MOLECULAR PHARMACOLOGY, 12, 177-184

Inhibition of Growth Rate and Deoxynucleoside Triphosphate Concentrations in Cultured Leukemia L1210 Cells

JEFFREY K. LOWE[1] AND GERALD B. GRINDEY[2]

Roswell Park Memorial Institute, James T. Grace Cancer Drug Center, Buffalo, New York 14263

(Received January 24, 1975)

SUMMARY

LOWE, JEFFREY K. & GRINDEY, GERALD B. (1976) Inhibition of growth rate and deoxynucleoside triphosphate concentrations in cultured Leukemia L1210 cells. *Mol. Pharmacol.*, 12, 000-000.

Thymidine and deoxyguanosine produce antiproliferative effects on murine Leukemia L1210 cells when added to the growth medium, and these effects can be prevented by the addition of one or a combination of deoxynucleosides. This study utilized these toxic deoxynucleosides to induce growth rate inhibition in L1210 cells in order to investigate the relationship between growth rate and deoxynucleoside triphosphate pool size in mammalian cells. Deoxyguanosine toxicity of these cells was manifested by deoxynucleoside triphosphate concentration changes which were correlated with growth rate. Both dTTP and dCTP decreased in parallel in a linear manner, while dATP remained unaffected and dGTP increased. In thymidine-inhibited cells, although both dCTP and dATP decreased, only their sum diminished in proportion to growth inhibition.

INTRODUCTION

Deoxynucleosides are toxic to numerous types of mammalian cells in culture (1-5), and this toxicity has been attributed to inhibition of the synthesis of the other deoxynucleoside triphosphates which are required by proliferating mammalian cells for continued DNA biosynthesis. These compounds, after anabolism to the triphosphate level, may behave as natural feedback effectors of ribonucleotide reductase, thus causing a depletion in one or more of the other DNA triphosphate precursors (3, 5, 6).

On the basis of enzymological studies, it is believed that the levels of the four deoxynucleoside triphosphates are normally maintained within certain limits by a complex system of feedback and feedforward loops in mammalian cells (7-9), and the intent of this study was to evaluate the extent to which this regulatory system operates in cultured L1210 cells. The approach taken was to study the antiproliferative effects of thymidine and deoxyguanosine, to analyze cellular deoxynucleoside triphosphate concentrations, and to correlate the two parameters.

This study was supported by Research Grants CA-05298 and CA-13038 from the National Cancer Institute.

[1] Present address: Cancer Research Unit (McEachern Laboratory), University of Alberta, Edmonton, Alberta, Canada T6G 2E1.

[2] To whom requests for reprints should be addressed.

MATERIALS AND METHODS

Cell culture and extraction procedures. The origin and maintenance of the line of murine Leukemia L1210 used in these studies were previously described (10), except that dialyzed fetal calf serum (Grand Island Biological Company) and the organic buffers (11) *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (20 mM) and morpholinopropanesulfonic acid (10 mM) (Sigma Chemical Company) were used.

The growth kinetics of L1210 cells grown in medium containing these buffers were relatively independent of inoculum density and volume of the culture.

At least duplicate cultures were initiated for the growth rate experiments. These were set up with an inoculum of 2–10 × 10^5 cells/ml and were sampled at appropriate time intervals.

The exponentially growing L1210 cells were harvested by centrifugation at 1000 rpm in an International PR-2 centrifuge (head No. 269) for 8 min at 4°, and the cells were extracted by the addition of 1.5 ml of ice-cold 0.5 M $HClO_4$ to the cell pellet (12). The pellet was suspended in the acid by agitation and kept in an ice bath for 20 min before recentrifugation. The supernatant was retained, and to it were added 0.2 ml of 4 N KOH and 0.075 ml of 0.5 M Tris-HCl buffer, pH 8.3. These additions provided a neutralized extract of pH 7.5–8.5. The $KClO_4$ precipitate was removed by centrifugation, and the supernatant was either used immediately or stored for up to 2 weeks at −20° prior to deoxynucleoside triphosphate assay as described by Fridland (12).

DNA polymerase assay for deoxynucleoside triphosphates. The assay for deoxynucleoside triphosphates used was a modification of that described by Solter and Handschumacher (13). Typically, the reaction mixture contained the following in a final volume of 0.2 ml: 10 μmoles of Tris-HCl (pH 8.3), 10 μmoles of $MgCl_2$, 10 μg of undenatured calf thymus DNA, 300 pmoles of the deoxynucleoside triphosphates in excess (one of these being labeled at a specific activity of 800–1000 μCi/μmole), 0–25 pmoles of the limiting deoxynucleoside triphosphate, and 0.14 unit of *Escherichia coli* B DNA polymerase [fraction IV (14)] (P-L Biochemicals). The labeled deoxynucleoside triphosphate used was either [*methyl*-^{3}H]thymidine 5′-triphosphate or [8-^{3}H]deoxyadenosine 5′-triphosphate (Schwarz/Mann).

A mixture of all the components except the limiting deoxynucleoside triphosphate (partial reaction mixture) was made for the assay of dATP, dTTP, dCTP, and dGTP. The limiting deoxynucleoside triphosphate was added to chilled 12 × 75 mm assay tubes in the cold, either as the known standard dissolved in saturated $KClO_4$ solution, pH 8.3, or as the neutralized cellular extract. The reaction was initiated by adding 140 μl of the appropriate partial reaction mixture to the limiting deoxynucleotide in the assay tubes and was incubated in a 37° water bath for 25 min after mixing. The reaction was terminated by rapidly transferring the racks containing the tubes to a −20° freezer, where they were kept until the assay was continued (never more than 16 hr).

It was necessary to run standard curves for each deoxynucleoside triphosphate assay. Typically, the standard assays were run in duplicate whereas the unknowns were done in triplicate.

The thawed assay mixtures were spotted (85 μl) on numbered, silicone-treated (Siliclad, Becton-Dickinson and Company) Whatman No. 3MM paper discs 2.3 cm in diameter. The discs were then rapidly placed in ice-cold 5% trichloracetic acid–1% sodium pyrophosphate (approximately 20 ml/disc). The discs were agitated by pouring the solution and discs back and forth between two beakers 12 times. After the first wash, the discs were transferred to a fresh solution of trichloracetic acid–pyrophosphate and agitated in the same manner, followed by two washes each with 95% ethanol and anhydrous ether. The air-dried discs were placed in glass scintillation vials containing 10 ml of scintillation phosphor solution [2 parts toluene to 1 part Triton X-100 containing 5.0 g/liter of 2,5-diphenyloxazole and 0.1 g/liter of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (15)] and were counted in a Packard liquid scintillation spectrometer, model 3320.

The incorporation of tritiated deoxynucleoside triphosphate into acid-insoluble polymer was found to be linear over the range of 1–20 pmoles of limiting deoxynucleoside triphosphate. The amounts of each deoxynucleoside triphosphate in extracts of cells were measured by replacing the limiting deoxynucleoside triphosphate with an appropriate volume of extract, and the radioactivity incorporated into polymer corresponded to the amount of limit-

ing deoxynucleoside triphosphate in the extract. The incorporation of radioactivity into polymer was also linear with the number of cells extracted, up to 12 × 10^6 cells. All assays were therefore carried out with extracts of 8–10 × 10^6 cells, and appropriate dilutions were used when necessary to keep within the linear region of the standard curve. In addition, when deoxynucleoside triphosphates were determined in samples after the addition of known amounts of standard, only additive effects were seen.

RESULTS

Antiproliferative effects of deoxynucleosides in L1210 cells. The first phase of this study was to investigate the effects of deoxynucleosides on the growth of these cells. The concentration of TdR required to produce a 50% decrease (IC_{50}) in cell number compared to controls at the end of a 45-hr growth period was 35 μM. The antiproliferative effect of TdR was readily prevented by the simultaneous presence of deoxycytidine in the growth medium (Fig. 1). Progressive increases in the CdR concentration in the medium provided increasing protection against TdR toxcity. Concentrations of CdR above 10 μM supplied complete protection against the TdR antiproliferative effects. However, TdR when present above 1 mM caused a toxicity which could not be prevented by CdR. Quantitative evaluation of the progressive shifting of the dose-dependent antiproliferative effects of TdR to the right indicated that the ratio between the TdR concentration and the concentration of CdR required to reach 50% of control density [$(I/S)_{50}$] was not constant (Table 1). $(I/S)_{50}$ values are expected to remain relatively constant over a wide range of inhibitor and metabolite concentrations if a competitive interaction is involved (16).

Cytidine was also capable of overcoming the toxic effects of TdR in culture. Whittle (17) showed that the inhibitory effect of TdR on ^{32}P incorporation into DNA of rat thymus cells is completely prevented by 1 mM CR. Partial prevention of the dose-dependent TdR toxicity in L1210 cells by CR can be seen in Fig. 2. Once again, the

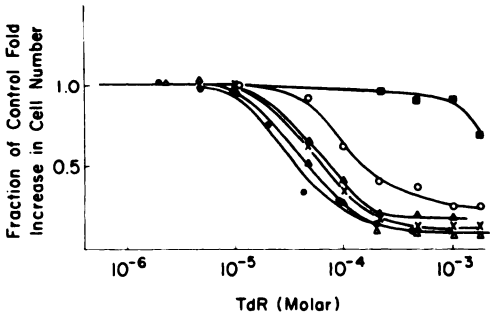

FIG. 1. *Reversal of antiproliferative effects of TdR by CdR*

Each point represents the average of duplicate cell cultures (2 ml). Control cultures increased 14.5-fold relative to the inoculum. ●——●, control; △——△, 0.108 μM CdR; ×——×, 0.5 μM CdR; ▲——▲, 1.08 μM CdR; ○——○, 2.3 μM CdR; ■——■, 10.8 μM CdR.

TABLE 1

Quantitative evaluation of reversal of TdR toxicity by CdR and CR

These data were calculated from the cell culture experiments shown in Figs. 1 and 2 by the method of Shrive (16).

Reversing metabolite	Concentration of TdR at 50% inhibition of growth	Concentration of reversing metabolite at 50% inhibition of growth	$(I/S)_{50}$
	μM	μM	
CdR	37	0	
	48	0.108	444
	65	0.5	130
	75	1.08	69
	155	2.3	67
	0	10.8	
CR	30	0	
	60	17.8	3.37
	92	56	1.64
	180	178	0.99
	300	560	0.54

$(I/S)_{50}$ values did not remain constant over the range studied (Table 1). CdR was obviously more efficient than CR in preventing TdR toxicity, as was reflected in their relative $(I/S)_{50}$ values.

Deoxyguanosine also produced potent antiproliferative effects in the L1210 cell line and had an IC_{50} of about 60 μM. The toxic manifestations of GdR were partially

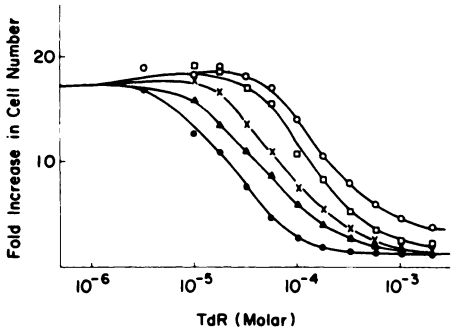

FIG. 2. *Reversal of TdR toxicity by CR*
Each point represents the average cell density of duplicate cultures (2 ml). ●——●, control; ▲——▲, 17.8 μM CR; ×——×, 56 μM CR; □——□, 178 μM CR; ○——○, 560 μM CR.

prevented with CdR or completely prevented with AdR and CdR.

The toxic effects of deoxynucleosides in L1210 cells were also quantitated by measurements of growth rate instead of extent of growth after 45–50 hr of incubation. TdR-inhibited L1210 cells grew exponentially, albeit at depressed rates (Fig. 3).[3] It is noteworthy that cells grown in the presence of 64 μM CdR or 90 μM TdR plus 32 μM CdR grew at the control growth rate whereas any concentration of TdR alone above 10 μM gave considerable inhibition of growth rate. The rapid onset of antiproliferative effects (as evidenced by the extrapolation of all slopes to the same cell density at zero time) resulted in linear plots and facile growth rate determinations.

In contrast, the growth rate of L1210 cells in the presence of added GdR was not linear with prolonged incubation (Fig. 4). Deoxynucleoside catabolism apparently caused the nonlinear growth kinetics; for this reason short-term incubations (8–12 hr) at higher cell densities (10^5 cells/ml) were used to produce GdR-inhibited cells for deoxynucleoside triphosphate measurement.

Deoxynucleoside triphosphate concentrations. The cellular content of deoxynucleoside triphosphates was analyzed during the course of the TdR and GdR experi-

[3] The controls increased in cell number with a generation time of 10.8 hr, and the cells exposed to 90 μM TdR exhibited a generation time of 28 hr.

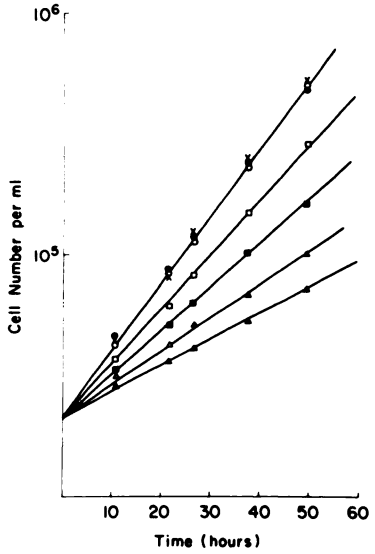

FIG. 3. *Effects of various concentrations of TdR on growth kinetics*
Cultures (40 ml) were initiated at 2 × 10^4 cells/ml and incubated at 37°. Each point represents the average of two to four cell densities. ●——●, no addition; ○——○, 64 μM CdR; ×——×, 90 μM TdR plus 32 μM CdR; □——□, 16 μM TdR; ■——■, 32 μM TdR; △——△, 60 μM TdR; ▲——▲, 90 μM TdR.

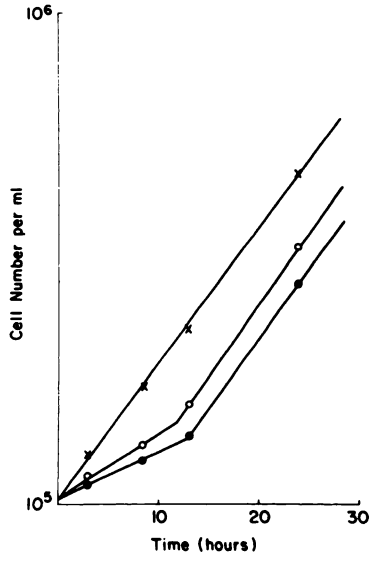

FIG. 4. *Effects of GdR on growth kinetics*
Duplicate cultures (30 ml) were initiated at 10^5 cells/ml with either no additions (×——×), 25 μM GdR (○——○), or 50 μM GdR (●——●).

ments previously mentioned. The values obtained for deoxynucleoside triphosphate pools of exponentially growing L1210 cells were 36 ± 6, 86 ± 9, 56 ± 12, and 36 ± 13 pmoles/10^6 cells for dATP, dTTP, dCTP, and dGTP, respectively, for a total of eight entirely separate extractions and determinations. Since some variation of control values for deoxynucleoside triphosphates occurred from one assay to the next, it seemed reasonable to express the data relative to controls (for that particular experiment). The deoxynucleoside triphosphate concentrations are expressed as a function of growth rate inhibition by TdR in Fig. 5. That dTTP levels increased with increasing inhibition of growth rate was not unexpected, since TdR was used to inhibit growth in these experiments. The intracellular concentrations of both dCTP and dGTP underwent change at low levels of growth inhibition and remained stable with increasing inhibition, although dCTP decreased and dGTP increased. The concentration of dATP decreased with increasing growth rate inhibition.

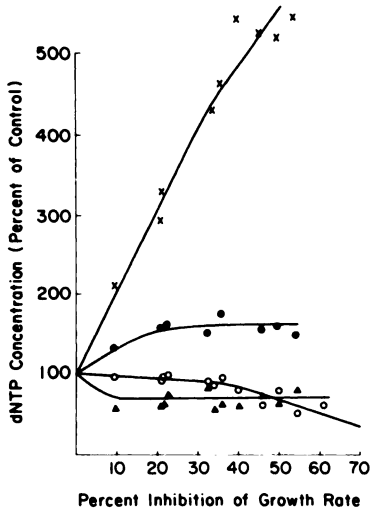

FIG. 5. *Relationship between growth rate inhibition and deoxynucleoside triphosphate (dNTP) pools induced by TdR*

Fractional inhibition of control growth rate was determined by comparison of inhibited with control slopes after 50 hr of incubation, and the cells were assayed at this time. ×——×, dTTP; ●——●, dGTP; ○——○, dATP; ▲——▲, dCTP.

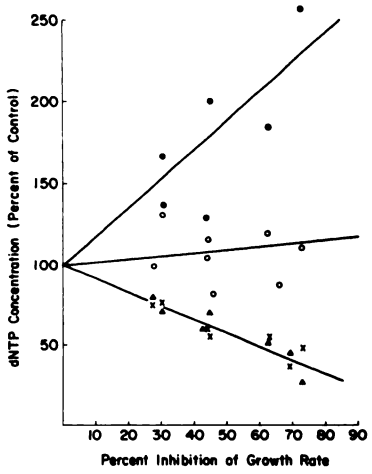

FIG. 6. *Relationship between growth rate inhibition and deoxynucleoside triphosphate (dNTP) pools induced by GdR*

These data were obtained in a manner similar to that described for Fig. 5, except that GdR was utilized to induce the observed perturbations and the cells were assayed after 8–12 hr of incubation. The symbols used are the same as those in Fig. 5.

In contrast to the results obtained with TdR, growth of cells in a medium containing GdR led to a decline in the concentrations of dTTP and dCTP as a linear function of inhibition of growth rate (Fig. 6). The intracellular concentration of dATP remained little affected by GdR inhibition, while the expected increase in dGTP resulted.

Since no direct relationship resulted between decreasing deoxynucleoside triphosphate concentrations and growth rate for TdR-inhibited cells, the possibility that more than a single deoxynucleotide might be of importance in determining the growth rate of cells (perhaps the DNA biosynthetic rate) was investigated. In this light, the available data were treated in a fashion similar to that used for *E. coli* RNA polymerase (18). The concentrations of the two decreasing deoxynucleoside triphosphates for each type of experiment were averaged, and this average was related to growth rate (Fig. 7). Most interestingly, the averaged concentrations of the decreasing nucleotides for either TdR- or GdR-inhibited cells fit the same relationship to growth rate.

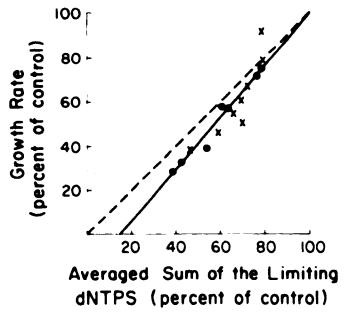

FIG. 7. *Relationship between growth rate and limiting deoxynucleoside triphosphates (dNTPS) in L1210 cells*

The data presented were derived from Figs. 5 and 6. The points due to TdR inhibition (×——×) represent the average of the sum of the decreases in dATP and dCTP, whereas the GdR inhibition symbols (●——●) represent the average of the sum of the decreases in dTTP and dCTP.

DISCUSSION

The initial phase of this investigation showed that the antiproliferative effects of TdR in L1210 cells could be prevented by CdR or CR. The CdR effect has been observed in numerous mammalian cells (3, 5, 19) and has been proposed to be a product reversal. The reduction of CDP by mammalian ribonucleotide reductase is inhibited by high levels of dTTP (7, 20); thus TdR has been proposed to induce a dCTP depletion (3, 5). The dCTP pool can be replenished with CdR through the appropriate series of kinases by a pathway which is downstream from the metabolic insult. The decreasing $(I/S)_{50}$ values (Table 1) with increasing CdR suggested that the interaction was not competitive, but neither was it a true noncompetitive product reversal because low levels of metabolite gave some reversal when an all-or-none type response might have been expected (16).

The CR effect was proposed by Whittle (17) to be due to an accumulation of intracellular CDP which can prevent the blockade of CDP reduction in a competitive manner. The changing $(I/S)_{50}$ values (Table 1) for increasing CR show that the relationship was not fully competitive. Neither CdR nor CR interaction with TdR in L1210 cells appeared to be fully noncompetitive or competitive, but the over-all appearance of the families of curves for the two metabolites differed (Figs. 1 and 2), suggesting that the less efficient CR effect was indeed due to CR and not contaminating CdR.

The antiproliferative effects of GdR in L1210 cells did not involve inhibition of purine biosynthesis *de novo*, since CdR plus hypoxanthine [at a level which allowed full reversal of all purine effects of methotrexate in L1210 cells (21)] did not fully prevent these effects[4] whereas CdR plus AdR provided complete protection. Thus it seems reasonable to assume that the metabolic insult caused by GdR or its metabolites involves the deoxynucleotide pathway.

The main purpose of this study was to investigate the regulation of deoxynucleotide metabolism in mammalian cells. The data obtained indicate that the deoxynucleoside triphosphate concentration fluctuations related to either TdR or GdR toxicity were understandable in view of what is known of the positive and negative feedback effects on ribonucleotide reductase, determined *in vitro* for Novikoff hepatoma enzyme (7). In the presence of physiological levels of ATP, the reductions of both CDP and ADP by the Novikoff enzyme are inhibited by dTTP while the reduction of GDP is activated. As the levels of dTTP increased (Fig. 5), the concomitant changes in the other three triphosphate pools were reflective of the effect of dTTP on the synthesis of those compounds *in vitro*. The dCTP change occurred at low levels of dTTP, followed by the intermediate sensitivity of the activation of GDP reduction and attendant increase in dGTP concentration; finally, at high concentrations of dTTP, the inhibition of ADP reduction became evident by the decrease in dATP pool size.

Regardless of the reason for the partial decrease in dCTP concentration, it is certain that these data were not consistent with a linear relationship between the antiproliferative effect of TdR and diminution of the dCTP pool, as has been proposed for Chinese hamster ovary cells (5).

[4] J. K. Lowe and G. B. Grindey, unpublished observations.

Bjursell and Reichard (5) found only the dCTP concentration to be decreased when the time course of TdR effects was studied in those cells. If the Chinese hamster ovary study and this report on L1210 cells can in any way be compared, one would have to believe that the two cell lines have different regulatory parameters. Tattersall *et al.* (22) recently reported the effects of a single concentration of TdR (40 μM) on the deoxyribonucleoside triphosphate pools in five mammalian cell lines. In accord with our L1210 data, they observed a decrease in the pools of both dCTP and dATP in three of the cell lines (including L1210) while dATP increased in the other two lines. Recent studies[5] have indicated that CCRF-CEM cells (12) respond to growth inhibition by TdR with a decrease only in dCTP and increased levels of the other deoxynucleoside triphosphates. Thus it seems reasonable that the generalization stating that TdR toxicity results from a depletion of only dCTP need not be true in all cells.

The pool size changes induced by GdR once again reflect the expected properties of ribonucleotide reductase. An excess of dGTP with the Novikoff enzyme has been shown to be inhibitory for the reduction of both pyrimidine ribonucleoside diphosphates (7), and in L1210 cells the decline of dCTP and dTTP are related in a linear fashion to growth rate (Fig. 6).

The limitation of availability of several of the requisite four precursors for DNA biosynthesis was a plausible explanation for the growth rate inhibition induced by TdR or GdR. However, for TdR inhibition no single compound decreased colinearly with growth rate, whereas for GdR inhibition both dTTP and dCTP decreased in a linear fashion. However, the assumption was made that the decrease in both deoxynucleoside triphosphates in each type of experiment was important, not the decrease in a single nucleotide; then, by averaging the concentrations of these two compounds, a linear correlation with growth rate resulted (Fig. 7). These observations indicated that perhaps in L1210 cells the DNA biosynthetic machinery may be substrate-limited, since no lag phase was evident between the decrease in the two limiting deoxynucleoside triphosphates and growth rate inhibition. The experimental data suggested a relationship more complex than simple substrate limitation, which might be represented by the dashed line in Fig. 7. Making the assumptions that the two deoxynucleoside triphosphates in each case are limiting for DNA biosynthesis and below K_m, and that the system behaves in a Michaelis-Menten fashion, one would expect the dashed line to represent simple substrate limitation in the first-order region of a substrate saturation curve. One possible reason for the discrepancy between the two lines of this figure might be separate compartmentalization of active and inactive pools of deoxynucleoside triphosphates. Recently Skoog and Bjursell (23) have presented evidence that dTTP is unequally distributed between the cytoplasm and nucleus of Chinese hamster ovary cells.

Although no concrete statements can be made concerning the mechanism for cellular antiproliferative effects of deoxynucleosides, evidence has been presented suggesting the regulatory nature of ribonucleotide reductase in intact L1210 cells.

[5] M. C. Wang and G. B. Grindey, unpublished observations.

REFERENCES

1. Klenow, H. (1962) *Biochim. Biophys. Acta*, **61**, 885–896.
2. Hakala, M. T. & Taylor, E. (1959) *J. Biol. Chem.*, **234**, 126–128.
3. Morris, N. R., Reichard, P. & Fischer, G. A. (1963) *Biochim. Biophys. Acta*, **68**, 93–99.
4. Karnofsky, D. A. & Lacon, C. R. (1961) *Biochem. Pharmacol.*, **7**, 154–158.
5. Bjursell, G. & Reichard, P. (1973) *J. Biol. Chem.*, **248**, 3904–3909.
6. Blakley, R. L. & Vitols, E. (1968) *Annu. Rev. Biochem.*, **37**, 201–224.
7. Moore, E. C. & Hulbert, R. B. (1966) *J. Biol. Chem.*, **241**, 4802–4809.
8. Maley, G. F. & Maley, F. (1964) *J. Biol. Chem.*, **239**, 1168–1176.
9. Scarano, E., Rossi, M. & Geraci, G. (1968) in *The Regulation of Enzyme Activity and Allosteric Interactions* (Kvamme, E. & Pihl, A., eds.), pp. 145–165, Universitetsforlaget, Oslo.
10. Grindey, G. B. & Nichol, C. A. (1972) *Cancer Res.*, **32**, 527–531.

11. Eagle, H. (1971) *Science*, **174**, 500–503.
12. Fridland, A. (1974) *Cancer Res.*, **34**, 1883–1888.
13. Solter, A. W. & Handschumacher, R. E. (1969) *Biochim. Biophys. Acta*, **174**, 585–590.
14. Richardson, C. C., Schildkraut, C. L., Aposhian, H. V. & Kornberg, A. (1964) *J. Biol. Chem.*, **239**, 222–232.
15. Benson, R. H. (1966) *Anal. Chem.*, **38**, 1353–1356.
16. Shrive, W. (1950) *Ann. N. Y. Acad. Sci.*, **52**, 1212–1234.
17. Whittle, E. D. (1966) *Biochim. Biophys. Acta*, **119**, 410–412.
18. Nazar, R. H., Tyfield, L. A. & Wong, J. T. F. (1972) *J. Biol. Chem.*, **247**, 798–804.
19. Whittle, E. D. (1966) *Biochim. Biophys. Acta*, **114**, 44–60.
20. Murphree, S., Moore, E. C. & Beall, P. T. (1968) *Cancer Res.*, **28**, 860–863.
21. Moran, R. G. (1974) Thesis, State University of New York, Buffalo.
22. Tattersall, M. H. N., Jackson, R. C., Jackson, S. T. M. & Harrap, K. R. (1974) *Eur. J. Cancer*, **10**, 819–826.
23. Skoog, L. & Bjursell, G. (1974) *J. Biol. Chem.*, **249**, 6434–6438.