containing compounds corresponding to 6-aminohexanol 1-pyrophosphate (R_f 0.58) and inorganic phosphate (R_f 0.37), respectively, as the only products. Chromatography of a sample of compound IV incubated with snake venom for 15 min revealed a trace amount of dGMP (R_f 0.45) in addition to the above compounds.

Preparation of the Sepharose Adsorbent. Coupling of compound IV to Sepharose 4B was carried out in batches using the following quantities and the procedures of Cuatrecasas (1970). A slurry of Sepharose 4B consisting of 600 g of firm gel and 300 ml of water was allowed to react with 120 g of CNBr. The well-washed, activated gel was quickly mixed with 2 mmol of compound IV in 300 ml of water at pH 10.0. The mixture was adjusted to pH 10.0 and stirred at 4°C for 18 hr. The gel was washed extensively with water in a column at room temperature and contained 2.4 μmol of ligand covalently bound per ml of packed wet gel, as estimated by the loss of ultraviolet absorbance.

Chromatography on dGTP-Sepharose. Experiments to determine the optimum conditions for purification of ribonucleotide reductase on dGTP-Sepharose were conducted

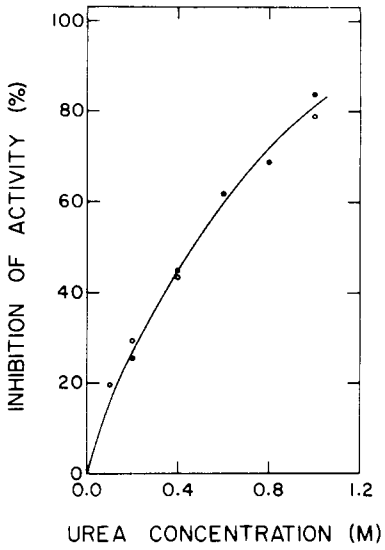

FIGURE 5: Inhibition of ribonucleotide reductase by urea and hydroxyurea. The activity of the enzyme (4.3 units), in the presence of various concentrations of urea (●) and hydroxyurea (○), was determined by the spectrophotometric assay.

using two sizes of column. Columns of dimension 1 × 13 cm (10 ml) were operated at a flow rate of 30 ml/hr and 1-ml fractions were collected, while 1 × 65 cm (50 ml) columns were eluted at 55 ml/hr; 3-ml fractions were collected. Preparative columns (2.5 × 100 cm) were operated at 120–150 ml/hr and 20-ml fractions were collected.

Quantities of enzyme applied to affinity gel were always sufficiently below the capacity of the adsorbent so that greater than 90% of the enzyme activity was retained by the adsorbent. Columns of adsorbent, which were eluted with nucleotide, were washed with 2 *M* urea before reuse. When not in use, dGTP–Sepharose was equilibrated with 0.1 *M* sodium phosphate buffer (pH 7.3) and stored at 4°C. The affinity gel has been used for more than a year without any detectable change in capacity or chromatography.

Results

Synthesis of the Affinity Adsorbent. A nucleoside affinity adsorbent has been prepared by coupling dGTP to Sepharose via a six-carbon spacer attached to the γ-phosphate. The necessary triphosphate compound was synthesized by condensing suitably protected 6-aminohexanol 1-pyrophosphate with dGMP using the imidazolide method of Staab et al. (1959), as developed by Hoard and Ott (1965) (Figure 1). The ligand was then coupled to cyanogen bromide treated agarose through the free amino group of the hexanolamine moiety.

In preliminary work it was found that P^3-(6-*N*-trifluoroacetyl)aminohex-1-yl)-dGTP (III) was formed in greater yield (49%) from the imidazolide of dGMP (Figure 1) than by an alternate route involving the imidazolide of II (32%). Synthesis via the alternate route resulted in substantial hydrolysis of the starting reactant.

N-Trifluoroacetyl-6-aminohexanol 1-pyrophosphate (II) was prepared from *N*-trifluoroacetyl-6-aminohexanol 1-phosphate (I) and inorganic phosphate by the imidazolide method (Figure 1). Purification of the compound was achieved on Dowex-1 (Figure 2A). Of the other compounds eluted peak A contained methyltri-*N*-octylammonium chloride and some dimethylformamide which produced color in the phosphate assay, while peak B was unreacted inorganic

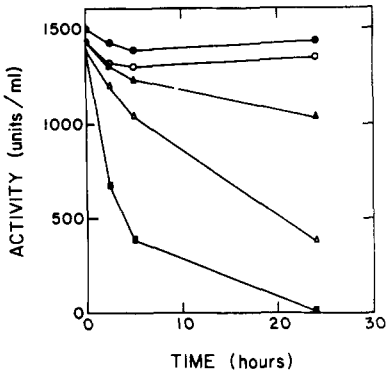

FIGURE 6: Stability of ribonucleotide reductase in the presence of urea. Solutions of enzyme in 0.1 *M* sodium phosphate buffer were adjusted to pH 6.3 or 7.3 and solid urea added to give the desired concentration. The solutions were incubated at 4 or 24°C, and 1-μl samples were assayed for activity by the colorimetric method at various times. Activities were corrected for dilution of the enzyme solutions, and inhibition by urea: (●) no urea, pH 7.3 or 6.3 at 4 or 24°C (control); (○) 2.0 *M* urea, pH 6.3, 4 or 24°C; (▲) 2.0 *M* urea, pH 7.3, 4°C; (△) 3.0 *M* urea, pH 6.3, 24°C, and 2.0 *M* urea, pH 7.3, 24°C; (■) 4.0 *M* urea, pH 6.3, 24°C.

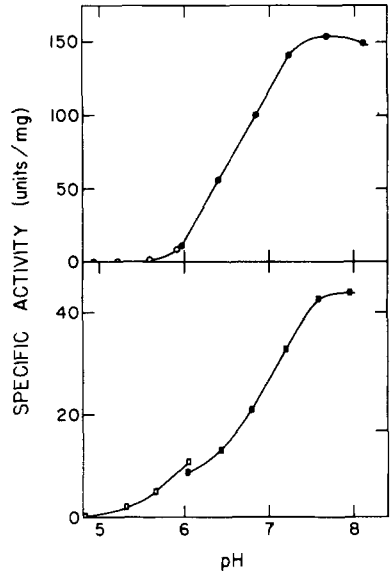

FIGURE 7: The activity of ribonucleotide reductase at different hydrogen ion concentrations. The activity of the enzyme was determined in 0.05 *M* sodium phosphate (●,■) or sodium citrate (○,□) buffer at various pH values in the presence (upper panel) or absence (lower panel) of 1.0 *M* sodium acetate. The colorimetric assay was used, and prior to addition of chloroacetamide mixtures were adjusted to pH 7.5 by the addition of 0.1 ml of NaOH of appropriate concentration.

phosphate. Peak C (Figure 2A) is presumably reactant I, but this and other minor by-products (peaks E and F) were not identified.

A fivefold excess of carbonyldiimidazole is necessary to obtain III in good yield from II. With a 1.5-fold excess (as in the synthesis of II) by-product formation is increased and the yield of III only 12–15%. III was not pure after chromatography on Dowex-1 (Figure 2B, peak D), but after hydrolysis to the free amine (IV), chromatography on DEAE-cellulose (Figure 3) gave a pure product. The compounds in peaks A and B eluted from Dowex-1 (Figure 2B) were identified as the two starting reactants, dGMP and II, respectively; 76% of the unreacted II was recovered. Peaks C and E (Figure 2B) contained mixtures of by-products which were not investigated further.

Both III and IV activate ATP reduction, but not CTP reduction, confirming that they are dGTP derivatives (Vitols et al., 1967). The apparent activation constants are 88 ± 14 μM and 181 ± 68 μM, respectively (cf. 45 ± 4 μM for dGTP) and increases in apparent V_{max} are the same as for dGTP. The value of the activation constant for IV suggested that an affinity adsorbent prepared from this compound should bind ribonucleotide reductase.

Factors Affecting Affinity Chromatography of Ribonucleotide Reductase on dGTP–Sepharose.

Specificity. Ribonucleotide reductase is adsorbed by dGTP–Sepharose and specifically eluted by dGTP (Table I). A representative elution profile is shown in Figure 4. GTP was less effective as an eluent and no elution occurred with ATP. The enzyme could also be eluted with urea (Table I). Although urea is not a specific eluting agent and might elute contaminants also, it elutes the enzyme in a small volume (Table I) and, unlike dGTP, does not interfere with absorbance measurements.

Stability and Activity in Urea. Ribonucleotide reductase is inhibited by urea or hydroxyurea (Figure 5), but allosteric activation of the enzyme is not impaired by these concentrations of urea. In this study the enzyme activity was determined spectrophotometrically because urea inhibits color formation in the diphenylamine colorimetric assay, whereas the activity of the thioredoxin system is unaffected by 1.0 *M* urea. In 1.0 *M* urea (which caused 80% inhibition) dGTP (0.1 m*M*) caused an 8.4-fold increase in the rate of ATP reduction. The specificity of activation (Vitols et al., 1967) was retained since dATP showed no effect on ATP reduction. Urea (1.0 *M*) decreases the affinity of the enzyme for dGTP, the apparent activation constant increasing from 45 ± 4 to 156 ± 17 μM. This is consistent with the ability of urea to elute the enzyme from the affinity adsorbent.

The stability of ribonucleotide reductase is dependent on the concentration of urea and the pH. Figure 6 shows the increased stability to 2 *M* urea caused by lowering the pH from 7.3 to 6.3, and the decrease in stability on raising the temperature from 4 to 24°C or on increasing the urea concentration.

Since lower pH enhances stability to urea, the profile of activity vs. pH was determined (Figure 7). Although 1 *M* sodium acetate activates the enzyme about fourfold, it does not appreciably alter the shape of the curve. The optimum (about pH 7.8) is the same as reported earlier for a less pure enzyme preparation (Ghambeer and Blakley, 1966).

In investigations of urea removal, the specific activity of the dialyzed enzyme was unchanged when the dialysis buffer concentration was varied from 0.02 to 0.50 *M*, the pH was varied from 5.6 to 7.3, or when 5 m*M* EDTA was added to the buffer, but was about 10% higher when sodium citrate buffer was used rather than sodium phosphate buffer. The specific activity was the same whether urea was dialyzed away rapidly or slowly.

Temperature. Since the affinity of ribonucleotide reductase for dGTP–Sepharose increases markedly with decreasing temperature, it is essential to operate columns of the adsorbent near room temperature in order to elute enzyme in a small volume (Table II). At 4°C the enzyme is eluted by 2 *M* urea but in a much larger volume than at 24°C; dGTP elutes very little enzyme at 4°C. Most of the contaminating proteins were also bound tightly to the adsorbent at 4°C.

pH. Reductase affinity for dGTP–Sepharose increases with decreasing pH (Table III). At pH 5.3 2 *M* urea cannot

Table II: Effect of Temperature on the Elution of Ribonucleotide Reductase from dGTP–Sepharose.[a]

Eluent	Temp of Sample Application (°C)	Temp of Elution (°C)	Vol of Pooled Fractions (Bed Vol)	Recovery of Act. Applied (%)
dGTP	4	4	2.0	6
dGTP	4	24	2.0	53
dGTP	24	24	2.0	56
Urea	24	24	0.4	82
Urea	24	4	1.0	78

[a] Elution with dGTP (0.5 m*M* in 0.2 *M* potassium phosphate, pH 6.3) was on 1 × 13 cm columns of affinity gel. Elution with urea (2.0 *M* in 0.02 *M* sodium citrate, pH 6.3) was on 1 × 65 cm columns of affinity gel.

Table III: Effect of pH on the Elution of Ribonucleotide Reductase from dGTP–Sepharose.[a]

pH	% of Act. Applied, Eluted with: Buffer	Urea	Sp Act. (Units/mg)
7.3	10	66	117
6.3	2	82	139
5.8	0	58	90
5.3	0	<1	20

[a] Columns (1 × 65 cm) of affinity adsorbent were equilibrated and washed (after sample application) with 0.1 *M* sodium phosphate at pH 7.3 or with 0.1 *M* sodium citrate buffer at the lower pH values. Enzyme samples were adjusted to the appropriate pH before application to the column and elution was performed with 2.0 *M* urea in 0.02 *M* buffer at the appropriate pH. Columns were operated at 24°C.

release enzyme. Although most of the protein can be subsequently eluted with 2 *M* urea at pH 6.3, only 6% of the applied activity is recovered, so that the enzyme apparently denatures on the column at pH 5.3. Elution at pH 6.3 gives the best recovery and enzyme specific activity. Lower recovery and specific activity at pH 7.3 are probably due to instability of the reductase at this pH (cf. Figure 6). The low specific activity at pH 5.8 can be attributed to some enzyme denaturation on the column and to greater retention of contaminating proteins at this pH.

Ionic Strength. The effect of ionic strength was determined in two types of experiment: (1) ionic strength varied only during sample application and washing; (2) ionic strength varied only during elution of enzyme with 2 *M* urea.

When application and washing are carried out in 0.5 *M* citrate, protein impurities are retarded, and urea subsequently elutes 30% more protein than at lower salt concentrations (0.02 or 0.1 *M*), resulting in enzyme of lower specific activity. A recovery of 80% of the applied activity was obtained at each buffer concentration. The volume in which the enzyme is eluted by urea or dGTP increases with ionic strength (Table IV) and 2 *M* urea gives best recovery in 0.1 *M* buffer. These results demonstrate that the affinity of both protein impurities and of ribonucleotide reductase for dGTP-Sepharose is increased at higher ionic strength.

Urea Concentration. The use of urea concentrations less than 2.0 *M* for elution results in the enzyme appearing in a broader peak. With 1.0 *M* urea, for example, activity was

Table IV: Effect of Ionic Strength during Elution of Ribonucleotide Reductase from dGTP–Sepharose with Urea.[a]

Eluent	Concn of Citrate Buffer in Eluent (*M*)	Vol of Pooled Fractions (Bed Vol)	Recovery of Act. Applied (%)
Urea	0.02	0.4	79
Urea	0.10	0.4	90
Urea	0.50	1.0	75
dGTP	0.01	0.9	65
dGTP	0.10	2.0	53

[a] Columns (1 × 65 cm) of dGTP–Sepharose were equilibrated and washed (after sample application) with 0.1 *M* sodium citrate buffer (pH 6.3). Elution was with 2.0 *M* urea or 0.5 m*M* dGTP in sodium citrate buffer (pH 6.3) at the concentrations indicated below. Columns were operated at 24°C.

still present in the elutate after elution with 1.5 column volumes and only 43% of the applied activity had been recovered to that point.

Final Procedure. The procedure adopted for affinity chromatography of the reductase is as follows. An enzyme sample (60 units/ml of packed dGTP–Sepharose) having a specific activity of about 50 units/mg and a protein concentration of 8–10 mg/ml is adjusted to pH 7.3 and applied in 0.1 *M* sodium phosphate buffer (pH 7.3) to a column of adsorbent equilibrated in the same buffer. The column is washed with the buffer until the nonabsorbed protein is eluted as judged by absorbance at 280 nm. The pH is lowered by washing the adsorbent with 0.5 column volume of 0.1 *M* sodium citrate buffer (pH 6.3), after which the enzyme is eluted with 2.0 *M* urea in 0.1 *M* sodium citrate buffer (pH 6.3). Fractions containing the activity are pooled and dialyzed against 500 vol of 0.1 *M* sodium citrate buffer (pH 5.6), at 4°C over a total of 24 hr. The enzyme is concentrated by pressure dialysis to about 10 mg/ml and stored at 0°C. Ribonucleotide reductase obtained in this manner had a specific activity of 140–150 units per mg of protein or 170–180 units per mg after correction of protein concentration values for the high color value given by ribonucleotide reductase in the biuret assay (see below).

Homogeneity and Properties. After affinity chromatography ribonucleotide reductase gave a single band on polyacrylamide gel electrophoresis at a loading of 100 μg of protein per gel. A single symmetrical schlieren peak was obtained in the ultracentrifuge and the enzyme exhibited the same concentration dependence of $s_{20,w}$ as previously reported (Panagou et al., 1972).

Automated Edman degradation confirmed that Ser is at the amino terminus (Panagou et al., 1972) and permitted identification of seven of the first eight amino-terminal residues (Table V). The Ala obtained in step 1 from hydrolysis of Ser phenylthiazolinone was accompanied by Lys, Asp, Glu, Gly, and Leu in amounts small enough to be attributed to adventitious contamination. However, with only the usual background correction Ala still constituted 79% of the total amino acids present, and the largest contaminant (Gly) was only 7% of the total. Thus, the protein had a minimum purity of 79% and no single contaminant amounted to more than 7% of the total. However, the results are consistent with complete homogeneity of the protein.

A solution containing 1 mg/ml of homogeneous enzyme had an absorbance at 280 nm of 1.33 ± 0.01 when protein concentration was determined by amino acid analysis (Pan-

Table V: Amino Acid Sequence of N-Terminal Region of Ribonucleotide Reductase.

sequence: Ser-Glu—Ile-$\frac{Ser}{Cys}$-Leu-$\frac{Ser}{Cys}$-Ala

Step	Amino Acid Identified: HI	NaOH	TLC	Assignment	Yield (%)
1	Ala	N.D.[a]		Ser	88
2	Glx		Glu	Glu	100
3					
4	Ile + *a*Ile			Ile	92
5	Ala	N.D.		Ser[b]	111
6	Leu			Leu	113
7	Ala	N.D.		Ser[b]	117
8	Ala	Ala		Ala	95

[a] No residue detected. [b] Or *S*-carboxymethylcysteine.

agou et al., 1972) or by refractive index in the ultracentrifuge (Babul and Stellwagen, 1969). For ease of comparison with previous reports (Panagou et al., 1972; Orr et al., 1972; Chen et al., 1974), protein concentrations are expressed as determined by the biuret method using bovine serum albumin as standard, although ribonucleotide reductase gives a greater color yield than bovine serum albumin, per milligram of protein, in both the biuret (1.18 times) and the Lowry (1.35 times) determinations.

A comparison of the kinetic constants (Table VI) and the circular dichroic (CD) spectra (Figure 8) of ribonucleotide reductase eluted from the affinity adsorbent with dGTP or urea indicated that no gross alteration in the structure or catalytic activity of the enzyme had occurred as a result of exposure to 2 *M* urea. Even in the presence of 2 *M* urea no significant changes in the CD spectrum could be detected (Figure 8).

Discussion

Choice of Ligand and Its Mode of Attachment to Sepharose. Of several possible ligands (substrate, coenzyme, modifier) the modifier dGTP was chosen for preparation of the affinity adsorbent for the following reasons. The deoxyribonucleotide activators have high affinity for ribonucleotide reductase (Chen et al., 1974; Morley, 1968), whereas the ribonucleotide substrates show only weak binding to the enzyme in the absence of other reactants (Chen et al., 1974). An adsorbent containing bound coenzyme (AdoCbl) was considered unsuitable because dithiol must be present for adsorption of the enzyme (Yamada and Hogenkamp, 1972) and this leads to subsequent enzyme instability.[2] Furthermore, AdoCbl-Sepharose is photolabile.

dGTP was esterified with hexanolamine and then attached to Sepharose for the following reasons. Affinity adsorbents in which the ligand is extended from the solid matrix through a linear side chain usually bind proteins better (Steers et al., 1971), since steric hindrance between the protein and matrix is decreased. Coupling of a well-characterized ligand, previously derivatized with the spacer group, is preferable to coupling of spacer followed by derivatization with ligand, since only the former method produces a single type of functional group.

The point of attachment of the spacer group to the ligand is important, since unsuitable substitution would decrease enzyme binding. Coupling of dGTP to agarose through the purine moiety (Trayer et al., 1974; Brodelius et al., 1974; Lee et al., 1974) or a ribose hydroxyl (Jackson et al., 1973) seemed more likely to decrease enzyme binding, since the specificity of activation of the enzyme by deoxyribonucleotides implies that the sugar and base moieties must be in intimate contact with the protein. Attachment of dGTP to agarose via the terminal phosphate seemed most promising, especially since an affinity adsorbent prepared from dATP *p*-aminophenyl ester has been used successfully in the purification of ribonucleotide reductase from *Escherichia coli* (Thelander, 1973) and phage T_4 (Berglund, 1972).

Chemical Synthesis. Synthesis of *N*-trifluoroacetyl-6-aminohexanol 1-pyrophosphate permitted the subsequent synthesis of P^3-(6-aminohex-1-yl)-dGTP (IV) from dGMP using the imidazolide method. IV was subsequently allowed to react with CNBr-activated Sepharose to yield the nucleotide affinity adsorbent. This method, based on that of Barker et al. (1972), should be generally applicable to the preparation of an affinity adsorbent with any nucleoside triphos-

[2] D. Panagou and R. L. Blakley, unpublished results.

Table VI: Kinetic Constants of Ribonucleotide Reductase Eluted from dGTP-Sepharose with dGTP or Urea.[a]

Substrate or Activator	Apparent K_m or K_{act}[b]: dGTP-Eluted Enzyme	Urea-Eluted Enzyme
GTP	0.17 ± 0.01 m*M*	0.16 ± 0.01 m*M*
CTP	0.32 ± 0.01 m*M*	0.33 ± 0.02 m*M*
ATP	0.95 ± 0.04 m*M*	1.08 ± 0.05 m*M*
dGTP	66 ± 0.3 μM	51 ± 0.5 μM
dATP	46 ± 3 μM	43 ± 2 μM

[a] For determination of the apparent K_m of the ribonucleotide substrates the concentration of AdoCbl was 12 μM. For determination of the apparent activation constant of the allosteric activators, dGTP and dATP, the concentrations of ATP and CTP were 1.0 and 0.33 m*M*, respectively, and the concentration of AdoCbl was 4 μM. Concentrations of other reagents of the spectrophotometric assay were as described under Materials and Methods. [b] Activation constant.

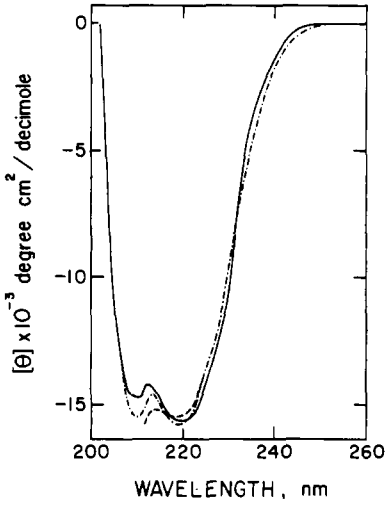

FIGURE 8: Circular dichroism of ribonucleotide reductase. The spectrum of the enzyme eluted from dGTP-Sepharose by dGTP (—) or urea (---) was recorded in 0.1-cm lightpath cells at 24°C. Enzyme solutions (0.13-0.16 mg/ml in 0.01 *M* sodium phosphate buffer, pH 6.0) were passed through a Millipore HA 0.45 μm filter and equilibrated with N_2 in a desiccator at 4°C overnight, prior to measurement. The spectrum of ribonucleotide reductase in the presence of 2.0 *M* urea (- - -) was obtained in the same manner.

phate linked to agarose via a linear alkyl chain through the terminal phosphate.

Synthesis of IV via the diphenylphosphoryl intermediate was avoided because of the large quantities of undesirable symmetrical diphosphates produced with purine nucleotides (Barker et al., 1972) and an anticipated low yield of the desired product (Berglund and Eckstein, 1972). Trayer et al. (1974) have reported the synthesis of 6-aminohexanol 1-pyrophosphate from the amino alcohol and crystalline pyrophosphoric acid. Reaction of 6-aminohexanol 1-pyrophosphate with ethyl trifluorothiol acetate would give II by an alternative route.

Conditions during Affinity Chromatography. The effect of temperature on enzyme elution is so dramatic that recovery when elution with dGTP is used at 4°C is negligible. Unfortunately the affinity of impurities for the adsorbent is also greater at 4°C so that when the sample is loaded at 4°C, enzyme of low specific activity is subsequently recovered. The tighter binding of ribonucleotide reductase to dGTP-Sepharose at 4°C is consistent with the increased binding of dGTP to the enzyme observed at lower temperatures.[1]

The ion-enhanced binding of ribonucleotide reductase to the nucleotide adsorbent was also contrary to expectation since the opposite is usually assumed (Cuatrecasas and Anfinsen, 1971), but concentrated buffer solutions did not elute phage T_4 ribonucleotide reductase from dATP-Sepharose (Berglund and Eckstein, 1972). Shaltiel et al. (1974) took advantage of similar behavior to purify histidinolphosphate aminotransferase.

Purification of Ribonucleotide Reductase on dGTP-Sepharose. The use of dGTP-Sepharose has permitted rapid, large-scale purification of ribonucleotide reductase, this step replacing the final Sephadex-G-100 gel filtration, ammonium sulfate fractionation, and polyacrylamide gel electrophoresis steps of the previous procedure (Panagou et al., 1972). The enzyme has been consistently obtained with a specific activity equal to the highest obtained previously (2). Overall recovery of activity is about 80% compared to 22% by the previous procedure (Panagou et al., 1972; Orr et al., 1972).

Under certain conditions, ribonucleotide reductase is resolved into multiple forms on the affinity adsorbent (Hoffmann and Blakley, 1974). The conditions under which this occurs and the significance of these forms will be reported elsewhere.

References

Ames, B. N., and Dubin, D. T. (1960), *J. Biol. Chem. 235,* 769–775.

Babul, J., and Stellwagen, E. (1969), *Anal. Biochem. 28,* 216–221.

Barker, R., Olsen, K. W., Shaper, J. H., and Hill, R. L. (1972), *J. Biol. Chem. 247,* 7135–7147.

Berglund, O. (1972), *J. Biol. Chem. 247,* 7270–7275.

Berglund, O., and Eckstein, F. (1972), *Eur. J. Biochem. 28,* 492–496.

Brandursky, R. S., and Axelrod, B. (1951), *J. Biol. Chem. 193,* 405–410.

Brodelius, P., Larsson, P-O., and Mosbach, K. (1974), *Eur. J. Biochem. 47,* 81–89.

Chen, A. K., Bahn, A., Hopper, S., Abrams, R., and Franzen, J. S. (1974), *Biochemistry 13,* 654–661.

Cuatrecasas, P. (1970), *J. Biol. Chem. 245,* 3059–3065.

Cuatrecasas, P., and Anfinsen, C. B. (1971), *Methods Enzymol. 22,* 345–378.

Ghambeer, R. K., and Blakley, R. L. (1966), *J. Biol. Chem. 241,* 4710–4716.

Gleisner, J. M., Peterson, D. L., and Blakley, R. L. (1975), *J. Biol. Chem. 250,* 4937–4944.

Gornall, A. G., Bardawill, C. S., and David, M. M. (1949), *J. Biol. Chem. 177,* 751–766.

Hartree, E. F. (1972), *Anal. Biochem. 48,* 422–427.

Hoard, D. E., and Ott, D. G. (1965), *J. Am. Chem. Soc. 87,* 1785–1788.

Hoffmann, P. J., and Blakley, R. L. (1974), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33,* 1533 Abstr.

Jackson, R. J., Wolcott, R. M., and Shiota, T. (1973), *Biochem. Biophys. Res. Commun. 51,* 428–435.

Jaenicke, L. (1974), *Anal. Biochem. 61,* 623–627.

Koerner, J. F., and Sinsheimer, R. L. (1957), *J. Biol. Chem. 228,* 1049–1062.

Lee, C.-Y., Lappi, D. A., Wermuth, B., Everse, J., and Kaplan, N. O. (1974), *Arch. Biochem. Biophys. 163,* 561–569.

Letters, R., and Michelson, A. M. (1963), *Bull. Soc. Chim. Biol. 45,* 1353–1362.

Levy, A. L., and Chung, D. (1953), *Anal. Chem. 25,* 396–399.

Morley, C. G. D. (1968), Ph.D. Thesis, Australian National University, Canberra, Australia.

Orme-Johnson, W. H., Beinert, H., and Blakley, R. L. (1974), *J. Biol. Chem. 249,* 2338–2343.

Orr, M. D., Blakley, R. L., and Panagou, D. (1972), *Anal. Biochem. 45,* 68–85.

Panagou, D., Orr, M. D., Dunstone, J. R., and Blakley, R. L. (1972), *Biochemistry 11,* 2378–2388.

Rosen, H. (1957), *Arch. Biochem. Biophys. 67,* 10–15.

Shaltiel, S., Henderson, G. B., and Snell, E. E. (1974), *Biochemistry 13,* 4330–4338.

Staab, H. A., Schaller, H., and Cramer, F. (1959), *Angew. Chem. 71,* 736.

Steers, E., Jr., Cuatrecasas, P., and Pollard, H. B. (1971), *J. Biol. Chem. 246,* 196–200.

Thelander, L. (1973), *J. Biol. Chem. 248,* 4591–4601.

Trayer, I. P., Trayer, H. R., Small, D. A. P., and Bottomley, R. C. (1974), *Biochem. J. 139,* 609–623.

Vitols, E., Brownson, C., Gardiner, W., and Blakley, R. L. (1967), *J. Biol. Chem. 242,* 3035–3041.

Yamada, R-H., and Hogenkamp, H. P. C. (1972), *J. Biol. Chem. 247,* 6266–6270.