Inhibition of Ribonucleotide Reductase from Ehrlich Tumor Cells by RNA

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SUMMARY

Ribonucleotide reductase activity from Ehrlich ascites cells was inhibited by RNA. Inhibition of cytidine 5'-diphosphate reduction was observed with Ehrlich tumor cell transfer RNA, ribosomal RNA, yeast transfer RNA, and synthetic polycytidylylate. Polyguanylylate and polyuridylylate were only slightly effective as inhibitors, while polyadenylylate did not inhibit at all. The RNA's and polycytidylylate appeared to be competitive inhibitors with respect to cytidine 5'-diphosphate as substrate. Adenosine 5'-diphosphate reduction was inhibited by transfer RNA but was only slightly inhibited by polyadenylylate.

INTRODUCTION

RNA has been shown to have a direct effect on DNA synthesis. It was demonstrated (2, 23) that RNA can direct the synthesis of DNA via the "reverse transcriptase" found in oncogenic RNA viruses. It has also been reported (24) that single-stranded polyribonucleotides at very low concentrations inhibit DNA polymerase of Rauscher leukemia virus.

In this report, we present data that show a way in which RNA indirectly affects DNA synthesis. These data show that RNA markedly inhibits ribonucleotide reductase from Ehrlich ascites tumor cells, at concentrations which could be of physiological significance. Previous studies showed that there is a direct relationship between tumor growth rates and ribonucleotide reductase levels (7) and between the level of ribonucleotide reductase and levels of DNA synthesis in vivo (5). The role of ribonucleotide reductase in normal DNA synthesis is, therefore, very important. The factors that control the in vivo activity of ribonucleotide reductase must be elucidated.

MATERIALS AND METHODS

Ehrlich ascites cells were taken from mice (ICR/Dub) 7 days after transplantation. The crude extract was prepared from these cells essentially by the method of Moore (17), except that all solutions contained 1 mM dithioerythritol. The pH 5.2-insoluble fraction, after redissolving in 0.05 M Tris-HCl, pH 8.0, was made up to 0.25% in protamine sulfate with 1% solution of protamine sulfate. The insoluble material was collected by centrifugation, and the supernatant fluid was discarded. The ribonucleotide reductase activity was eluted from the insoluble pellet with 0.02 M sodium phosphate, pH 8.0 (one-half volume of the original pH 5.2 fraction).

The standard CDP reductase assay mixture contained 14C-labeled CDP, 5 X 10^-5 M, (6.67 mCi/mmol); ATP, 1.9 mM; magnesium acetate, 3.9 mM; dithioerythritol, 5.7 mM; potassium phosphate buffer, 7.7 mM, pH 7; and 100 µl of the protamine sulfate fraction (0.6 to 0.9 mg protein) in a final reaction volume of 150 µl. The reactions were initiated by the addition of substrate and were carried out for 30 min at 37°. The reactions were terminated by heating for 4 min in a boiling-water bath. Carrier dCMP (0.3 µmole), 2 mM MgCl2, and 6 mM Tris-Cl (pH 8.8) in 50 and 25 µl snake venom suspension (Crotalus adamanteus, 40 mg/ml) were added, and the reactions were carried out for at least 2.5 hr at 37° and then were stopped by heating. Water (0.8 ml) was added to each tube, and the insoluble protein was removed by centrifugation. The supernatant fluid was passed over a column of Dowex 1-borale (21). The standard ADP reductase assay mixture contained 14C-labeled ADP, 1.9 X 10^-5 M (17.7 mCi/mmol); dithioerythritol, 5.7 mM; magnesium acetate, 3.9 mM; dGTP, 9.6 X 10^-4 M; potassium phosphate buffer, 7.7 mM, pH 7; and 100 µl of the protamine sulfate fraction (0.6 to 0.9 mg protein) in a final reaction volume of 150 µl. The reactions were initiated by the addition of substrate, were carried out for 30 min at 37° and were terminated by heating for 4 min in a boiling-water bath. Carrier dAMP (0.3 µmole), 2 mM MgCl2, and 6 mM Tris-Cl (pH 8.8) in 50 and 25 µl snake venom suspension (C. adamanteus, 40 mg/ml) were added; reactions were carried out for at least 2.5 hr at 37° and were stopped by heating. Aliquots (100 µl) were spotted on Whatman No. 3MM paper (9 x 22 inches), and the chromatogram was developed overnight with a solvent system containing isopropyl alcohol:ammonium hydroxide:0.1 M boric acid (70:10:20) (8). The deoxyadenosine areas were cut out and eluted with 0.01 N HCl, and aliquots were taken for 14C-labeled deoxyadenosine measurements. Heated controls served as blanks.

1 This work was supported by Grant CA-10380 from the USPHS, National Cancer Institute.

2 Recipient of Research Career Development Award CA-17199 from USPHS, National Cancer Institute.

Received July 21, 1972; accepted February 2, 1973.

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The assays were run either in duplicate or triplicate. The data are expressed in terms of nmoles of deoxyadenosine per 30 min. The duplicate or triplicate tubes for ADP reductase activity assayed by this paper chromatographic method usually varied by 10 to 15% of the average value of the samples.

RNA was extracted from the tumor cell homogenate with phenol, according to the procedure of Kirby (13). The tRNA was separated from rRNA with 1 M NaCl (27). The RNA and tRNA prepared by these procedures had absorbance ratios at 260:280 nm of 1.92 and 1.74, respectively.

To prepare RNA-14C, we gave tumor-bearing mice injections of cytidine-14C (0.2 µCi) at 12-hr intervals 2 days before sacrifice. The tumor cells were isolated and the RNA was prepared as before. The specific activity of the isolated tRNA was 2 × 10^6 cpm/mg RNA. RNase activity in the protamine sulfate fraction was determined by measurement of the radioactivity released as acid-soluble material after incubation at 37° for 30 min, essentially by the method of Kalnitsky et al. (11), except that the RNase activity was determined at the pH of the reductase assay.

Protein concentrations were estimated by the method of Lowry et al. (16), with egg albumin (crystallized 5 times) as the standard. RNA was determined by the orcinol method (10) or by use of a nomograph (26). Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer.

The 14C-labeled CDP (338 mCi/m mole), 14C-labeled cytidine (374 mCi/m mole), and 14C-labeled ADP (17.7 mCi/m mole) were purchased from New England Nuclear, Boston, Mass. The rest of the biochemicals used in these studies were purchased from Sigma Chemical Company, St. Louis, Mo. The mice were purchased from Flow Research Animals, Inc., Dublin, Va.

RESULTS

Inhibition of Ribonucleotide Reductase by RNA. Ribonucleotide reductase activity was inhibited by tRNA and rRNA from Ehrlich ascites cells, by yeast tRNA, and by poly(C). Poly(A) was not inhibitory, while poly(I), poly(G), and poly(U) were only slightly inhibitory. These data are summarized in Table 1. Poly(I) and poly(G) did not alter the inhibition caused by poly(C).

Chart 1 shows the effect of yeast tRNA concentration on the inhibition of ribonucleotide reductase. Similar data were obtained for the other RNA's. In the case of each of the RNA's used in this study, the degree of inhibition obtained leveled off at 70 to 75%. However, the concentration of RNA required to reach this inhibition was quite low. A concentration of approximately 5 µM was required to give 50% inhibition.

Since the assay method for determining ribonucleotide reductase activity involves the use of a 2nd enzymatic hydrolysis step (phosphodiesterase and phosphomonoesterase activities in snake venom), the effect of RNA on this portion of the assay method was tested. The standard assay for reductase was set up, allowed to proceed for 30 min, and terminated by heating; then the RNA and snake venom were added and the procedure was carried out in the usual manner. It was observed that, with concentrations as high as 625 µg of poly(C) or yeast tRNA per ml, there was only a 10% decrease in activity. Therefore, at the concentrations of RNA used as inhibitors in these experiments, the effects observed were the result of inhibition of ribonucleotide reductase and not of the snake venom enzymes.

Effect of RNase Treatment on Inhibition by RNA. Yeast tRNA was hydrolyzed by either pancreatic or T1 RNase, or by a mixture of both. The hydrolysis of RNA by these nucleases did not affect the extent of inhibition (Table 2). Paper chromatography of the RNase-treated RNA samples showed that there was extensive hydrolysis, while chromatography of the T1 RNase-treated RNA sample (over Sephadex G-25) showed that approximately 17% eluted with the void volume, 40% eluted in the region of M.W. 1300 to 1800, and the rest eluted as smaller molecular-weight compounds.

Since it was observed that there was little alteration in inhibition by RNase treatment of the tRNA, the effect of 2'(3')-nucleotides on ribonucleotide reductase activity was determined. These data are shown in Table 3. From these data, it seems that 2'(3')-CMP is the monoribonucleotide responsible for the inhibition of ribonucleotide reductase, although a synergistic effect is obtained when the other 3 nucleotides are added. However, the concentration of mononucleotide required to obtain the limited inhibition observed was about 1 mM, which is approximately 10^6 times higher than was required for yeast or tumor cell tRNA.

Table 3 also presents data showing a comparison of the inhibition of CDP reductase activity by 2'(3')-CMP, CpC, CpCpC, poly(C), and yeast tRNA. On either a weight or molarity basis, poly(C) was most inhibitory. Addition of 5'-CMP to the assay mixture at the same final concentration of 2'(3')-CMP caused an "apparent" inhibition of 27%. It is known that the protamine sulfate fraction contains nucleotide kinases which phosphorylate some of the 5'-CMP to 5'-CDP during the reaction period and would cause isolate dilution of the CDP-14C substrate.

Effect of Preincubation on Inhibition by RNA. Since RNase-treated RNA inhibited CDP reductase activity, the possibility existed that it was not RNA but the nuclease-generated products that were inhibitory. To test this theory, we incubated yeast tRNA and poly(C) with the protamine sulfate fraction under 2 different sets of conditions. In the 1st set of experiments, the protamine sulfate fraction was incubated with or without tRNA and poly(C) for 20 hr at 0-5°. The substrate (CDP-14C) and the RNA or poly(C) were added to the appropriate tubes, and the reductase assay was carried out at 37° as usual. The extent of inhibition with either tRNA or poly(C) was the same (40 and 60%, respectively) whether the RNA was preincubated with the enzyme fraction or added at the time of substrate addition. In the 2nd set of experiments, the enzyme fraction was incubated in the presence and absence of tRNA and poly(C) at 37° for 30 and 60 min. Substrate and RNA were added, and the reaction was carried out for an additional 30 min at 37°. Again, preincubation of
Inhibition of Ribonucleotide Reductase by RNA

Table 1
Effect of various RNA's on CDP reduction

<table>
<thead>
<tr>
<th>RNA</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor tRNA</td>
<td>40</td>
</tr>
<tr>
<td>Tumor rRNA</td>
<td>42</td>
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<tr>
<td>Yeast tRNA</td>
<td>55</td>
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<tr>
<td>Poly(C)</td>
<td>60</td>
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<td>Poly(A)</td>
<td>2</td>
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<td>Poly(U)</td>
<td>19</td>
</tr>
<tr>
<td>Poly(G)</td>
<td>15</td>
</tr>
<tr>
<td>Poly(I)</td>
<td>20</td>
</tr>
<tr>
<td>Poly(C) + poly(I)</td>
<td>60</td>
</tr>
<tr>
<td>Poly(C) + poly(G)</td>
<td>60</td>
</tr>
</tbody>
</table>

The assays were carried out as described in “Materials and Methods” with the addition of the RNA to the standard assay mixture with a final reaction volume of 150 μl. The RNA's were added at a final concentration of 25 μg/reaction. Poly(I) and poly(G) (25 μg) were added to poly(C) (25 μg) before addition of enzyme and substrate. The enzyme activity for CDP reduction in absence of added RNA was 1.44 nmoles/30 min.

Table 2
Effect of pancreatic and T₁ RNases on CDP reduction

<table>
<thead>
<tr>
<th>RNA sample</th>
<th>Activity remaining (%)</th>
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<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Yeast tRNA</td>
<td>56</td>
</tr>
<tr>
<td>Pancreatic RNase-treated RNA</td>
<td>64</td>
</tr>
<tr>
<td>T₁ RNase-treated RNA</td>
<td>61</td>
</tr>
<tr>
<td>Pancreatic and T₁ RNase-treated RNA</td>
<td>64</td>
</tr>
</tbody>
</table>

Note: a The controls containing no RNA had an initial velocity of 1.64 nmoles of deoxycytidine per 30 min.

Table 3
Effect of 2'-(3')-mononucleotides and oligonucleotides on CDP reduction

<table>
<thead>
<tr>
<th>Experiment</th>
<th>2'(3')-nucleotide(s)</th>
<th>Activity remaining (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CMP, UMP, GMP, AMP</td>
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</tr>
<tr>
<td></td>
<td>CMP</td>
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<tr>
<td></td>
<td>CMP, UMP</td>
<td>74</td>
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<tr>
<td></td>
<td>CMP, GMP</td>
<td>64</td>
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<tr>
<td></td>
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<tr>
<td></td>
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<tr>
<td></td>
<td>GMP</td>
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<tr>
<td></td>
<td>UMP</td>
<td>87</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Experiment</th>
<th>2'(3')-nucleotide(s)</th>
<th>Activity remaining (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2'(3')-CMP</td>
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</tr>
<tr>
<td></td>
<td>CpC</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>CpCpC</td>
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</tr>
<tr>
<td></td>
<td>Poly(C)</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>Yeast tRNA</td>
<td>54</td>
</tr>
</tbody>
</table>

Chart 1. Effect of yeast tRNA on ribonucleotide reductase activity. Ribonucleotide reductase activity was measured in the presence of varying concentrations of yeast tRNA. The assays were carried out in duplicate as described in “Materials and Methods.” The data represent 3 different protamine sulfate preparations from different pH 5.2-insoluble fractions (c, *, and e). The enzyme activity of the controls containing no RNA were 1.60, 1.53, and 1.42 nmoles/30 min, respectively.

The inhibition by RNA was also shown to be reversible by the graphic method of Ackermann and Potter (1) in which the velocity was measured as a function of enzyme concentration.

the RNA or poly(C) with the protamine sulfate fraction did not increase the extent of inhibition.

Finally, the extent of inhibition as a function of time of reaction was followed. Control and inhibited reactions were stopped at 10, 20, 30, and 60 min; in the presence of tRNA, the inhibitions were 47, 48, 49, and 45%, respectively. The degree of inhibition did not change with time.

Nature of Inhibition by RNA and Poly(C). The nature of the inhibition of ribonucleotide reductase by RNA was determined by the method of Dixon (6). From the data shown in Chart 2, A to D, it can be seen that the inhibition of reductase activity by RNA is competitive with Kᵢ's of 145, 39, 300, and 350 μg/ml for the yeast tRNA, poly(C), tumor cell tRNA, and tumor cell rRNA, respectively. For yeast and tumor cell tRNA, these values represent Kᵢ's of approximately 5.8 and 12 μM, respectively, assuming a molecular weight of 25,000 for the soluble RNA. The poly(C) was shown to be heterogeneous by chromatography over Sephadex G-100 (22), eluting in a broad peak in the region following the elution of yeast tRNA from the column.

The inhibition by RNA was also shown to be reversible by the graphic method of Ackermann and Potter (1) in which the velocity was measured as a function of enzyme concentration.
Chart 2. Dixon plots of the effect of various RNA's on ribonucleotide reductase activity. The enzyme was assayed as described in "Materials and Methods," except that the substrate concentration was altered. The final reaction volume was 155 µl. In each of the graphs (A to D), the final substrate concentrations were 1 x 10^{-5} M (●) and 5 x 10^{-5} M (●). A, B, C, and D, data for tumor cell tRNA, tumor cell rRNA, yeast tRNA, and poly(C), respectively. The data are plotted as 1/v, in terms of the reciprocal of nmoles deoxycytidine per 30 min versus the µg of exogenous RNA per reaction mixture.

in the presence and absence of inhibitor. These data are shown in Chart 3. At the lowest concentration of enzyme, the activities in both the control and inhibited samples were not proportional to the enzyme concentration, but this was probably due to a dilution effect and is commonly seen for ribonucleotide reductase (3, 9). However, the presence of the exogenous inhibitor did not titrate out the enzyme activity, as would be expected for an irreversible inhibitor.

Effect of Poly(A), Poly(C), and tRNA on ADP Reduction. Since it was observed that poly(C) was a competitive inhibitor with respect to CDP as substrate and that poly(A) did not inhibit CDP reduction, the effect of poly(A), poly(C), and tRNA on ADP reduction was studied. The percentage inhibition of ADP reductase activity by these polycytidylates at a final concentration of 167 µg/ml was 20, 11, and 40%, respectively for poly(A), poly(C), and tRNA. Although poly(A) inhibited ADP reduction, it inhibited appreciably less than did yeast tRNA. Because of the nature of the assay for ADP reduction, the study was not extended. It is questionable whether poly(C) inhibits ADP reduction.

RNase Activity in the Protamine Sulfate Fraction. tRNA-14C (100 µg) prepared from Ehrlich tumor cells was incubated with the protamine sulfate fraction for 30 min at 37°. Less than 2% of the radioactivity was released to the acid-soluble fraction, indicating slight RNase activity in the enzyme fraction.

Effect of Protamine Sulfate Fractionation on RNA Concentration. The treatment of the pH 5.2-insoluble fraction (which contains the ribonucleotide reductase activity) with protamine sulfate results in a 10-fold increase in reductase-specific activity with a corresponding 43-fold decrease in the RNA content of the protamine sulfate fraction. When sufficient RNA is added to the protamine sulfate fraction to approximate the concentration of RNA found in the pH 5.2-insoluble fraction, a marked decrease in enzyme activity occurs, and the activity approaches that of the pH 5.2 fraction. These data are shown in Table 4. The specific activity in terms of enzyme units per mg protein was still much higher in the protamine sulfate fractions to which exogenous RNA was added than the specific activity of the pH 5.2 fraction.
Inhibition of Ribonucleotide Reductase by RNA

The data do show that RNA at relatively low concentrations was an effective inhibitor of ribonucleotide reductase. The tRNA from yeast and tumor cells had $K_i$'s of 5.8 and 12 $\mu$M, respectively. Poly(C) was even more potent on a $\mu$g basis than either of these.

A comparison of the inhibition by poly(C), CpCpC, CpC, and $2'(3')$-CMP again showed that poly(C) was the most effective inhibitor on a weight basis. The inhibition of ribonucleotide reductase by RNase-treated RNA was therefore probably principally due to the larger molecular-weight oligonucleotides and undigested RNA.

The reduction of ADP by ribonucleotide reductase of Ehrlich tumor cells was inhibited by tRNA and, to a lesser extent, by poly(A). The inhibition of ADP reduction by poly(C) was minimal.

Since preincubation of the RNA with the reductase fraction did not increase the extent of inhibition and the extent of inhibition was not time dependent, it is doubtful that nucleases were responsible for the inhibition. It was observed that the extent of inhibition by cytidine nucleotides decreased with decreasing chain length [the order being poly(C) > CpCpC > CpC = 2'(3')-CMP].

Ribonucleotide reductase activity has been shown in both mammalian (18) and bacterial (15) systems to be carefully regulated by nucleotides that can act as allosteric activators or inhibitors, depending on the particular substrate. These data showing that RNA inhibits ribonucleotide reductase activity may be related to the allosteric nature of this enzyme system and may also explain why the inhibition by RNA is not strictly competitive.

Whether the intracellular RNA (or a specific RNA molecule) is one of the factors involved in controlling the in vivo activity of ribonucleotide reductase remains to be shown. In this connection, we have observed that mitomycin C [which reportedly lowers the RNA concentration of Sarcoma 180 ascites cells (20)], when injected into mice bearing Ehrlich tumor cells, caused a 23 to 37% increase in specific activity of ribonucleotide reductase in the crude cell-free extracts.

Table 4

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Endogenous RNA (mg/ml)</th>
<th>Reductase activity (nmoles/30 min)</th>
<th>% activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 5.2-insoluble</td>
<td>5.22</td>
<td>0.49</td>
<td></td>
</tr>
<tr>
<td>Protamine sulfate</td>
<td>0.12</td>
<td>1.59</td>
<td>100</td>
</tr>
<tr>
<td>Protamine sulfate + rRNA (5.1 mg)</td>
<td>0.12</td>
<td>0.63</td>
<td>40</td>
</tr>
<tr>
<td>Protamine sulfate + yeast tRNA (5.1 mg)</td>
<td>0.12</td>
<td>0.35</td>
<td>22</td>
</tr>
</tbody>
</table>

DISCUSSION

The level of ribonucleotide reductase activity has been shown to vary (a) with the growth cycle of the cell (19, 25), (b) in regenerating liver (12), (c) with the rate of tumor growth (7), and (d) with the level of DNA synthesis (5). What controls the alteration in reductase activity in vivo is not known. It has been reported that the increase in enzyme activity depends on protein synthesis (7), while it has also been reported that there are naturally occurring inhibitors of ribonucleotide reductase in Ehrlich tumor cells (4).

In this report, data are presented that show that RNA markedly inhibited ribonucleotide reductase in vitro. The inhibition by RNA appeared to be dependent on the base composition of the RNA, since yeast tRNA, tumor cell tRNA, rRNA, and poly(C) were effective inhibitors of ribonucleotide reductase, but poly(A), poly(G), poly(I), and poly(U) were much less active inhibitors. The addition of poly(G) or poly(I) to poly(C) had little or no effect on the inhibition caused by poly(C), indicating that poly(C) did not have to be single stranded to be an effective inhibitor of ribonucleotide reductase. The inhibition of ribonucleotide reductase by RNA appeared to be reversible and did not appear to be time dependent.

Treating the data graphically by the method of Dixon (6), we found that the RNA appeared to be a competitive inhibitor with respect to the substrate CDP. Increasing the concentration of CDP did lower the degree of inhibition observed. However, the ribonucleotide reductase system is much too complicated to express with such simple enzyme kinetics, and the data do not fit the case of a strictly competitive inhibition. That it is not a simple case of competitive inhibition is also shown from the data presented in Chart 1. The physical interpretation of these $K_i$ data cannot be simply stated, but...
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prepared from these tumor cells over the specific activity of reductase in the nontreated tumor cells (unpublished observations). Since ribonucleotide reductase was demonstrated to be a cytoplasmic enzyme in regenerating liver (14) and in Ehrlich tumor cells (unpublished observations), it is entirely possible that a RNA fraction of the cytoplasm is involved in the regulatory control of ribonucleotide reductase.

The observation that RNA markedly inhibits ribonucleotide reductase in vitro may also be important in the studies involving the purification of the enzyme from mammalian cells, since the concentration of RNA found in the cell-free extracts will inhibit the reductase activity present (Table 4).

ACKNOWLEDGMENTS

The expert technical assistance of Miss Eileen L. Monley and Mrs. Mary M. Mansell is gratefully acknowledged.

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*Cancer Res* 1973;33:993-998.

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