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Glutathione-dependent hydrogen donor system for calf thymus ribonucleoside-diphosphate reductase

(glutaredoxin/thioredoxin/DNA synthesis/enzyme mechanisms)

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ABSTRACT **Purified calf thymus ribonucleoside-diphosphate reductase (2′-deoxyribonucleoside-diphosphate:oxidized-thioredoxin 2′-oxidoreductase, EC 1.17.4.1), showed an absolute requirement for a dithiol as hydrogen donor, whereas the natural monothiol glutathione (GSH) was inactive *per se*. However, a protein partially purified from thymus coupled the oxidation of GSH to the formation of deoxyribonucleotides by ribonucleotide reductase. In analogy with the ribonucleotide reductase system of *Escherichia coli* this protein was called glutaredoxin [Holmgren, A. (1976) *Proc. Natl. Acad. Sci. USA* 73, 2275–2279]. Thymus glutaredoxin had the following properties: (*i*) its molecular weight determined by gel chromatography was about 12,000; (*ii*) it was active with ribonucleotide reductase in the presence of GSH, NADPH, and glutathione reductase but had no activity with NADPH and thioredoxin reductase; and (*iii*) it was immunologically different from thioredoxin because it did not bind to antithioredoxin immunoadsorbents. Experiments on the crossreactivity of thymus and *E. coli* ribonucleotide reductases and the corresponding thioredoxin and glutaredoxin systems showed essentially no specificity for the homologous thioredoxin but a high species specificity for the homologous glutaredoxin.**

In mammalian cells, as in *Escherichia coli*, the *de novo* synthesis of deoxyribonucleotides by ribonucleotide reductase (ribonucleoside-diphosphate reductase; 2′-deoxyribonucleoside-diphosphate:oxidized-thioredoxin 2′-oxidoreductase, EC 1.17.4.1) occurs by replacement of the 2′-OH group of the ribose moiety in the four ribonucleoside diphosphates by hydrogen, with NADPH as the ultimate hydrogen donor (1). The first-isolated immediate hydrogen-donor substrate in this reaction was reduced thioredoxin [thioredoxin-$(SH)_2$], which is kept in the reduced state by NADPH and the specific enzyme thioredoxin reductase (1). However, the true physiological function of thioredoxin was questioned recently, because a mutant of *E. coli* (2, 3) lacking detectable thioredoxin (4) exhibited unabated ribonucleotide reduction *in vivo* and *in vitro* (5). Subsequently, a novel hydrogen-donor system, consisting of NADPH, reduced glutathione (GSH), glutathione reductase, and a small protein called glutaredoxin, was discovered in *E. coli* (5). Recently, glutaredoxin was purified from *E. coli* B (6).

Glutaredoxin from *E. coli* has a molecular weight (12,000) that is similar to thioredoxin. However, the two proteins have entirely different tryptic peptide maps (6), consistent with large differences in amino acid sequences. Also, thioredoxin and glutaredoxin have different catalytic properties. Glutaredoxin exhibits a K_m value for ribonucleotide reductase *in vitro* that is 1/10th that of thioredoxin (7). Glutaredoxin is not reduced by *E. coli* thioredoxin reductase, and thioredoxin is not reduced by GSH (6). Furthermore, glutaredoxin couples the reduction of ribonucleotides by ribonucleotide reductase to the oxidation of GSH to oxidized glutathione (GSSG) (7). One similarity of thioredoxin and glutaredoxin is the presence of a functional cystine disulfide bridge at the active center of both proteins (6).

Ribonucleotide reductase has been purified 3400-fold from calf thymus (unpublished results). Calf liver (8) and rat Novikoff hepatoma (9) thioredoxin and the corresponding thioredoxin reductase (10, 11) have been obtained in highly purified form. We have used the thymus ribonucleotide reductase to search for a glutaredoxin system in calf thymus, and this paper provides the evidence for its existence. In addition we have studied the crossreactivity of thioredoxin, glutaredoxin, and ribonucleotide reductase from calf thymus and *E. coli*.

MATERIALS AND METHODS

The thymus glands from 2 to 4-month-old calves were obtained from local slaughter houses. NADPH, CDP, ATP, dithiothreitol, 5,5′-dithiobis-(2-nitrobenzoic acid), GSH, and crystalline yeast glutathione reductase (240 units/mg) were from Sigma. [^{3}H]-CDP was from Schwarz/Mann. DEAE-cellulose was from Whatman. Glutaredoxin, thioredoxin, and thioredoxin reductase from *E. coli* and phage T4 thioredoxin were homogeneous preparations (6, 12–14). Thioredoxin from calf thymus, which was a preparation of better than 80% purity, and homogeneous calf liver thioredoxin were obtained as described (8, 10). Thioredoxin reductase from rat liver was a near-homogeneous preparation (unpublished results). Ribonucleotide reductases from calf thymus and *E. coli* KK546 (λdnrd) were purified (unpublished results and ref. 15). The gamma globulin fraction of pooled rabbit antisera against calf liver thioredoxin was obtained as described (16).

Assay for Glutaredoxin. Glutaredoxin was determined in a coupled assay with ribonucleotide reductase (5). The incubation mixture was supplemented with 0.5 mM NADPH, 4 mM GSH, and 10 μg of yeast glutathione reductase per ml.

Assay of Ribonucleotide Reductase. This was determined by measuring the conversion of [^{3}H]CDP to [^{3}H]dCDP by techniques described for *E. coli* ribonucleotide reductase (6) or thymus ribonucleotide reductase (unpublished results).

Other Methods. The amounts of thioredoxin in thymus extracts were measured either by thioredoxin reductase-mediated reduction of disulfide bonds in insulin (Method 2) (10) or by four-point double-antibody radioimmunoassay (16). The activity of thioredoxin reductase was measured by NADPH-dependent reduction of 5,5′-dithiobis-(2-nitrobenzoic acid) as described earlier (10).

The antithioredoxin-Sepharose immunoadsorbent was prepared from the gamma globulin fraction of antisera against calf liver thioredoxin and used as described (17).

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Abbreviations: GSH, reduced glutathione; thioredoxin-$(SH)_2$, reduced thioredoxin.

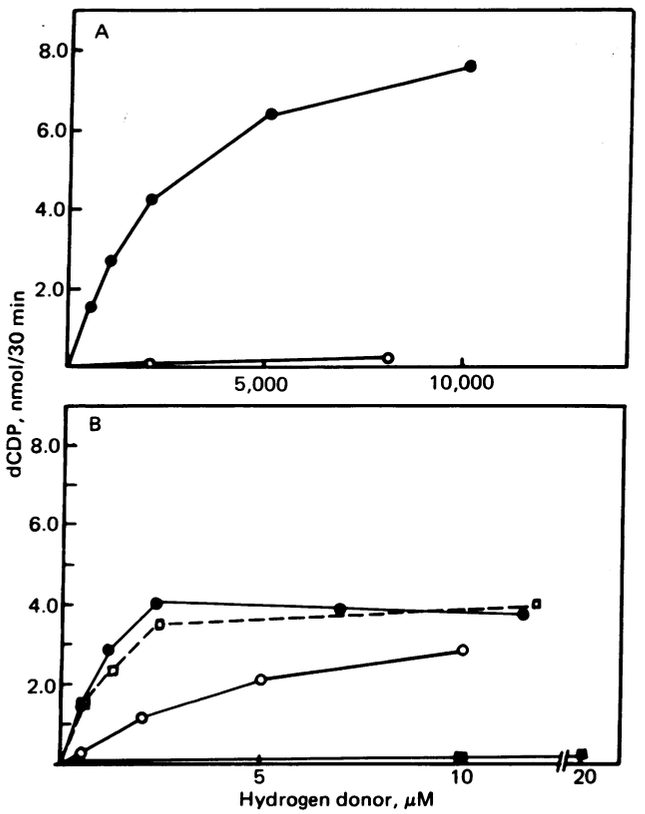

FIG. 1. Hydrogen donor requirements for purified calf thymus ribonucleotide reductase. (*A*) ●, Dithiothreitol; ○, GSH, 1 mM NADPH, and glutathione reductase (0.75 μg). (*B*) The experiments were performed in the presence of 1 mM NADPH and rat liver thioredoxin reductase (2 μg) in combination with calf thymus thioredoxin (●); *E. coli* thioredoxin reductase (2 μg) in combination with *E. coli* thioredoxin (□); *E. coli* thioredoxin reductase (2 μg) and T4 thioredoxin (■); or GSH (2 mM), glutathione reductase (0.75 μg), and *E. coli* glutaredoxin (○). In all experiments 10 μg of purified calf thymus ribonucleotide reductase was used.

Purification of Thymus Glutaredoxin. A total of 3 kg of thymus was homogenized in 9 liters of 50 mM Tris·HCl, pH 7.6, and this extract was centrifuged for 30 min at 20,000 × *g*. The supernatant fraction (crude extract) was saved, and nucleic acids were removed by addition of streptomycin sulfate to 0.65%. After centrifugation, the precipitate was discarded and ammonium sulfate was added to the supernatant fraction to 40% saturation to precipitate ribonucleotide reductase. The remaining protein was precipitated by adding ammonium sulfate to 85% saturation. After dialysis of the dissolved precipitate (40–85% saturation) against 50 mM Tris·HCl, pH 7.5/1 mM EDTA, aliquots of the solution were incubated with 2 mM dithiothreitol for 30 min at 25°C and chromatographed on a large column of Sephadex G-50 coarse. The fractions corresponding to 10,000–15,000 daltons were pooled (thioredoxin as marker). This material (1030 mg of protein in 8000 ml) was incubated with 2 mM dithiothreitol and applied to a column of DEAE-cellulose (400 ml) equilibrated with 50 mM Tris·HCl, pH 7.5/1 mM EDTA. The column was eluted with a linear salt gradient (total volume, 4 liters). The unadsorbed protein (flow-through fraction) was saved, and the eluted protein was divided in three main pools (fractions II–IV, Table 2). After about 25-fold concentration by flash evaporation of all fractions, the salt and dithiothreitol were removed by extensive dialysis against 50 mM Tris·HCl, pH 7.5/0.1 mM EDTA. The fractions were then further concentrated by flash evaporation, incubated with dithiothreitol, and desalted on Sephadex G-25. Assays for thioredoxin and thioredoxin reductase revealed significant levels in all fractions.

Table 1. NADPH-dependent hydrogen donor activity for thymus ribonucleotide reductase in crude extract

Addition	Activity, nmol dCDP per 30 min: 2 mM NADPH	2 mM NADPH + 4 mM GSH*
Extract, 0.2 mg†	0.39	1.69
Heat-treated extract, 0.02 mg‡	0.18	0.73
None	0.03	0.06

* The assay was performed with glutathione reductace (0.5 μg).
† The experiments were carried out in crude extracts in which SH groups in thioredoxin and thioredoxin reductase are easily oxidized (16), inactivating the system. The relative activity of the thioredoxin system (only NADPH) may thus be underestimated.
‡ The crude extract was heated for 2 min at 65°C, precipitated protein was removed by centrifugation, and the supernatant solution was concentrated by ammonium sulfate precipitation to 85% saturation followed by desalting on a column of Sephadex G-25.

To remove possible interference from the thioredoxin system in the glutaredoxin assay, a sample from each fraction was passed through an antithioredoxin-Sepharose column (16, 17). The thioredoxin content of the effluent from the column was measured by radioimmunoassay. The flow-through fraction from DEAE-cellulose, after passage through the antithioredoxin column, was concentrated by vacuum filtration with Millipore filter units to a protein concentration of approximately 6 mg/ml, and this solution was used as the thymus glutaredoxin preparation.

RESULTS

Hydrogen Donor Requirements for Thymus Ribonucleotide Reductase. The assay for thymus ribonucleotide reductase during purification utilizes the reduction of [^{3}H]CDP to [^{3}H]dCDP, using 10 mM dithiothreitol as hydrogen donor

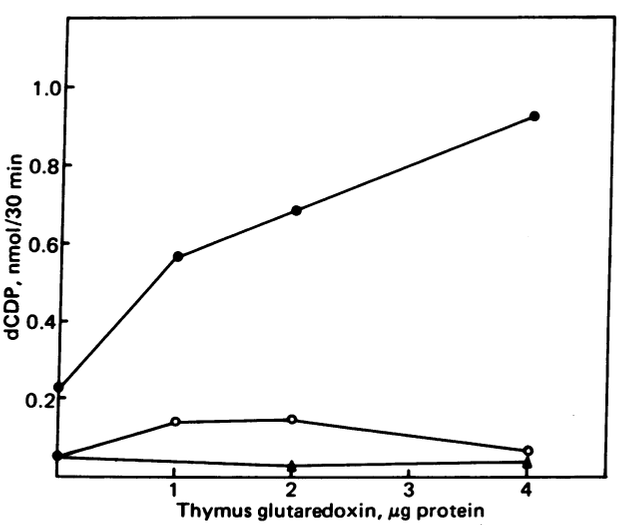

FIG. 2. Activity of the thymus glutaredoxin fraction as hydrogen donor for calf thymus ribonucleotide reductase. Assay with NADPH only (▲), NADPH and rat liver thioredoxin reductase (○), NADPH, GSH, and yeast glutathione reductase (●).

Table 2. Separation of thymus thioredoxin and glutaredoxin by DEAE-cellulose and antithioredoxin-Sepharose chromatography

Concentrated DEAE fractions	Total glutaredoxin activity, units	Before antithioredoxin-Sepharose*		After antithioredoxin-Sepharose†	
		Thioredoxin, μg/ml	Glutaredoxin activity units/ml	Thioredoxin, μg/ml	Glutaredoxin activity, units/ml
Fraction I (flow-through)	6240	17	75	<0.1	42
Fraction II (0.05–0.20 M acetate)	1770‡	550	131‡	<1.0	4
Fraction III (0.20–0.35 M acetate)	680	170	46	<0.3	2
Fraction IV (0.35–0.50 M acetate)	125	40	11	<0.3	6

One unit of glutaredoxin activity corresponds to 1 nmol of dCDP formed per 30 min. Thioredoxin was determined by radioimmunoassay (16).

* The dialyzed DEAE fractions were concentrated, incubated with 1 mM dithiothreitol for 30 min at 25°C, and desalted on Sephadex G-25.

† An aliquot of the peak fraction from Sephadex G-25 was passed through an antithioredoxin-Sepharose column (5 ml), and the effluent was analyzed.

‡ Total glutaredoxin activity also includes activity of the thioredoxin system, because this fraction had thioredoxin and detectable thioredoxin reductase.

substrate (unpublished results). The apparent K_m value for dithiothreitol was 3 mM, whereas the monothiol GSH, together with excess NADPH and glutathione reductase, was completely inactive at these concentrations (Fig. 1*A*). The thymus ribonucleotide reductase preparation thus showed the same *in vitro* specificity for a dithiol as was described for the enzymes from *E. coli* and *Lactobacillus leichmannii* (1). Furthermore, no significant amounts of thioredoxin were present in the ribonucleotide reductase preparation as tested by radioimmunoassay.

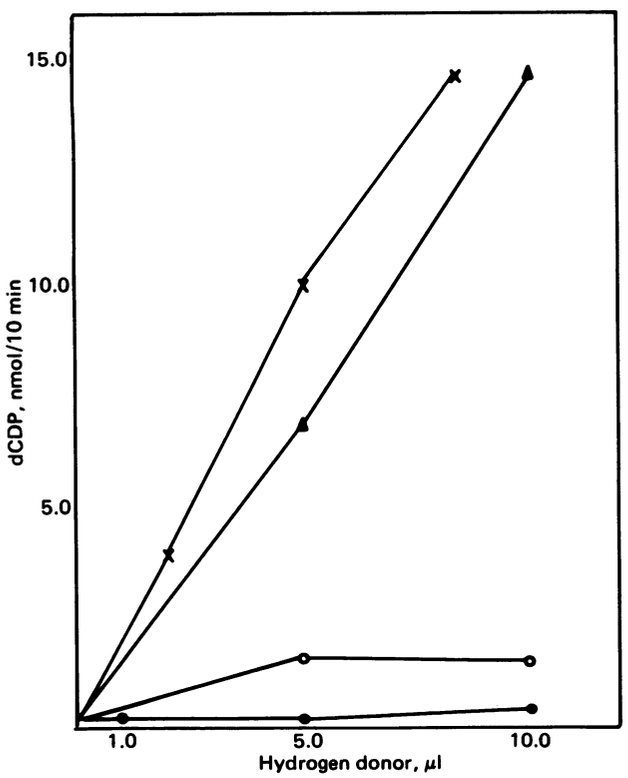

FIG. 3. Efficiency of different hydrogen donor systems with *E. coli* ribonucleotide reductase. The activity tests were performed in a final volume of 120 μl (5) with 10 μg of enzyme. Additions: X, *E. coli* thioredoxin (90 μM) plus 1 mM NADPH and *E. coli* thioredoxin reductase; ▲, calf thymus thioredoxin (90 μM) plus 1 mM NADPH and rat liver thioredoxin reductase; O, calf thymus thioredoxin (90 μM) plus 4 mM GSH, 1 mM NADPH, and yeast glutathione reductase; ●, thymus glutaredoxin (6 mg/ml) plus 4 mM GSH, 1 mM NADPH, and yeast glutathione reductase.

The effect of different natural hydrogen donor systems on the enzyme activity of thymus ribonucleotide reductase is shown in Fig. 1*B*. The thioredoxin systems from calf thymus and *E. coli* were essentially equally active, and the V_{max} values were similar to that obtained with dithiothreitol. These thioredoxin systems showed K_m values of about 2 μM. The phage T4 thioredoxin system containing NADPH and *E. coli* thioredoxin reductase was totally inactive. The *E. coli* glutaredoxin system was less active than the thioredoxin system, because the apparent K_m value for glutaredoxin was several-fold higher than for *E. coli* thioredoxin. Thus, thymus ribonucleotide reductase can catalyze glutathione-dependent ribonucleotide reduction by using the *E. coli* glutaredoxin system, but with a low efficiency.

Evidence for a Thymus Glutaredoxin System. Thymus contains both thioredoxin and thioredoxin reductase (8, 10, 16). In the search for a glutaredoxin system in thymus, we assayed crude extracts with ribonucleotide reductase by using either NADPH alone or NADPH plus 4 mM GSH and glutathione reductase. As shown in Table 1, addition of NADPH alone resulted in a low activity, whereas the addition of both NADPH and GSH stimulated the activity about 4-fold, indicating the presence of a GSH-dependent system. The activity was heat-stable because the same degree of stimulation was observed after heating the extract to 65°C for 2 min.

In separate experiments, desalted crude extract was heated to 65°C, precipitated protein was removed by centrifugation, and the supernatant solution was incubated with dithiothreitol (16) and chromatographed on a Sephadex G-50 column (data not shown). The GSH-dependent hydrogen donor activity for ribonucleotide reductase eluted under the thioredoxin peak, suggesting the existence of a thymus glutaredoxin with a molecular weight similar to that of thioredoxin (12,000).

Partial Purification of Thymus Glutaredoxin. Thymus

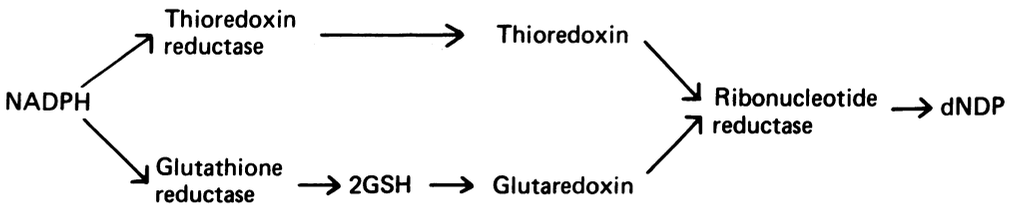

FIG. 4. Hydrogen transport systems *in vitro* for the enzymatic formation of deoxyribonucleotides (dNDP) in calf thymus.

glutaredoxin was purified from crude extract by fractionation with ammonium sulfate (40–85% saturation) and chromatography on Sephadex G-50. Proteins with molecular weights of 10,000–15,000, including thymus thioredoxin, were pooled and chromatographed on a column of DEAE-cellulose with a salt gradient (Table 2). This chromatography separated thioredoxin and glutaredoxin, because almost all of the glutaredoxin activity was found in the flow-through fraction. In contrast, most of the thioredoxin was found in fraction II. To eliminate thioredoxin, a sample from all the fractions was passed over a calf thioredoxin immunoadsorbent Sepharose column. After this treatment no significant level of thioredoxin remained, as determined by radioimmunoassay, whereas most of the glutaredoxin activity was recovered in good yield in the flow-through fraction. This material was used in subsequent studies as the partially purified thymus glutaredoxin.

Characterization of Thymus Glutaredoxin Activity. As shown in Fig. 2, thymus glutaredoxin was hydrogen donor for ribonucleotide reductase from thymus in the presence of GSH, NADPH, and glutathione reductase. The glutaredoxin fraction supplemented with NADPH or NADPH plus rat liver or thymus thioredoxin reductase gave negligible activity.

Crossreactivity of Calf Thymus Glutaredoxin and Thioredoxin with Ribonucleotide Reductase from *E. coli*. The reduction of CDP by homogeneous *E. coli* ribonucleotide reductase was measured with different hydrogen donor systems (Fig. 3). Calf thymus thioredoxin was a good hydrogen donor when coupled to NADPH and thioredoxin reductase but was essentially inactive with GSH and NADPH plus glutathione reductase. The calf thymus glutaredoxin system gave very little activity with *E. coli* ribonucleotide reductase. These results show that the interaction of glutaredoxin with ribonucleotide reductase is highly species specific, whereas thioredoxin exhibits very low species specificity.

DISCUSSION

The results of this paper show that two different hydrogen donor systems of bovine thymus couple the formation of deoxyribonucleotides by purified thymus ribonucleotide reductase to the oxidation of NADPH (Fig. 4). One system is the well known thioredoxin system (8, 10, 16). The other system is a GSH-dependent system that requires the catalytic activity of a small, heat-stable protein identified as a thymus glutaredoxin and partially purified here. The thymus glutaredoxin resembles the *E. coli* glutaredoxin (6, 7) in several properties: (*i*) it has an apparent molecular weight of about 12,000 as determined by gel chromatography; (*ii*) it has no activity with NADPH and thioredoxin reductase but is active with GSH, NADPH, and glutathione reductase; and (*iii*) it is immunologically different from bovine thioredoxin because it is not bound to antithioredoxin-Sepharose.

Glutaredoxin from *E. coli* and thymus shows a pronounced species specificity in crossreactivity experiments with ribonucleotide reductase. The pure *E. coli* glutaredoxin was almost 10-fold more active than thioredoxin in its reaction with the bacterial ribonucleotide reductase. In contrast, 2 μM *E. coli* glutaredoxin was less than 30% as active as 2 μM *E. coli* thioredoxin in the reaction with thymus ribonucleotide reductase. Similarly, the thymus glutaredoxin preparation showed no activity with ribonucleotide reductase from *E. coli*. This observation is of general importance for attempts to purify corresponding glutaredoxin systems in other organisms.

In contrast to glutaredoxin, the thioredoxins from *E. coli* and calf thymus are essentially equally active as hydrogen donors for ribonucleotide reductase in the crossreactivity experiments. Thioredoxins from *E. coli* and calf liver have homologous amino acid sequences, and both are equally active as protein disulfide reductases in the reduction of insulin disulfides (unpublished results). In *E. coli*, the mechanism of ribonucleotide reductase with thioredoxin as hydrogen donor involves the reduction of a functional cystine disulfide of the enzyme by thioredoxin-$(SH)_2$ in a ping-pong mechanism (18). No ternary complex is thus formed. It seems likely that thymus ribonucleotide reductase reacts with thioredoxin in a similar way. The mechanism for the glutaredoxin-dependent reaction is not known.

It is not yet possible to understand the biological significance of the existence of the two *in vitro* pathways of hydrogen transport for ribonucleotide reductase. From studies of *E. coli* mutants in the gene for either thioredoxin (4, 5) or thioredoxin reductase (19), it is evident that thioredoxin is not an obligatory intermediate in ribonucleotide reduction. Furthermore, *E. coli* thioredoxin was recently shown to be present in logarithmic phase cells in a very labile phosphorylated form with a thiol-phosphate bond (20, 21) that blocks a cysteine residue (Cys-32) of the active center (22). This active cysteine is located in a protrusion in the three-dimensional structure of the molecule (23). In mammalian tissues, thioredoxin is an abundant protein which occurs in all cells examined, irrespective of ongoing cell divisions or DNA synthesis (16, 24), whereas ribonucleotide reductase shows a strong correlation to growth rate and DNA synthesis (unpublished results and ref. 24). Further studies are required to relate the activity of the glutaredoxin pathway to the rate of ribonucleotide reduction and DNA synthesis in normal and malignant cells.

Note Added in Proof. The purification of calf thymus ribonucleotide reductase was as described (25).

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