MOLECULAR PHARMACOLOGY, 13, 585–597

Mechanism of Polyribonucleotide Inhibition of Ribonucleic Acid Tumor Virus Replication: Effect of Poly(inosinic acid) on the Synthesis of Proviral Deoxyribonucleic Acid of Murine Leukemia Virus

S. K. ARYA

Department of Medical Viral Oncology, Roswell Park Memorial Institute, and Graduate Faculty in Microbiology, State University of New York at Buffalo, Roswell Park Division, Buffalo, New York 14263

(Received December 9, 1976)
(Accepted March 8, 1977)

SUMMARY

ARYA, S. K. (1977) Mechanism of polyribonucleotide inhibition of ribonucleic acid tumor virus replication: effect of poly(inosinic acid) on the synthesis of proviral deoxyribonucleic acid of murine leukemia virus. *Mol. Pharmacol.*, **13**, 585–597.

Single-stranded polyribonucleotides inhibit the replication of RNA tumor viruses in cultured cells with little or no effect on their growth rates or viability. They also inhibit the virion-associated RNA-directed DNA polymerase *in vitro*. The several polyribonucleotides investigated exhibit a reasonably good correlation between their effects on virus replication in cell culture and on virion-associated RNA-directed DNA polymerase *in vitro*. These observations suggested that the inhibition of virus replication by polyribonucleotides may be related to their effect on intracellular viral RNA-directed DNA synthesis. Several other observations also indirectly support this notion. For example, polyribonucleotides are more effective when added prior to or just after virus infection; this suggests that an early step in virus infection is affected. The present study concerns the effect of poly(inosinic acid) on viral as well as cellular DNA synthesis in cultured JLS-V9 cells infected with murine leukemia virus. Poly(I) inhibits the synthesis of proviral DNA with little or no effect on cellular DNA synthesis. Furthermore, poly(I), at a concentration inhibitory for proviral DNA synthesis, apparently does not interfere with the uptake of labeled murine leukemia virus by cultured JLS-V9 cells. The effects of poly(I) on partially purified murine leukemia virus and JLS-V9 cell DNA polymerases *in vitro* were compared. Analysis of the kinetics of inhibition shows that the apparent inhibitory potency of poly(I) toward viral DNA polymerase is 10–50 times that for cellular DNA polymerases. These results support the hypothesis that the inhibition of RNA tumor virus replication by polyribonucleotides is due, at least in part, to their inhibition of proviral DNA synthesis. This is more likely to be due to their effect on viral RNA-directed DNA synthesis than on DNA-directed DNA synthesis.

INTRODUCTION

Single-stranded polyribonucleotides inhibit the replication of RNA tumor viruses in cell culture (1–6); however, the mechanism of this inhibition is not yet established. Several indirect observations suggest that the inhibition of RNA tumor virus replication by polyribonucleotides may be related to their effect on viral RNA-directed DNA synthesis. (a) These polyribonucleotides strongly inhibit virion-associated RNA-directed DNA polymerase *in*

This work was supported in part by United States Public Health Service Center Grant CA 14801 in Viral Chemotherapy and Regulation from the National Cancer Institute.

ISSN 0026-895X

vitro (6–11). The several polyribonucleotides investigated by us have exhibited a reasonably good correlation between their effects on murine leukemia virus replication in cell culture and on virion-associated MuLV[1] RNA-directed DNA polymerase *in vitro* (5, 6, 9, 10). (b) They are more effective when added prior to or just after virus infection; this suggests that an early step in virus infection is affected (1–3). (c) They lose much of their effectiveness as inhibitors of virus replication in persistently infected cells.[2] (d) They do not suppress the activation of endogenous virus(es) by halogenated pyrimidines in virus-free cultured cells (2, 12). The latter two observations indicate that polyribonucleotides affect a step prior to, or including, the integration of proviral DNA into the cell genome. (e) They do not affect the replication of some lytic RNA viruses, presumably those viruses which do not require RNA-directed DNA synthesis for their replication (2, 3).

To investigate the mechanism of inhibition more directly, the effect of poly(inosinic acid) on viral as well as cellular DNA synthesis in JLS-V9 cells infected with MuLV has been examined. Poly(I) inhibits the synthesis of proviral DNA of MuLV with little or no effect on cellular DNA synthesis. Furthermore, poly(I), at a concentration inhibitory for proviral DNA synthesis, does not interfere with the uptake of radiolabeled MuLV by cultured JLS-V9 cells. In addition, the effects of poly(I) on partially purified MuLV and JLS-V9 cell DNA polymerases have been compared. Analysis of inhibition kinetics shows that poly(I) is 10–50 times more potent as an inhibitor of viral enzyme than of cellular enzymes. These results support the notion that the inhibition of MuLV replication by polyribonucleotides is due, at least in part, to their effect on the synthesis of proviral DNA.

[1] The abbreviations used are: MuLV, Moloney murine leukemia virus; PFU, plaque-forming units; PBS, phosphate-buffered saline (0.02 M sodium phosphate–0.15 M NaCl, pH 7.4); SDS, sodium dodecyl sulfate; phosphate buffer, equimolar mixture of mono- and disodium hydrogen phosphate, pH 6.8; SSC, 0.15 M NaCl–0.015 M sodium citrate, pH 7.0.

[2] S. K. Arya, unpublished observations.

MATERIALS AND METHODS

Polynucleotides. Poly(I) (mol wt > 100,000), poly(A) (8.1 S), poly[d(A-T)] (15.1 S), and oligo(dT)$_{12-18}$ were obtained from Miles Laboratories. The concentration of polynucleotide in 0.01 M NaCl–0.01 M Tris-HCl (pH 7.2) was determined spectrophotometrically by using the following absorptivities for a 1 mg/ml solution: poly(I), 27.5 at 248 nm; poly(A), 28.5 at 258 nm; poly[d(A-T)], 20.8 at 260 nm. Tritium-labeled thymidine triphosphate (52.5 Ci/mmole) and uridine (26 Ci/mmole) were obtained from New England Nuclear Corporation, and unlabeled nucleoside triphosphates, from P-L Biochemicals.

Cells and virus. Mouse bone marrow-derived JLS-V9 cells were grown as monolayers in plastic flasks, using RPMI-1640 culture medium containing 10% fetal calf serum (Grand Island Biological Company). The Moloney strain of murine leukemia virus was obtained from cultured JLS-V9 cells persistently infected with this virus. The culture medium from such cells, after being clarified by slow-speed centrifugation, was used as a virus inoculum for uninfected cells. The preparations of virus thus obtained contained 8–12 × 10^6 PFU/ml of virus when measured by XC plaque assay (13).

Purified virus was obtained from the medium of the persistently infected JLS-V9 cells harvested on a continuous basis as described before (9). This involved rate-zonal sucrose gradient centrifugation of the virus preparation, followed by banding to equilibrium in sucrose gradients.

Preparation of labeled virus. Subconfluent monolayers of JLS-V9 cells persistently infected with MuLV were grown for 18 hr in culture medium plus fetal calf serum containing 100 μCi/ml of [^{3}H]uridine (26 Ci/mmole). The monolayers were then washed three times with PBS and incubated in fresh culture medium plus fetal calf serum but without labeled uridine. Two hours later, the culture medium was harvested and monolayers were incubated again for 2 hr with fresh unlabeled culture medium plus fetal calf serum. The culture medium was harvested, and the process was repeated two more times to obtain a total of four har-

vests containing virus no more than 2 hr old.

The labeled virus in the culture medium was purified by isopycnic sucrose gradient centrifugation. The harvested culture medium was clarified by slow-speed centrifugation and layered on a 15–60% sucrose gradient. It was centrifuged for 18 hr at 25,000 rpm in a Beckman SW 27.1 rotor at 4°. The fractions were collected from the bottom of the tube, and aliquots were precipitated with trichloracetic acid and counted for radioactivity. Aliquots of the fractions were also used to determine the density of sucrose in the fractions. A profile of density gradient distribution of labeled MuLV is shown in Fig. 1. The fractions containing radioactivity and banding at a density of 1.15–1.17 g/ml (fractions 10–12, Fig. 1) were collected and diluted 10–20-fold with culture medium plus fetal calf serum. For experiments with labeled virus, the preparation of labeled MuLV was used the same day it was purified, and a minimum of time was allowed to elapse between harvest of the culture medium and purification of the virus.

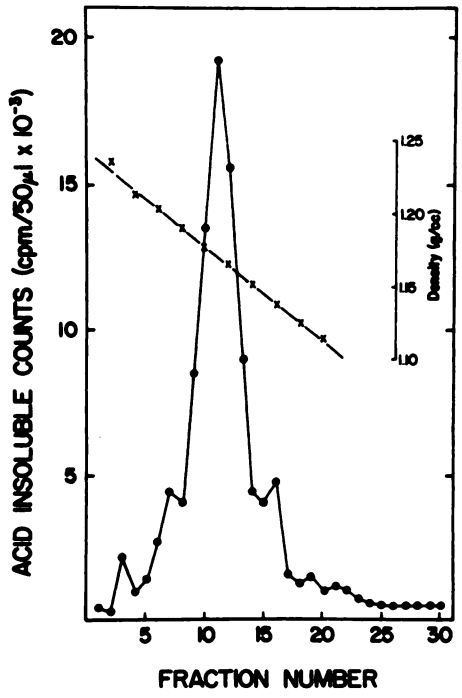

FIG. 1. *Isopycnic sucrose density gradient centrifugation of MuLV*

The culture medium harvested from monolayers of persistently infected JLS-V9 cells and containing [^{3}H]uridine-labeled MuLV was clarified by slow-speed centrifugation. It was then layered on a 15–60% sucrose gradient and centrifuged for 18 hr at 25,000 rpm in a Beckman SW 27.1 rotor at 4°. Aliquots of the fractions, collected from the bottom of the tube, were precipitated with trichloracetic acid. The precipitate was collected and counted for radioactivity. Aliquots were also used to determine the density of sucrose in the fractions.

Preparation of labeled MuLV cDNA. The labeled cDNA of MuLV was prepared by endogenous reaction of the purified virus (14, 15). A preparation of purified virus (200 µg of protein per milliliter) was incubated in a reaction mixture containing 0.01 M Tris-HCl (pH 7.9), 0.06 M NaCl, 0.02 M dithiothreitol, 0.006 M magnesium acetate, 0.01% NP-40, a 1 mM concentration each of dATP, dGTP, and dCTP, 200 µCi/ml of [^{3}H]TTP (52.5 Ci/mmole), 10 mM phosphoenolpyruvate, 50 µg/ml of pyruvate kinase, and 50 µg/ml of actinomycin D. The reaction mixture was incubated at 37° under an atmosphere of nitrogen for 4 hr. At this time, an additional 200 µCi/ml of [^{3}H]TTP were added and the incubation was continued for 4 hr more. The reaction was terminated by the addition of SDS to 2%. The mixture was made 0.2 M in NaCl and extracted with phenol at room temperature. It was passed over a column of Sephadex G-50, and the column flow-through fractions containing radioactivity were collected and applied to hydroxylapatite in a column maintained at 60°. The column was eluted stepwise with 0.06 M, 0.12 M, and 0.4 M phosphate buffer. Aliquots of the fractions were precipitated with trichloracetic acid and counted for radioactivity. The elution profile thus obtained is shown in Fig. 2. Apparently only a little more than 50% of the product synthesized by the endogenous reaction of this MuLV preparation, despite the presence of actinomycin D, was eluted with 0.12 M phosphate buffer and thus was single-stranded cDNA (16). The rest of the product was eluted with 0.4 M phosphate and thus was double-stranded cDNA (16). For the experiments presented in this report, only single-stranded cDNA was used. Any residual viral RNA in single-stranded cDNA preparations was eliminated by

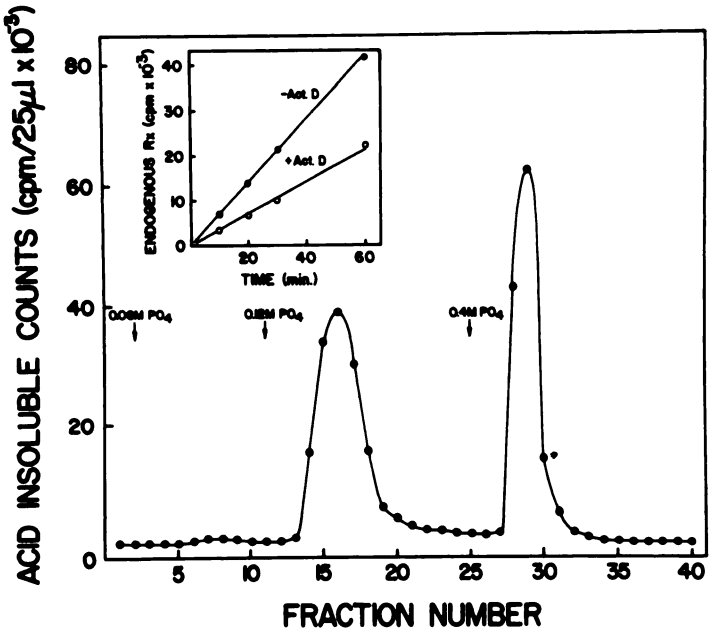

FIG. 2. *Hydroxylapatite column chromatography of MuLV cDNA synthesized by endogenous reaction of MuLV in the presence of actinomycin D*

MuLV cDNA was synthesized by endogenous reaction in the presence of actinomycin D (50 μg/ml), using a purified preparation of MuLV as described in MATERIALS AND METHODS. The reaction mixture was extracted wtih chloroform and precipitated with ethanol. The precipitate was dissolved in 0.06 M phosphate buffer and passed over a column of Sephadex G-50. The column flow-through fractions containing radioactivity were applied to a column of hydroxylapatite maintained at 60°. The column was then eluted stepwise with 0.06 M, 0.12 M, and 0.4 M phosphate buffer. Aliquots of the fractions were precipitated with trichloracetic acid and counted for radioactivity. The inset shows the effect of actinomycin D on DNA synthesis in an endogenous reaction of purified MuLV. The reaction conditions were the same as used for the preparation of MuLV cDNA.

making the solution 0.3 M in NaOH and boiling for 10 min, followed by neutralization with acetic acid. The resulting solution was rechromatographed on Sephadex G-50, and the fractions containing radioactivity were collected. The specific activity of this cDNA, estimated from the specific activity of the added [^{3}H]TTP, was about 27×10^6 cpm/μg.

Poly(I) treatment and virus infection. Duplicate or triplicate monolayers of JLS-V9 cells were treated for 30 min with DEAE-dextran (10 μg/ml), washed with PBS, and incubated for 2 hr with culture medium without fetal calf serum and containing poly(I) (20 μg/ml). The control cultures received culture medium without poly(I). The monolayers were washed with PBS and incubated with the MuLV inoculum for 1 hr. They were then processed according to the requirements of the experiment; further details are given in the legends to the figures. This procedure of poly(I) treatment and virus infection is the same in its essential details as used previously for studying the effect of polynucleotides on progeny virus synthesis in infected cells (5, 6).

Extraction of proviral DNA and hybridization with viral cDNA. The proviral DNA from MuLV-infected JLS-V9 cells was selectively extracted by the procedure of Hirt (17) as described by Gianni *et al.* (18). Briefly, monolayers were lysed with 0.6% SDS–0.01 M EDTA (pH 7.5), and the lysate was gently poured into centrifuge tubes, made to 1 M in NaCl, and allowed to stand at 0° for 12–18 hr. It was then centrifuged at 18,000 × *g* for 45 min, and the supernatant, containing proviral DNA, was collected. It was extracted twice with chloroform–isoamyl alcohol (24:1, v/v), and

the nucleic acids were precipitated by the addition of 2 volumes of ethanol. The precipitate was dissolved in 0.01 M EDTA (pH 7.5), made 0.3 M in NaOH, and boiled for 15 min to remove contaminating RNA. After neutralization with acetic acid, DNA was precipitated with ethanol and the precipitate was dissolved in 0.01 × SSC.

For hybridization with cDNA, aliquots of DNA in 0.01 × SSC were mixed with single-stranded MuLV cDNA and lyophilized to dryness. The mixture was then dissolved in 2 × SSC, sealed in siliconized glass capillary tubes, heated at 100° for 5 min, and incubated at 65° for 50 hr. The contents of the capillary tubes were then placed in 20 volumes of 0.025 M sodium acetate–0.1 mM zinc sulfate (pH 4.5), 20 μg of denatured salmon sperm DNA were added, and the mixture was digested with S-1 nuclease by incubation at 37° for 1 hr. (The amount of S-1 nuclease required for more than 90% digestion of single-stranded DNA was determined empirically.) After the incubation, the mixture was chilled, 100 μg of carrier yeast RNA were added, and the hybrids were precipitated with cold 10% trichloracetic acid. The precipitate was collected on Millipore filters, washed with cold 5% trichloracetic acid, and counted for radioactivity.

Polyribonucleotide inhibition of DNA polymerase activities. The partially purified preparations of MuLV DNA polymerase and JLS-V9 cell DNA polymerases were obtained as described before (6). DNA polymerase assay with MuLV DNA polymerase was performed in a reaction mixture (50 or 100 μl) containing 0.05 M Tris-HCl (pH 7.9), 0.06 M NaCl, 0.001 M manganese chloride, 0.02 M dithiothreitol, 50 μM [^{3}H]TTP (2600 cpm/pmole), 5–100:0.5–10 μM poly(A):oligo(dT) (molar ratio, 10:1), various concentrations of poly(I), and partially purified MuLV DNA polymerase. The cellular DNA polymerase assays utilizing poly(A):oligo(dT) as a template were performed in a similar reaction mixture, except that KCl replaced NaCl. The reaction mixture for cellular DNA polymerase utilizing poly[d(A-T)] as a template contained 0.05 M Tris-HCl (pH 7.9), 0.06 M KCl, 0.006 M magnesium acetate, 0.02 M dithiothreitol, 50 μM [^{3}H]TTP (2600 cpm/pmole), 100 μM dATP, 10–100 μM poly[d(A-T)], various concentrations of poly(I), and the enzyme preparation. The reaction mixture was incubated at 37°, and the radioactivity incorporated into acid-insoluble material was determined as previously described (6, 10). Under these conditions, the reaction mixture contained limiting concentration of the enzyme and saturating concentrations of deoxyribonucleoside triphosphates.

The kinetic data were treated according to Lineweaver and Burk to construct $1/v$ vs. $1/[S]$ plots (19). The concentration of the template was used as the substrate concentrations ($[S]$), and the incorporation of the precursor deoxynucleoside triphosphate was taken as a measure of the template utilization or velocity of the reaction ($1/v$). The Michaelis constant, K_m, was estimated from the intercept on the $1/[S]$ axis, and the inhibition constant, K_i, from the slopes of the lines representing the uninhibited and inhibited reaction (19).

RESULTS

Most of the studies presented in this report were performed with a poly(I) concentration of 20 μg/ml. This concentration of poly(I) yields about 80% inhibition of progeny virus synthesis in JLS-V9 cells infected with MuLV when the cells have been treated previously with poly(I) for 2 hr and extracellular progeny virus is scored 40 hr after infection (5). For the results presented in this report, cultured JSL-V9 cells were also previously treated with poly(I) for 2 hr, followed by infection with MuLV.

Effect on virus uptake. Among the possible ways in which treatment of cells with polyribonucleotides might result in the observed inhibition of progency virus synthesis is their interference with virus uptake by the host cells. Monolayers of cultured cells were first treated with polyribonucleotide, washed, and then infected with the virus. Thus polyribonucleotide and infecting virus did not come in an overt extracellular contact with each other as free entities in solution. However, this does not rule out the possibility that the polyribo-

nucleotide could interfere with of virus uptake by the cultured cells. For example, polyribonucleotide adsorbed to the cellular membrane might affect the subsequent attachment and penetration of the virus particles. Therefore the effect of poly(I) on the uptake of [^{3}H]uridine-labeled MuLV by JLS-V9 cells was investigated.

Monolayers of JLS-V9 cells were treated with DEAE-dextran and poly(I) (20 μg/ml) and then exposed to labeled MuLV for 1 hr. After thorough washing, the labeled virus taken up by the cells was assayed (Fig. 3). Over a 10-fold concentration range of added virus, there was no apparent significant difference in virus uptake by control and poly(I)-treated cultures. In addition, no difference in the uptake by control and poly(I)-treated cultures was noted when the amount of added labeled virus was kept constant and the number of cells in the culture was varied (data not shown). These results suggest that, at least at 20 μg/ml, poly(I) does not interfere with the uptake of MuLV by JLS-V9 cells. In a similar study, Tennant *et al.* (2) reported that poly(2′-*O*-methyladenylic acid), at high concentration (100 μg/ml), inhibited the uptake of labeled AKR virus by Swiss mouse embryo cells. However, at low concentration (10 μg/ml), this polyribonucleotide did not affect virus uptake while strongly inhibiting the infection of Swiss mouse embryo cells by MuLV. Thus it appears that interference with virus uptake is not a factor in polyribonucleotide inhibition of virus replication.

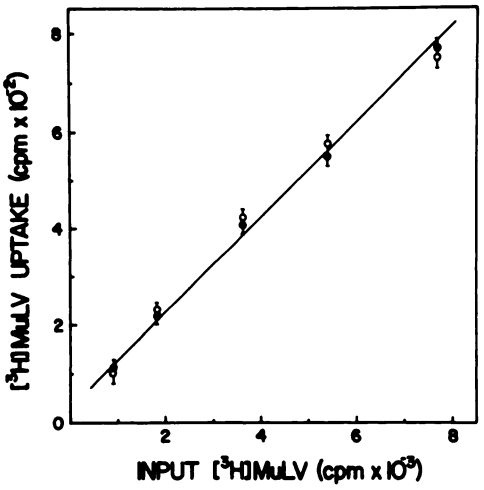

FIG. 3. *Effect of poly(I) on uptake of purified [^{3}H]uridine-labeled MuLV by JLS-V9 cells*

Duplicate monolayers of JLS-V9 cells (2 × 10^6 cells/35-mm dish) were treated for 30 min with 1 ml of DEAE-dextran (10 μg/ml), washed with PBS, and incubated for 2 hr with 1 ml of culture medium lacking (●——●) or containing (○——○) poly(I) (20 μg/ml). They were washed with PBS and incubated for 1 hr with 1 ml of purified [^{3}H]uridine-labeled MuLV (1200–13,000 cpm/ml) in culture medium plus fetal calf serum. The monolayers were then quickly washed four times with culture medium plus fetal calf serum, and the cells were scraped and suspended in PBS and precipitated with cold 10% trichloracetic acid. The precipitate was collected on glass fiber filters, washed with cold 5% trichloracetic acid, and counted for radioactivity. The culture dish without cells did not adsorb labeled virus when processed as above.

This conclusion, however, may need to be qualified. In any given preparation of RNA tumor virus, the number of virus particles that are infectious (for example, as measured by XC plaque assay) is rather low compared with the number of total particles (as measured by electron microscopy); it varies from 1 particle/1000 to 1 particle/10,000 or more. The results presented in Fig. 3 pertain to all particles, whether or not they are infectious. Thus the conclusion that interference with virus uptake is not a factor assumes that the infectious particles behave in a manner similar to the noninfectious particles in terms of their cellular adsorption and penetration. On the other hand, this conclusion is supported by the observation that polyribonucleotides are effective as inhibitors of virus replication even when added 2–4 hr after virus infection (1, 3). Indirect additional support is provided by the lack of effect of polyribonucleotides on the infection of cells by some lytic RNA viruses (1–3).

Effect on host cell DNA synthesis. The synthesis of cellular DNA and its replication apparently are required for the establishment of RNA tumor virus infection of cultured cells (see ref. 20 for a review). Thus the agents which inhibit host cell DNA synthesis will also affect the infection of cells by these viruses. Therefore the effects of poly(I) on cellular DNA synthesis

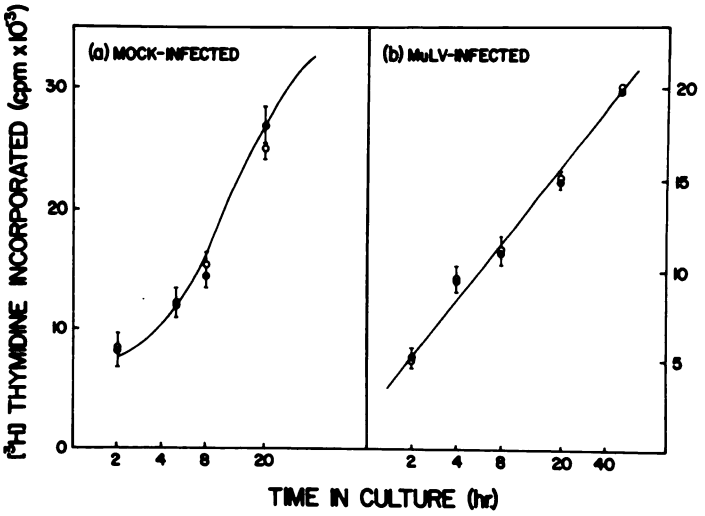

FIG. 4. *Effect of poly(I) on DNA synthesis in mock-infected (a) and MuLV-infected (b) JLS-V9 cells*
Triplicate monolayers of JLS-V9 cells (1.0–1.2 × 10^6 cells/35-mm dish) were treated for 30 min with 1 ml of DEAE-dextran (10 μg/ml), washed with PBS, and incubated for 2 hr with 1 ml of culture medium lacking (●——●) or containing (○——○) poly(I) (20 μg/ml). They were washed with PBS and infected for 1 hr with MuLV (2 PFU/cell) or were mock-infected. The monolayers were washed again wtih PBS and incubated with 2 ml of culture medium plus fetal calf serum containing [^{3}H]thymidine (2 μCi/ml) with (○——○) or without (●——●) poly(I) (20 μg/ml). At specified times thereafter, the monolayers were washed three times with culture medium plus fetal calf serum, and the cells were scraped and suspended in PBS. They were washed once with PBS by centrifugation, suspended in 0.01 M EDTA, and precipitated with cold 10% trichloracetic acid. The precipitate was collected on glass fiber filters, washed with cold 5% trichloracetic acid, and counted for radioactivity.

in MuLV-infected and mock-infected JLS-V9 cells were studied by determining its effect on the incorporation of labeled thymidine into acid-insoluble material. As the data in Fig. 4 show, poly(I) at 20 μg/ml had no significant effect on DNA synthesis in either mock-infected or MuLV-infected JLS-V9 cells. In the case of MuLV-infected cells, thymidine incorporation was followed over a period of 40 hr, which corresponds to about two generations for these cells. Thus poly(I) (20 μg/ml) apparently does not affect host cell DNA synthesis over a span of at least two generations. These observations are consistent with our previous report that poly(I) and other polyribonucleotides, at concentrations inhibitory for MuLV replication, do not affect the population growth rates of JLS-V9 cells (5).

Effect on proviral DNA synthesis. Weinberg and co-workers (18) reported the kinetics of proviral DNA synthesis in JLS-V9 cells infected with MuLV. They selectively extracted viral DNA from infected cells early after infection by the procedure of Hirt (17) and hybridized it with labeled MuLV cDNA or RNA. These studies indicated that free proviral DNA is synthesized early after infection and reaches a plateau 8–10 hr after infection. They also showed that the amount of cDNA hybridized was related to the number of cells used for the extraction of DNA containing virus-specific sequences. Similar results are shown in Fig. 5. It is apparent that doubling the number of cells used for DNA extraction nearly doubled the amount of cDNA hybridized.

To investigate the effect of poly(I) on the synthesis of proviral DNA, duplicate monolayers of JLS-V9 cells were treated for 2 hr with poly(I) (20 μg/ml) as before and infected for 1 hr with MuLV. At 5 and 10 hr after the infectious period, nucleic acids containing proviral DNA were selectively extracted from untreated infected and poly(I)-treated infected cultures as well as from mock-infected cultures. After alkaline digestion and ethanol precipitation,

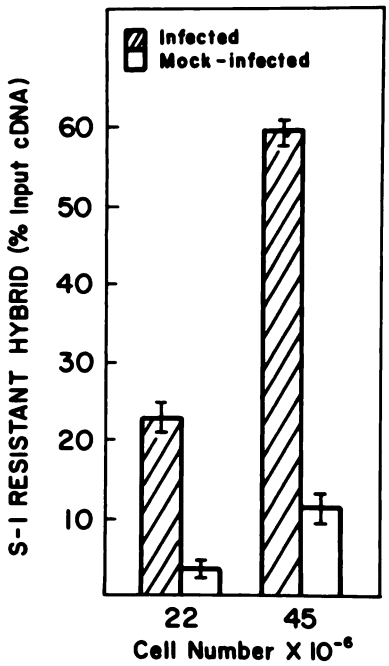

FIG. 5. *Detection of MuLV-specific DNA in extract of MuLV-infected JLS-V9 cells*

Monolayers of JLS-V9 cells were treated for 30 min with DEAE-dextran (10 μg/ml) and infected for 1 hr with MuLV (2 PFU/cell) or were mock-infected. Ten hours later, monolayers were washed with PBS, and the cells were detached from the substratum by mild trypsinization and counted. Aliquots of the cell suspension containing the indicated number of cells were centrifuged, and the pelleted cells were suspended in 0.01 M EDTA (pH 7.5) and lysed by making the suspension 0.6% in SDS. The nucleic acids were extracted by the procedure of Hirt (17), digested with alkali, and precipitated with ethanol as described in MATERIALS AND METHODS. The precipitate was dissolved in 150 μl of 0.01 × SSC, and 50-μl aliquots were mixed with tritium-labeled single-stranded MuLV cDNA and lyophilized to dryness. They were dissolved in 25 μl of 2 × SSC and transferred to siliconized glass capillary tubes, which were sealed, heated at 100° for 5 min, and incubated at 65° for 50 hr. The hybrids were assayed by determining the amount resistant to S-1 nuclease digestion as described in MATERIALS AND METHODS.

samples of DNA were hybridized with single-stranded labeled MuLV cDNA. The results (Fig. 6) show that the DNA obtained from poly(I)-treated infected cultures hybridized with MuLV cDNA to a significantly lower extent than the DNA from untreated infected cultures. For example, about 60% of the added cDNA hybridized with DNA from untreated infected cultures (5 hr after infection) at the higher cDNA input shown in Fig. 6. The corresponding figure for DNA from poly(I)-treated infected cultures is about 35%. This implies that the virus-specific DNA synthesized in poly(I)-treated cultures at 5 hr after infection was about 60% of that synthesized in control cultures. Similarly, at 10 hr after infection, the virus-specific DNA in poly(I)-treated cultures was about 50% of that in control cultures.

The data in Figs. 5 and 6 also show that the DNA from mock-infected cultures hybridized significantly with MuLV cDNA. Similar results have been reported by Weinberg and co-workers (18). This is not a nonspecific hybridization, since no significant amount of S-1 nuclease-resistant radioactivity was observed when MuLV cDNA was incubated with heterologous salmon sperm DNA (Fig. 6). The latter observation also indicates that MuLV cDNA did not self-reanneal; it was largely single-stranded or behaved so under the hybridization conditions used. The hybridization of DNA from mock-infected cultures with MuLV cDNA indicates that it contains some virus-related DNA sequences. These sequences are presumably derived from the endogenous virus genome(s). The JLS-V9 cells can be induced to produce virus particles by treatment with halogenated pyrimidines (21), and their cell genome contains sequences at least partially homologous to MuLV genome.[2] If we assume that the mock-infected cell DNA sequences are equally represented in the untreated infected and poly(I)-treated infected cell DNA, the virus-specific DNA in poly(I)-treated cultures would be 20–40% of that in the control cultures.

Effect on viral and cellular DNA polymerases. We have previously reported the inhibition by polyribonucleotides of detergent-activated RNA-directed DNA polymerase activity associated with MuLV (6, 9, 10). Those studies have now been extended to partially purified MuLV DNA polymerase. To determine whether these polyribonucleotides would display a degree of

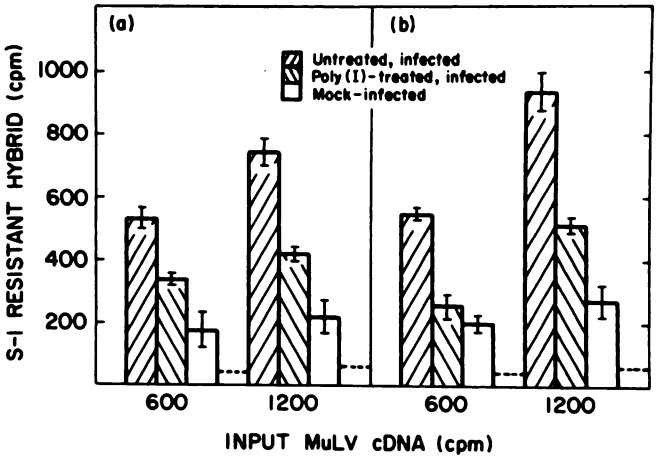

FIG. 6. *Effect of poly(I) on synthesis of proviral DNA in MuLV-infected JLS-V9 cells*

Duplicate monolayers of JLS-V9 cells (35–40 × 10^6 cells/150-cm^2 flask) were treated for 30 min with 15 ml of DEAE-dextran (10 μg/ml), washed with PBS, and incubated for 2 hr with 20 ml of culture medium lacking or containing poly(I) (20 μg/ml). They were washed with PBS and infected for 1 hr with MuLV (2 PFU/cell) or were mock-infected. The monolayers were washed again with PBS and reincubated with 20 ml of fresh culture medium plus fetal calf serum with or without poly(I) (20 μg/ml). At 5 and 10 hr after the infection, monolayers, containing 45–50 × 10^6 cells each, were washed with PBS and lysed with 0.6% SDS in 0.01 M EDTA (pH 7.5). The nucleic acids were extracted and hybridized with single-stranded MuLV cDNA, and hybrids were assayed by S-1 nuclease digestion as described in the legend to Fig. 5. - - -, MuLV cDNA incubated with heterologous salmon sperm DNA (2 mg/ml).

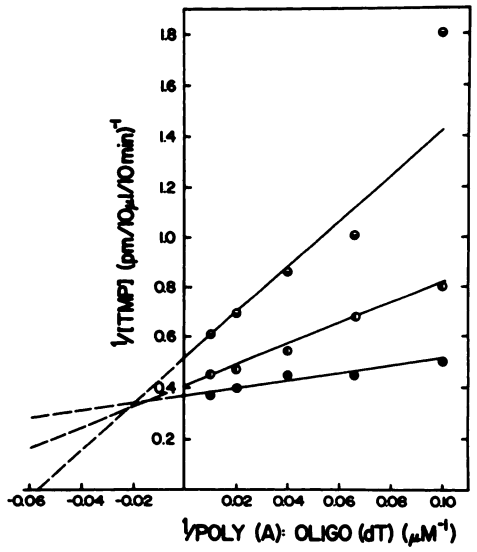

FIG. 7. *Kinetics of poly(I) inhibition of partially purified MuLV DNA polymerase*

The reaction mixture contained 0.05 M Tris-HCl (pH 7.9), 0.06 M NaCl, 0.02 M dithiothreitol, 0.001 M manganese chloride, 50 mM [^{3}H]TTP (2600 cpm/pmole), 5–100:0.5–10 μM poly(A):oligo(dT) (molar ratio, 10:1), 0, 0.5, or 2 μM poly(I), and DNA polymerase. The reaction mixture was incubated at 37° for 10 min, and aliquots were precipitated with trichloracetic acid. The radioactivity incorporated into acid-insoluble materials was counted. ●——●, incorporation in the absence of poly(I); ◐——◐, in the presence of 0.5 μM poly(I); ⊖——⊖, in the presence of 2 μM poly(I).

selectivity for viral DNA polymerase as compared with cellular DNA polymerases, their effects on partially purified host cell DNA polymerases were also investigated.

Figure 7 shows the kinetics of poly(I) inhibition of partially purified MuLV DNA polymerase directed by poly(A):oligo(dT). Judging from these Lineweaver-Burk plots, the kinetics of inhibition apparently is consistent with neither simple competitive nor noncompetitive inhibition. It appears to be a mixed type of inhibition, possibly partially competitive (19); both the K_m and V_{max} of the reaction are affected by poly(I). Similar results were noted by us previously for virion-associated DNA polymerase activity (9, 10). Regardless of the complex kinetics, the kinetic constants can be obtained from these plots (19). The values of K_m, estimated from the intercept on the 1/[*S*]axis of the line representing the reaction in the absence of inhibitor, and K_i, estimated from

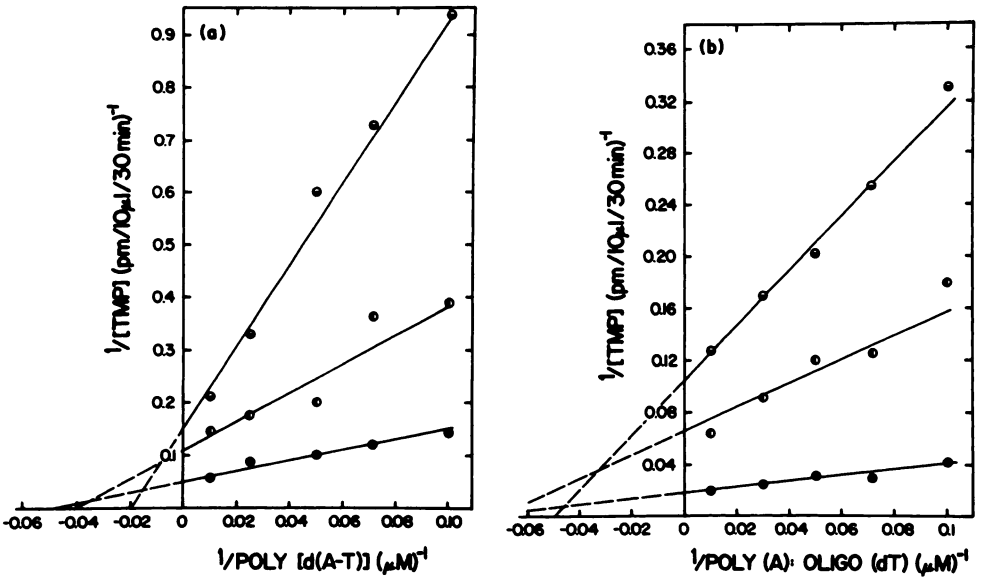

FIG. 8. *Kinetics of poly(I) inhibition of partially purified JLS-V9 cell DNA polymerases I (a) and III (b)* The reaction mixture for polymerase I contained 0.05 M Tris-HCl (pH 7.9), 0.06 M KCl, 0.02 M dithiothreitol, 0.006 M magnesium acetate, 50 μM [^{3}H]TTP (2600 cpm/pmole), 100 μM dATP, 10–100 μM poly[d(A-T)], 0, 35, or 70 μM poly(I), and cell DNA polymerase. The reaction mixture for polymerase III was the same as described for Fig. 6, except that NaCl was replaced by KCl and it contained 0, 10, or 35 μM poly(I). The reaction mixture was incubated at 37° for 30 min, and aliquots were precipitated with trichloracetic acid. The radioactivity incorporated into acid-insoluble material was counted. ●——●, incorporation in the absence of poly(I); ◐——◐, incorporation in the presence of 35 μM (a) or 10 μM (b) poly(I); ⊖——⊖, incorporation in the presence of 70 μM (a) or 35 μM (b) poly(I).

the relative slopes of lines representing the uninhibited and unhibited reaction (19), are listed in Table 1.

The K_m of poly(A):oligo(dT) and the K_i of poly(I) for partially purified MuLV RNA-directed DNA polymerase are about 4 μM and 0.3 μM, respectively (Table 1). These values are to be compared with those previously reported for detergent-activated, virion-associated MuLV DNA polymerase activity; the K_m of poly(A):oligo(dT) and K_i of poly(I) were found to be 18 ± 2 μM and 0.7 ± 0.2 μM, respectively (10). Similar differences were also noted for poly(X) and poly(G) (6). The reasons for the differences in the K_m of the templates and K_i of inhibitors for virion-associated and partially purified MuLV DNA polymerase activities are not clear; they may be related to the interaction of the templates and inhibitors with extraneous proteins and other macromolecules present in the detergent-disrupted virus preparation. Similar differences in the K_m of the templates for detergent-disrupted and partially purified avian myeloblastosis virus DNA polymerase activities have been reported (11).

The effect of poly(I) on partially purified host cell DNA polymerase activities is presented in Fig. 8. Three apparent DNA polymerase activities have been isolated from JLS-V9 cells, and two of these activities were tested for inhibition by poly(I). These two activities, DNA polymerases I and III, show some preference for DNA and RNA templates, respectively (see MATERIALS AND METHODS). Figure 8a shows the kinetics of poly(I) inhibition of the poly[d(A-T)]-directed reaction of DNA polymerase I, and Fig. 8b presents similar data for the poly(A):oligo(dT)-directed reaction of DNA polymerase III. As with viral DNA polymerase, the kinetics of inhibition is consistent with neither simple competitive nor noncompetitive inhibition; both the K_m and V_{max} of the reaction are

TABLE 1

Kinetic parameters for poly(I) inhibition of MuLV and JLS-V9 cell DNA polymerases

K_m and K_i values were estimated from Lineweaver-Burk ($1/v$ vs. $1/[S]$) plots. At least five concentrations of the template:primer and two concentrations of poly(I) were used. The results are expressed as micromolar concentrations of nucleotide residues in the single strand.

DNA polymerase	K_m of template	K_i of poly(I)
	μM	μM
Partially purified MuLV DNA polymerase	4.0[a]	0.30 ± 0.05
Partially purified JLS-V9 DNA polymerase I	9.4[b]	16.3 ± 4.8
Partially purified JLS-V9 DNA polymerase III	8.3[a]	3.8 ± 0.3

[a] The template:primer was poly(A):oligo(dT) (molar ratio, 10:1).

[b] The template:primer was poly[d(A-T)].

affected. The kinetic constants estimated from these plots are listed in Table 1; the K_i values of poly(I) for JLS-V9 cell DNA polymerases I and III are about 16.3 μM and 3.8 μM, respectively. It is notable that the RNA-directed DNA polymerase activity (DNA polymerase III) of cellular DNA polymerases is affected by poly(I) more than the DNA-directed DNA polymerase activity (DNA polymerase I). We have previously reported similar observations for these two polymerase activities regarding their inhibition by poly(X) and poly(G) (6).

DISCUSSION

Single-stranded polyribonucleotides inhibit the replication of MuLV in cultured JLS-V9 cells (4–6). At concentrations inhibitory for virus synthesis, these polymers display no apparent effects on growth rates and viability of normal uninfected or MuLV-infected JLS-V9 cells (4–6). In addition, the inhibition of virus synthesis appears not to be related to their possible effect on the uptake of virus by the cultured cells (Fig. 3) (2). These observations suggested that the molecular target(s) of polyribonucleotide inhibition is probably an intracellular function(s) specific for virus synthesis and is not a normal cellular biosynthetic activity. Furthermore, polyribonucleotides inhibit the virion-associated MuLV RNA-directed DNA polymerase *in vitro* (9, 10). This raised the possibility that the inhibition of virus replication by polyribonucleotides may be related to their effect on intracellular viral RNA-directed DNA synthesis.

The results in this report suggest that poly(I) diminishes the synthesis of proviral DNA of MuLV in infected JLS-V9 cells. For example, if one takes into account the virus-related DNA sequences contributed by the endogenous virus genome(s), poly(I), at a concentration of 20 μg/ml, inhibits the synthesis of proviral DNA to the extent of 60–80% relative to the control cultures (Fig. 6). This inhibition appears to be specific for viral DNA synthesis. Poly(I), at 20 μg/ml, apparently does not affect cellular DNA synthesis in either mock-infected or MuLV-infected JLS-V9 cells (Fig. 4). Thus these results suggest that the inhibition of virus synthesis by polyribonucleotides is due, at least in part, to their specific effect on proviral DNA synthesis. Whether this inhibition is due solely to their effect on viral RNA-directed DNA synthesis or also involves viral DNA-directed DNA synthesis remains to be clarified.

It is to be noted that the synthesis of proviral DNA involves both RNA-directed as well as DNA-directed DNA synthesis (see ref. 22 for a review). The viral genomic RNA (positive strand) of the infecting virus first serves as a template for the synthesis of a negative strand of viral DNA. This synthesis is catalyzed by the viral RNA-directed DNA polymerase. The negative strand of viral DNA then serves as a template for the synthesis of the positive DNA strand, resulting in proviral DNA. This latter synthesis is evidently catalyzed by DNA-directed DNA polymerase, which may or may not be virus-specific. We have used a single-stranded viral cDNA probe, synthesized *in vitro*, to evaluate the impact of poly(I) on proviral DNA synthesis. This probe presumably is composed largely of the negative strand of viral DNA copied from the viral template

RNA. It thus scores the synthesis of the positive strand of viral DNA, which is not the direct product of the viral RNA-directed DNA synthesis. Obviously, the positive strand of proviral DNA cannot be synthesized unless the negative strand is first synthesized. Nonetheless, these results cannot rule out the possibility that the effect of poly(I) on DNA-directed DNA synthesis may also be involved. On the other hand, the likelihood that the observed effect on proviral DNA synthesis is due at least in part to the inhibition of RNA-directed DNA synthesis is suggested by two observations presented in this report. First, poly(I) does not affect cellular DNA synthesis; this suggests that poly(I) is not an effective inhibitor of DNA-directed DNA synthesis. Second, poly(I) is a considerably more potent inhibitor of viral RNA-directed polymerase *in vitro* than of cellular DNA-directed DNA polymerases (see below).

The inhibition constant of poly(I) for viral RNA-directed DNA polymerase, obtained by analysis of the kinetics of inhibition, is 0.3 ± 0.05 μM. The same constant for cellular DNA-directed DNA polymerase is 16.3 ± 4.8 μM (Table 1). Thus poly(I) is about 50 times more potent toward viral RNA-directed DNA polymerase than for cellular DNA-directed DNA polymerase. Interestingly, among the cellular DNA polymerases, poly(I) is more inhibitory for RNA-directed DNA polymerase than for DNA-directed DNA polymerase (Table 1). The significance of this observation is not yet clear, partly because a specific function has not yet been assigned to the latter cellular DNA polymerase.

Thus the results presented in this report support the notion that the inhibition of murine leukemia virus replication by polyribonucleotides is due, at least in part, to their inhibition of proviral DNA synthesis. This is more likely to be due to their effect on viral RNA-directed DNA synthesis than on DNA-directed DNA synthesis. This notion is further supported by the following additional observations. (a) Polyribonucleotides are more effective as inhibitors when added prior to or just after infection; this suggests an early function in virus replication as the molecular target (1-3). (b) They do not suppress the induction of endogenous viruses by halogenated pyrimidines (2, 12) and lose much of their effectiveness as inhibitors of virus synthesis in persistently infected cells; these observations suggest that the molecular target is an activity functioning prior to or including the integration of proviral DNA into the cellular genome. (c) They do not affect the replication of some lytic RNA viruses; these viruses presumably do not require RNA-directed DNA synthesis for their replication (2, 12).

ACKNOWLEDGMENTS

The author thanks Mr. R. Chawda, Ms. L. Job, and Mr. A. Cairo for technical assistance. The author also thanks Dr. B. I. Milavetz for providing a partially purified preparation of murine leukemia virus DNA polymerase.

REFERENCES

1. Tennant, R. W., Kenney, F. T. & Tuominen, F. W. (1972) *Nat. New Biol.*, 238, 51-53.
2. Tennant, R. W., Farrelly, J. G., Ihle, J. N., Pal, B. C., Kenney, F. T. & Brown, A. (1973) *J. Virol.*, 12, 1216-1225.
3. Pitha, P. B., Teich, N. M., Lowy, D. R. & Pitha, J. (1973) *Proc. Natl. Acad. Sci. U. S. A.*, 70, 1204-1208.
4. Arya, S. K., Carter, W. A., Alderfer, J. L. & Ts'o, P. O. P. (1975) *Mol. Pharmacol.*, 11, 501-505.
5. Arya, S. K., Carter, W. A., Alderfer, J. L. & Ts'o, P. O. P. (1976) *Mol. Pharmacol.*, 12, 234-241.
6. Arya, S. K., Helser, T. L., Carter, W. A. & Ts'o, P. O. P. (1976) *Mol. Pharmacol.*, 12, 844-853.
7. De Clercq, E., Billiau, A., Hobbs, J., Torrence, D. F. & Witkop, B. (1975) *Proc. Natl. Acad. Sci. U. S. A.*, 72, 284-288.
8. Tennant, R. W., Hanna, M. G. & Farelly, J. G. (1974) *Proc. Natl. Acad. Sci. U. S. A.*, 71, 3167-3171.
9. Arya, S. K., Carter, W. A., Alderfer, J. L. & Ts'o, P. O. P. (1974) *Biochem. Biophys. Res. Commun.*, 59, 608-615.
10. Arya, S. K., Carter, W. A., Alderfer, J. L. & Ts'o, P. O. P. (1975) *Mol. Pharmacol.*, 11, 421-426.
11. Erickson, R. J. & Grosch, J. C. (1974) *Biochemistry*, 13, 1987-1993.
12. Pitha, P. M., Pitha, J. & Rowe, W. P. (1975) *Virology* 63, 568-572.
13. Rowe, W. P., Pugh, W. E. & Hartley, J. W.

(1970) *Virology*, **42**, 1136–1139.
14. Fan, H. & Baltimore, D. (1973) *J. Mol. Biol.*, **80**, 93–117.
15. Varmus, H. E., Heasley, S. & Bishop, J. M. (1974) *J. Virol.*, **14**, 895–903.
16. Bernardi, G. (1971) *Methods Enzymol.*, **21**, 95–139.
17. Hirt, B. (1967) *J. Mol. Biol.*, **26**, 365–369.
18. Gianni, A. M., Smotkin, D. & Weinberg, R. A. (1975) *Proc. Natl. Acad. Sci. U. S. A.*, **72**, 447–451.
19. Webb, J. L. (1963) *Enzymes and Metabolic Inhibitors*, Vol. 1, pp. 149–180, Academic Press, New York.
20. Temin, H. M. (1974) *Adv. Cancer Res.*, **19**, 48–104.
21. Besmer, P., Smotkin, D., Haseltine, W., Fan, H., Wilson, A. T., Paskind, M., Weinberg, R. & Baltimore, D. (1974) *Cold Spring Harbor Symp. Quant. Biol.*, **39**, 1103–1107.
22. Green, M. & Gerard, G. F. (1974) *Prog. Nucleic Acid Res. Mol. Biol.*, **14**, 187–334.