ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1977, p. 946-951
Vol. 11, No. 6

Printed in U.S.A.

Inhibition of Influenza Virus Ribonucleic Acid Polymerase by Ribavirin Triphosphate

BERTIL ERIKSSON, ERIK HELGSTRAND, NILS GUNNAR JOHANSSON, ALF LARSSON, ALFONS MISIORNY, JAN OLOF NOREN, LENNART PHILIPSON, KJELL STENBERG, GORAN STENING, STIG STRIDH, AND BO ÖBERG*

Research and Development Laboratories, Astra Läkemedel AB, Södertälje, and Department of Microbiology, Biomedical Center, University of Uppsala, Uppsala, Sweden

Received for publication 27 January 1977

Ribavirin 5'-triphosphate (RTP), derived from the broad-spectrum antiviral compound ribavirin (Virazole), can selectively inhibit influenza virus ribonucleic acid polymerase in a cell-free assay. Ribavirin and its 5'-monophosphate have no effect on the polymerase. The inhibition is competitive with respect to adenosine 5'-triphosphate and guanosine 5'-triphosphate. RTP also inhibits ApG- and GpC-stimulated influenza virus ribonucleic acid polymerase. Since ribavirin is phosphorylated in the cell, the inhibition of influenza multiplication in the cell may also be caused by RTP.

The antiviral compound ribavirin (Virazole, 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) has been shown to inhibit the multiplication of several animal viruses (1a, 17). It can obviously be phosphorylated to the mono-, di-, and triphosphate within mammalian cells (11). The monophosphate inhibits a cellular inosine 5'-monophosphate (IMP) dehydrogenase (EC 2.1.1.14) (19). This effect has been assumed to be the basis for the antiviral activity of ribavirin (11).

On the other hand, it has been demonstrated that the synthesis of influenza virus proteins is inhibited at concentrations of ribavirin that do not suppress cellular protein synthesis (12). This could be explained if influenza virus ribonucleic acid (RNA) polymerase was selectively affected, resulting in a decrease in influenza virus messenger RNA. It has recently been reported that the synthesis of both virion RNA and complementary RNA (cRNA) is suppressed at ribavirin concentrations that do not block cellular RNA synthesis (18).

The present study describes experiments which reveal a selective inhibition of influenza virus RNA polymerase by ribavirin 5'-triphosphate (RTP). An analysis of the kinetics of inhibition and inhibition in the presence of dinucleotides has been carried out.

MATERIALS AND METHODS

Viruses. Influenza virus type A2 strain Aichi 2/68 was obtained as infected allantoic fluid from Statens Bakteriologiska Laboratorium, Stockholm, Sweden, and the virus was purified according to the method of Pons and Hirst (15), except that the purified virus was not treated with ribonuclease. Purified virus, 2 to 3 mg/ml, was kept frozen in aliquots at −70°C.

The Indiana serotype of vesicular stomatitis virus (VSV) was grown in HeLa cells and purified as described by Roy and Bishop (16).

Enzymes. Calf thymus RNA polymerase A and B (EC 2.7.7.6) were prepared according to Kedinger et al. (7), calf thymus deoxyribonucleic acid (DNA) polymerase α (EC 2.7.7.7) was purchased from Worthington, Freehold, N.J., and *Escherichia coli* RNA polymerase type II (EC 2.7.7.6) and *Micrococcus luteus* DNA polymerase (EC 2.7.7.7) were obtained from Sigma, St. Louis, Mo.

Chemicals and isotopes. Activated calf thymus DNA for the *M. luteus* and the calf thymus DNA polymerase assays was prepared according to Fansler and Loeb (4). *E. coli* Q-13 DNA for the *E. coli* RNA polymerase assay was prepared according to Marmur (10). Calf thymus DNA for the calf thymus RNA polymerase A and B assays was from Worthington. Adenosine 5'-triphosphate (ATP), cytidine 5'-triphosphate. (CTP), guanosine 5'-triphosphate (GTP), uridine 5'-triphosphate (UTP), deoxy ATP, deoxy CTP, deoxy GTP, deoxythymidine 5'-triphosphate, ApG, and GpC were from Sigma. Tritiated triphosphates, [^{3}H]GTP (5.6 Ci/mmol), [^{3}H]CTP (26.2 Ci/mmol), and [^{3}H]deoxythymidine 5'-triphosphate (40 Ci/mmol) were from New England Nuclear Corp., Boston, Mass. Samples of ribavirin, ribavirin 5'-monophosphate (RMP), and RTP were kindly supplied by R. Sidwell, ICN Pharmaceuticals Inc., Irvine, Calif.

Assay conditions. The methods for assaying different polymerase activities were essentially as described by the following: by Bishop et al. (2) for influenza virus polymerase, by Baltimore et al. (1) for VSV RNA polymerase, by Kedinger et al. (7) for calf thymus RNA polymerase A and B, by Holmes et al. (6) for calf thymus DNA polymerase α, by Harwood et al. (5) for *M. luteus* DNA polymerase, and

by Burgess (3) for *E. coli* RNA polymerase, with minor modifications. Where not otherwise noted, the nucleotide concentrations in the different assays were as indicated in Table 1.

Samples from the assay mixtures were precipitated with trichloroacetic acid on filter paper, washed, and counted in a toluene scintillation solution (Permablend III, Packard). One unit of enzyme activity corresponds to 1 nmol of [^{3}H]GMP, [^{3}H]-CMP, or [^{3}H]dTMP incorporated per h. Zero time incorporation was subtracted.

The specific activities of the enzymes and the amounts used in the assays were: influenza RNA polymerase, 3 U/mg, 100 μg/125 μl; thymus RNA polymerase A, 2.5 U/mg, 100 μg/125 μl; thymus RNA polymerase B, 30.6 U/mg, 100 μg/125 μl; *E. coli* RNA polymerase, 880 U/mg, 1.5 μg/125 μl; *M. luteus* DNA polymerase, 85 U/mg, 8.9 μg/125 μl; thymus DNA polymerase α, 2.5 U/200 μl; VSV RNA polymerase, 37 U/mg, 10 μg/125 μl.

Chromatographic procedures. Two chromatographic systems were used. In system A, silica gel thin-layer plates (Merck) were chromatographed in 2-propanol–concentrated aqueous ammonia–water (7:1:2). The eluted plates were sprayed with methanol containing 10% sulfuric acid and 2% *p*-methoxybenzaldehyde followed by heating at about 110°C. In system B, PEI-cel 200 thin-layer plates (Macherey-Nagel, 20 by 20 cm) were chromatographed in 1.4 M lithium chloride and then sprayed with a mixture of 5 ml of 60% (wt/wt) perchloric acid, 10 ml of 0.1 M hydrochloric acid, 25 ml of 4% (wt/vol) ammonium molybdate, and 60 ml of water.

RESULTS

Purity of the tested inhibitors. Ribavirin (mp, 166 to 168°C, literature mp, 166 to 168°C [20]; system A, R_f = 0.41) and RMP (system B, R_f = 0.70) were chromatographically pure. The sample of RTP contained some minor impurities.

A 1.1-mg (by weight) sample of RTP was purified by chromatography (system B, one plate). The appropriate band (R_f = 0.20) was cut out from the developed and dried plate and was washed with methanol (250 ml). Eluting the band with water and evaporation of the solvent gave a sample containing 0.6 mg of RTP (recovery of 55%, the amount determined by the ultraviolet light absorbance, $\lambda_{max}[H_2O]$ at 206 nm, $\epsilon = 8 \times 10^3$) which was chromatographically pure (system B). Some lingering lithium chloride was washed away from the dry sample with methanol.

No significant difference was found between the inhibitory activities of the crude and the purified samples of RTP. The experiments described below are pertinent to the crude sample.

Selective inhibition of influenza virus RNA polymerase. The polymerase activity of several DNA and RNA polymerases was tested in the presence of ribavirin, RMP, and RTP as shown in Table 2. Influenza virus RNA polymerase was selectively inhibited by the triphosphate. No inhibition was observed with ribavirin or its monophosphate. The triphosphate did not show any significant inhibition of calf thymus RNA polymerase A and B. VSV RNA polymerase, *M. luteus* DNA polymerase, and *E. coli* RNA polymerase were slightly inhibited by 500 μM RTP. Ribavirin and RMP had no inhibitory activity on any of the enzymes tested. A concentration of 100 μM RTP was required to obtain 50% inhibition of influenza virus RNA polymerase. No significant inhibition of any of the other enzymes was observed at this concentration (Table 2). Figure 1 shows the dose-response curve for inhibition of influenza virus RNA polymerase with RTP.

Competitive effect of purine triphosphates. The mechanism of inhibition of influenza virus RNA polymerase by RTP was studied by varying the concentration of nucleoside triphosphates in the polymerase assay system. The Lineweaver-Burk plots obtained for ATP, GTP, CTP, and UTP are presented in Fig. 2. RTP shows a competitive type of inhibition with varying concentrations of the purine triphosphates ATP and GTP (Fig. 2A and B). No competitive inhibition was found when the concentrations of the pyrimidine triphosphates CTP and UTP were varied (Fig. 2C and D).

TABLE 1. *Nucleotide concentrations in the polymerase assays*

Polymerase assay	Nucleotide concn (mM)							
	ATP	GTP	CTP	UTP	dATP[a]	dGTP[a]	dCTP[a]	dTTP[a]
Influenza virus RNA	0.50	0.11	0.10	0.10				
Thymus A RNA	0.50	0.11	0.10	0.10				
Thymus B RNA	0.50	0.11	0.10	0.10				
E. coli RNA	0.40	0.40	0.40	0.40				
M. luteus DNA					0.05	0.05	0.05	0.05
Thymus α DNA					0.10	0.10	0.10	0.10
VSV RNA	1.0	0.07	0.50	0.50				

[a] dATP, Deoxy ATP; dGTP, deoxy GTP; dCTP, deoxy CTP; dTTP, deoxythymidine 5'-triphosphate.

TABLE 2. *Effects of ribavirin, RMP, and RTP on different polymerases*

Polymerase	Inhibition (%)[a]					
	Ribavirin		RMP		RTP	
	100 μM	500 μM	100 μM	500 μM	100 μM	500 μM
Influenza RNA	−10	0, −8	−9	2, −4	50, 40	90, 82
Thymus A RNA	2	−4	−15	−12	−16	5, 3
Thymus B RNA	3	5, 5	9, 8	7, 13	15,9	15, 20, 1
E. coli RNA	4	−4	−1, 3	1, 0	5	20, 17
M. luteus DNA	0	2	1	−4	13	28, 38
Thymus α DNA						22
VSV RNA						36

[a] The different polymerases were assayed as described in Materials and Methods, and the inhibition of polymerase activity is expressed in percent relative to a control without added substance. A negative value refers to the percentage of enhancement observed in the assay. The figures are averages from duplicate incubations. Results from different experiments are given for some enzymes.

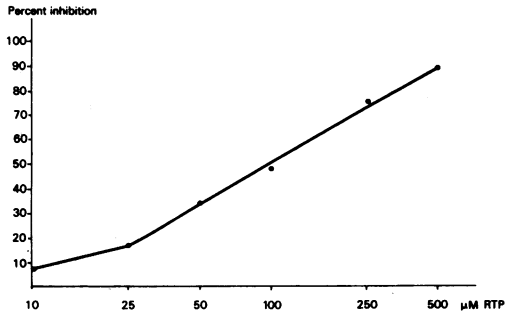

FIG. 1. *Inhibition of influenza virus RNA polymerase by RTP. Inhibition of influenza virus RNA polymerase at different concentrations of RTP was determined as described in Materials and Methods. All samples were in duplicate, and mean values are shown. The incubation time was 1 h, and more than 10^4 cpm was counted in each sample with [^{3}H]GTP as labeled precursor.*

Inhibition of influenza virus RNA polymerase stimulated with dinucleotides. The activity of influenza virus RNA polymerase could be stimulated by ApG or GpC as demonstrated by Plotch and Krug (14) and as also shown in Fig. 3. The polymerase activity in the stimulated system was also inhibited by RTP. The inhibition at 1 h of incubation was more than 80% when equimolar concentrations of RTP and ApG or GpC were used.

The mechanism of inhibition in this system was examined by varying the concentration of ApG and RTP. The result is shown in Fig. 4. When the data were transformed into a Lineweaver-Burk plot, the lines obtained were curved, and no conclusion could be made concerning the type of inhibition.

DISCUSSION

The selective inhibition of viral enzymes without affecting normal cellular functions is a highly desired property of an antiviral compound. Ribavirin has been considered to be a broad-spectrum antiviral substance with activity against several animal DNA and RNA viruses (1a, 17). Earlier reports about the function of ribavirin have indicated that the drug is metabolized to RMP, which inhibits cellular IMP dehydrogenase (10, 11). This inhibition has been shown to result in a decreased GTP pool in leukemic cells (13).

A selective effect on the synthesis of influenza virus proteins was observed by Oxford (12). Scholtissek (18) reported that the synthesis of influenza virus RNA was inhibited at concentrations of ribavirin that did not inhibit cellular RNA synthesis. These findings suggest a mechanism of inhibition that is distinct from the effect on IMP dehydrogenase. It has been reported that ribavirin is phosphorylated intracellularly not only to its monophosphate, but also to the di- and triphosphates (11), and that the intracellular concentration of RTP is of the same magnitude as that of ribavirin (11).

Our results (Table 2) demonstrate that RTP is a selective inhibitor of influenza virus RNA polymerase in a cell-free system. Ribavirin and RMP, on the other hand, do not inhibit the influenza virus RNA polymerase. From Fig. 2 it is evident that RTP shows a competitive inhibition only with ATP and GTP but not with UTP or CTP. The structure of RTP is more closely related to GTP and ATP than to UTP or CTP. It has been suggested that the influenza cRNA initiates with adenosine and guanosine (8). McGeoch and Kitron (9) showed a stimulation of influenza virus RNA polymerase by GpG and GpC, and Plotch and Krug have recently reported (14) that the in vitro initiation of influenza virus cRNA can be stimulated by the dinucleotides ApG and GpG, which are incorporated at the 5′ end of the cRNA. The enhancement of

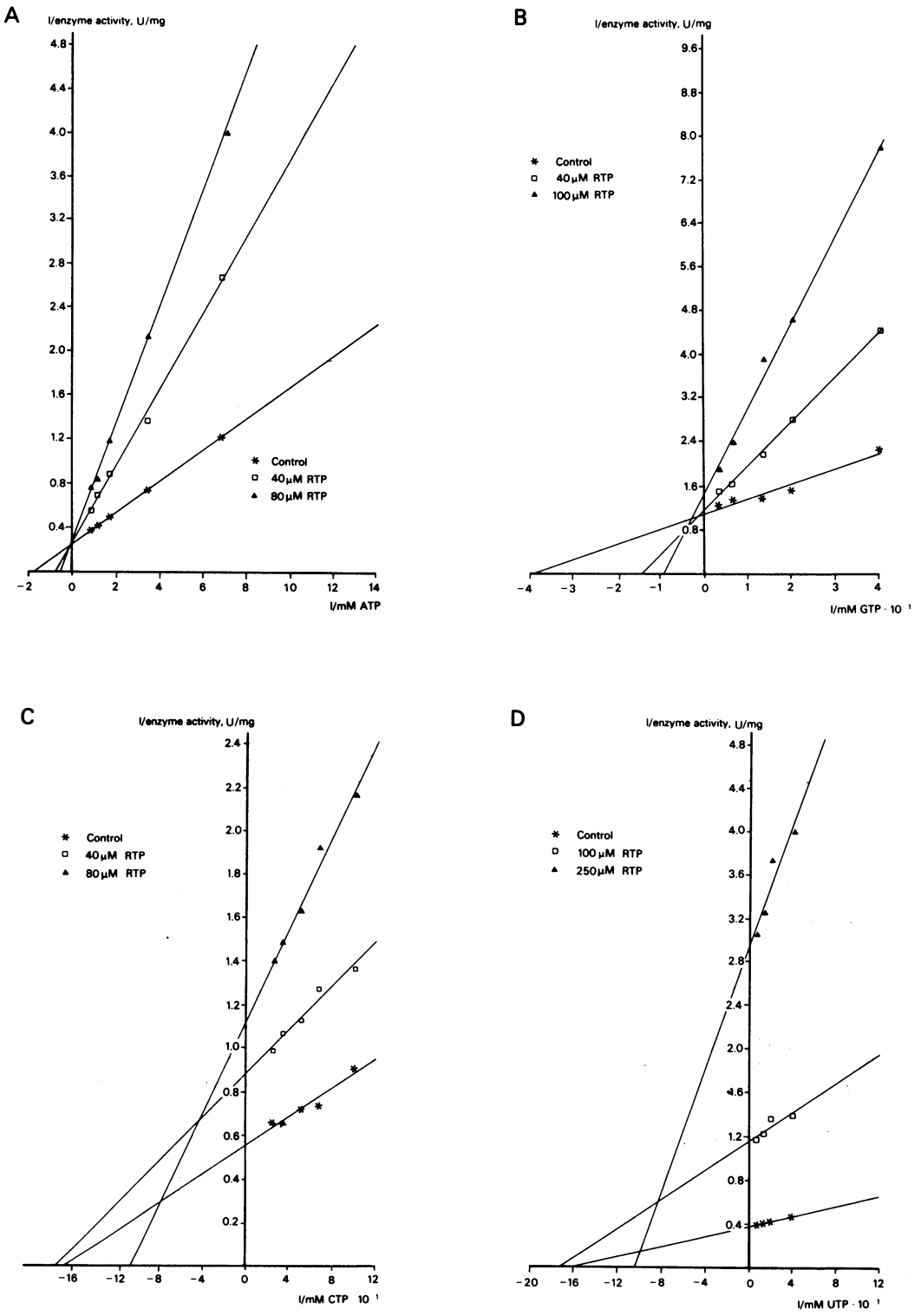

FIG. 2. *Effect of nucleoside triphosphates on the inhibitory activity of RTP. The activity of influenza virus RNA polymerase in the presence of RTP was determined at different concentrations of ATP, GTP, CTP, and UTP. The incorporation of [³H]GTP was measured when the concentration of ATP, CTP, or UTP was varied, and the incorporation of [³H]CTP was determined when the GTP concentration was varied. The data are presented as double reciprocal plots, utilizing a computerized least-squares method for regression analysis. The time of incubation was 1 h, and the nucleotide concentrations were the same as given in Table 1, apart from the one varied. (A) Incorporation at different ATP concentrations with 0, 40, and 80 μM RTP. (B) Incorporation at different GTP concentrations with 0, 40, and 100 μM RTP. (C) Incorporation at different CTP concentrations with 0, 40, and 80 μM RTP. (D) Incorporation at different UTP concentrations with 0, 100, and 250 μM RTP.*

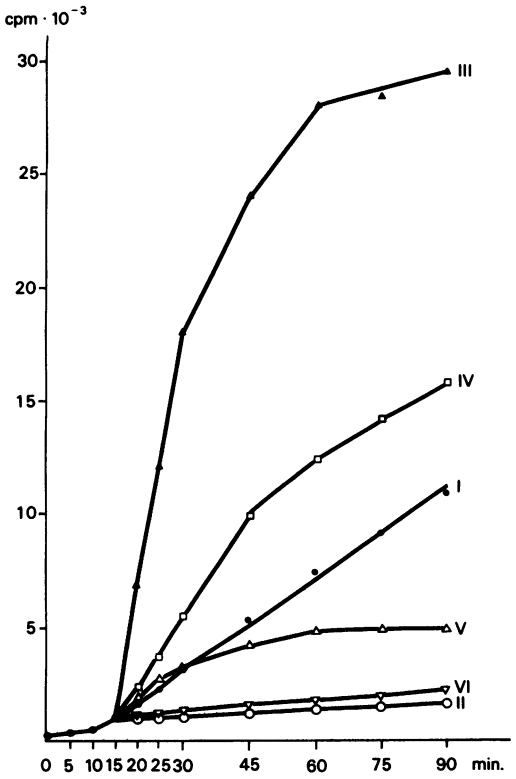

FIG. 3. *Inhibition of influenza virus RNA polymerase stimulated with ApG and GpC. Additions to the standard influenza virus RNA polymerase assay at 15 min of incubation with [³H]GTP as labeled precursor were: (I) control, no addition; (II) 500 μM RTP; (III) 500 μM ApG; (IV) 500 μM GpC; (V) 500 μM RTP and 500 μM ApG; (VI) 500 μM RTP and 500 μM GpC.*

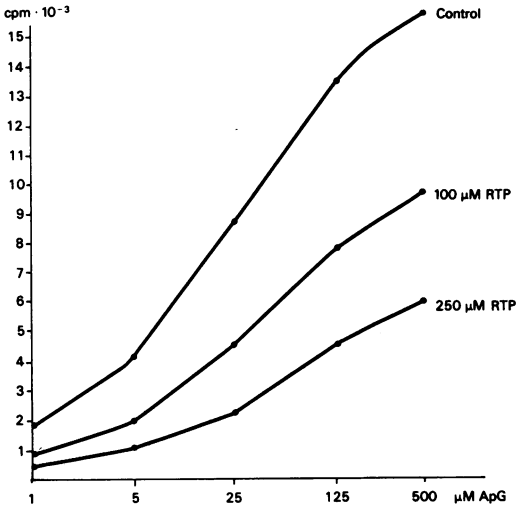

FIG. 4. *Inhibition of ApG-stimulated influenza virus RNA polymerase by RTP. The inhibition of influenza virus RNA polymerase was determined with varying concentrations of both RTP and ApG. The time of incubation was 15 min and [³H]GTP was used to label the product.*

polymerase activity by ApG and GpC can be abolished with RTP (Fig. 4). It could not be established whether the inhibition is competitive with respect to the dinucleotide. This may be due to the fact that the lag period before RNA synthesis assumes a linear rate varies with the concentration of the dinucleotide. It has not yet been determined whether RTP also inhibits the cytoplasmic influenza RNA polymerase synthesizing virion RNA, nor is it known whether the diphosphate has any inhibitory activity.

The lack of inhibition of calf thymus DNA polymerase α and RNA polymerase I and II by RTP has also recently been observed by Müller et al. (13).

It remains to be established whether the inhibition of influenza virus RNA polymerase by RTP also controls influenza virus replication in the infected cell. Considering the facts that both influenza virus RNA and protein synthesis are selectively inhibited by ribavirin (12, 18), it is entirely possible that the prevention of influenza virus multiplication depends on a selective inhibition of the RNA polymerase by the ribavirin metabolite RTP.

The suggested mechanism for inhibition of influenza virus RNA polymerase does not exclude concomitant effects by inhibition of IMP dehydrogenase.

LITERATURE CITED

1. **Baltimore, D., A. S. Huang, and M. Stampfer.** 1970. Ribonucleic acid synthesis of vesicular stomatitis virus. II. An RNA polymerase in the virion. Proc. Natl. Acad. Sci. U.S.A. 66:572–576.
1a. **Becker, Y.** 1976. Antiviral drugs, p. 57. *In* J. L. Melnick (ed.), Monogr. in virology, vol. II. S. Karger, Basel.
2. **Bishop, D., J. Obijeski, and R. Simpson.** 1971. Transcription of the influenza ribonucleic acid genome by a virion polymerase. I. Optimal conditions for in vitro activity of the ribonucleic acid-dependent ribonucleic acid polymerase. J. Virol. 8:66–73.
3. **Burgess, R. R.** 1969. A new method for the large scale purification of *E. coli* deoxyribonucleic acid-dependent ribonucleic acid polymerase. J. Biol. Chem. 244:6160–6167.
4. **Fansler, B. S., and L. A. Loeb.** 1974. Sea urchin nuclear DNA polymerase. Methods Enzymol. 29:53–70.
5. **Harwood, S. J., P. F. Schendel, and R. D. Wells.** 1970. Micrococcus luteus deoxyribonucleic acid polymerase. J. Biol. Chem. 245:5614–5624.
6. **Holmes, A. M., I. P. Hesslewood, and I. R. Johnston.** 1976. Evidence that DNA polymerase- α of calf thymus contains a subunit of molecular weight 155000. Eur. J. Biochem. 62:229–235.

7. **Kedinger, C., F. Gissinger, M. Gniazdowski, J-L. Mandel, and P. Chambon.** 1972. Animal DNA-dependent RNA polymerases. I. Large-scale solubilization and separation of A and B calf thymus RNA-polymerase activities. Eur. J. Biochem. 28:269–276.
8. **Krug, R. M., M. A. Morgan, and A. J. Shatkin.** 1976. Influenza viral messenger RNA contains internal N^6-methyladenosine and 5'-terminal 7-methylguanosine in cap structures. J. Virol. 20:45–53.
9. **McGeoch, D., and N. Kitron.** 1975. Influenza virion RNA-dependent RNA polymerase: stimulation by guanosine and related compounds. J. Virol. 15:686–695.
10. **Marmur, J.** 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. 3:208–218.
11. **Miller, J. P., L. J. Kigwana, D. G. Streeter, R. K. Robins, and L. N. Simon.** 1977. The relationship between the metabolism of ribavirin and its proposed mechanism of action. Ann. N. Y. Acad. Sci., in press.
12. **Oxford, J. S.** 1975. Inhibition of the replication of influenza A and B viruses by a nucleoside analogue (ribavirin). J. Gen. Virol. 28:409–414.
13. **Müller, W. E. G., A. Maidhof, H. Tashner, and R. K. Zahn.** 1977. Virazole (1-β-D-ribafuranosyl-1,2,4,-triazole-3-carboxamide) a cytostatic agent. Biochem. Pharmacol., in press.
14. **Plotch, S. J., and R. M. Krug.** 1977. Influenza virion transcriptase. The synthesis in vitro of large, poly A-containing complementary RNA. J. Virol. 21:24–34.
15. **Pons, M. and G. Hirst.** 1968. Polyacrylamide gel electrophoresis of influenza virus RNA. Virology 34:385–388.
16. **Roy, P., and D. Bishop.** 1972. Genome homology of vesicular stomatitis virus and defective T particles and evidence for the sequential transcription of the virion ribonucleic acid. J. Virol. 9:946–955.
17. **Sidwell, R. W., J. H. Huffman, G. P. Khare, L. B. Allen, J. T. Witkowski, and R. K. Robins.** 1972. Broad-spectrum antiviral activity of Virazole: 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide. Science 177:705–606.
18. **Scholtissek, C.** 1976. Inhibition of influenza RNA synthesis by Virazole (ribavirin). Arch. Virol. 50:349–352.
19. **Streeter, D. G., J. T. Witkowski, G. P. Khare, R. W. Sidwell, R. J. Bauer, R. K. Robins, and L. N. Simon.** 1973. Mechanism of action of 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (Virazole), a new broad spectrum antiviral agent. Proc. Natl. Acad. Sci. U.S.A. 70:1174–1178.
20. **Witkowski, J. T., R. K. Robins, R. W. Sidwell, and L. N. Simon.** 1972. Design, synthesis and broad spectrum antiviral activity of 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide and related nucleosides. J. Med. Chem. 15:1150–1154.