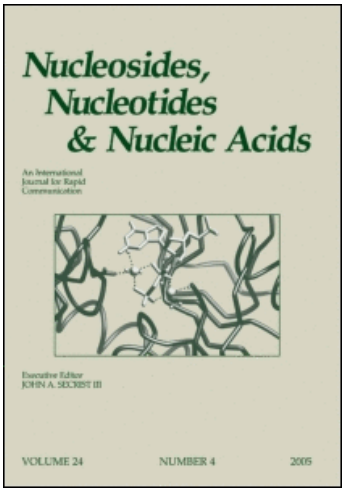

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

2',5'-Adenylate and Cordycepin Trimer Cores: Metabolic Stability and Evidence for Antimitogenesis without 5'-Rephosphorylation

Robert J. Suhadolnik[a]; Paul W. Doetsch[a]; Yair Devash[a]; Earl E. Henderson[b]; Ramamurthy Charubala[c]; Wolfgang Pfleiderer[c]
[a] Departments of Biochemistry, Temple University School of Medicine, Philadelphia, PA, USA [b] Microbiology and Immunology and the Fels Research Institute, Temple University School of Medicine, Philadelphia, PA, USA [c] Facultät für Chemie, Universität Konstanz, Federal Republic of Germany

To cite this Article Suhadolnik, Robert J. , Doetsch, Paul W. , Devash, Yair , Henderson, Earl E. , Charubala, Ramamurthy and Pfleiderer, Wolfgang(1983) '2',5'-Adenylate and Cordycepin Trimer Cores: Metabolic Stability and Evidence for Antimitogenesis without 5'-Rephosphorylation', Nucleosides, Nucleotides and Nucleic Acids, 2: 4, 351 — 366
To link to this Article: DOI: 10.1080/07328318308078869
URL: http://dx.doi.org/10.1080/07328318308078869

NUCLEOSIDES AND NUCLEOTIDES, 2(4), 351-366 (1983)

2',5'-ADENYLATE AND CORDYCEPIN TRIMER CORES: METABOLIC STABILITY AND EVIDENCE FOR ANTIMITOGENESIS WITHOUT 5'-REPHOSPHORYLATION

Robert J. Suhadolnik*‡, Paul W. Doetsch‡, Yair Devash‡, Earl E. Henderson§, Ramamurthy Charubala¶, and Wolfgang Pfleiderer¶

Departments of ‡Biochemistry and §Microbiology and Immunology and the Fels Research Institute, Temple University School of Medicine, Philadelphia PA 19140 USA and ¶Facultät für Chemie, Universität Konstanz, Postfach C-7750 Konstanz, Federal Republic of Germany

Abstract

The effect of the 2'.5'-adenylate and cordycepin trimer cores on DNA and protein synthesis in human umbilical cord lymphocytes, lymphoblasts, peripheral blood lymphocytes and Epstein-Barr virus infected lymphocytes and their metabolism in tissue culture medium have been studied. [^{32}P]Adenylate and [^{32}P]- and [^{3}H]cordycepin trimer cores were synthesized either enzymatically or chemically and added to cells in culture. The half-lives of the 2',5'-A_3 core and 2',5'-3'dA_3 core in tissue culture were 3 and 17 hr, respectively. Chromatographic analysis of the TCA-soluble extracts of the lymphocytes and lymphoblasts treated with 2',5'-[^{3}H]A_3 showed that 0.25% of the ^{32}P was identified as AMP, ADP, ATP and inorganic phosphate. By the more sensitive 2',5'-p_3A_4[^{32}P]pCp radiobinding assay, 2',5'-A_3 was detected in the TCA supernatants; however, there was no 5'-rephosphorylation. With the [^{3}H]- and [^{32}P]cordycepin trimer core, 0.55% and 1.3% of the radioactivity was in the TCA soluble extracts. Although there was no 5'-rephosphorylation as determined by radiobinding assay, the intact cordycepin trimer core was detected by tlc, radiobinding assay, and HPLC. Furthermore, in two experiments, the concentration of the cordycepin trimer core bound to or taken up by the lymphocytes was three-fold greater than the concentration in the medium. 2',5'-A_3 and 2',5'-3'dA_3 cores were both antimitogenic, but did not inhibit protein synthesis.

2',5'-A_n synthetase, found in lysates of rabbit reticulocytes and interferon-treated cells, produces 2',5'-oligoadenylates from ATP in the presence of dsRNA[1,2]. There is strong evidence suggesting that the 2',5'-oligoadenylates are important in the mechanism of antiviral action

 0732-8311/83/0204-0351$3.50/0

of interferon. The 2',5'-oligoadenylates activate a latent 2',5'-A_n dependent endoribonuclease (RNase L) in mammalian cells. When 2',5'-p_3A_n [the 5'-triphosphate] binds to the 2',5'-A_n dependent endonuclease, mRNA is hydrolyzed which then inhibits protein synthesis. Because 2',5'-p_3A_n is rapidly hydrolyzed by 2',5'-phosphodiesterase, the inhibition of protein synthesis is transient. Furthermore, Kerr and coworkers have reported that there are substantial concentrations of 2',5'-A_n core in interferon-treated mouse L cells[3]. They suggested that the 2',5'-adenylate cores may play an important role in DNA synthesis and cell growth.

A structurally modified 2',5'-p_3A_n that would inhibit protein synthesis and yet be resistant to hydrolysis by 2',5'-phosphodiesterase would be a useful analytical probe. There have been reports on the enzymatic and chemical synthesis of 2',5'-p_3A_n analogs and 2',5'-A_n core analogs, their increased metabolic stability, inhibition of cell growth, inhibition of protein synthesis, activation of the 2',5'-A_n dependent endonuclease, inhibition of vaccinia and tobacco mosaic virus infection, inhibition of reverse transcriptase, inhibition of Epstein-Barr virus (EBV-induced transformation of lymphocytes, and augmentation of natural killer (NK) cell activity[4-17]. It has been suggested that the 2',5'-A_n core and core analogs are either taken up intact[13], rephosphorylated at the 5'-hydroxyl position[5,10] or utilize a mechanism different from that of 2',5'-p_3A_n[6,8,14,18].

In 1965, we reported on the isolation of the 2',5'-phosphodiester bond following the addition of [^{3}H]cordycepin to H. Ep. #1 cells in culture[19]. More recently, we demonstrated that the cordycepin trimer core, which is metabolically more stable than the naturally occurring 2',5'-A_n core[20], inhibits HSV replication[14,15], inhibits the transformation of EBV-infected lymphocytes without prior treatment of the cells (e.g., calcium phosphate coprecipitation or permeabilization)[14], augments NK cell activity[21], inhibits the formation of the EBV-associated nuclear antigen[18], inhibits the replication of tobacco mosaic virus in tobacco plants[22], and inhibits tumor growth in animals[23]. In addition, the 5'-triphosphate tetramer [2',5'-$p_3$3'dA_4] has been shown to inhibit protein synthesis by 61% at 6.7×10^{-9} M in lysates from rabbit reticulocytes, to bind to and activate the 2',5'-A_n dependent endonuclease, to hydrolyze virus mRNA[24] and to inhibit protein synthesis in intact L cells and human fibroblasts[25]. In view of the broad bio-

logical activity exhibited by the 2',5'-oligonucleotide cores and analogs, it was critical to determine if the core molecules were affecting cellular reactions by a mechanism related to or independent of interferon. In the present investigation, 2',5'-[^{32}P]A_3 core and 2',5'-[^{32}P]3'dA_3 core were synthesized enzymatically and chemically and added to lymphocytes and lymphoblasts to determine if (i) the 2',5'-adenylate cores are taken up by cells, (ii) if they demonstrate differences in metabolic stability, (iii) if there is rephosphorylation of the 5'-hydroxyl group, (iv) if the nucleotides are recovered in the TCA soluble cytoplasmic pool, and (v) if the cores affect DNA and protein synthesis.

MATERIALS AND METHODS

Enzymatic and chemical synthesis of 2',5'-A_3 core and 2',5'-3'dA_3 core. 2',5'-[^{32}P]p_3A_n, 2',5'-[^{3}H]p_3A_n and 2',5'-[^{32}P]$p_3$3'dA_n were synthesized from [α-^{32}P]ATP (410 Ci/mmole; 500 μCi), [8-^{3}H]ATP (22 Ci/mmole; 500 μCi), and [α-^{32}P]3'dATP (3000 Ci/mmole, 1 mCi) with 600 nmoles of unlabeled ATP or 3'dATP and lysates from rabbit reticulocytes[24]. The isolation of the 5'-triphosphate trimers, 2',5'-p_3A_3 and 2',5'-$p_3$3'dA_3, was accomplished by DEAE cellulose chromatography, dialysis and isolation of the nucleotides with a charge of minus 6[24]. The [^{32}P]oligonucleotides were digested with bacterial alkaline phosphatase (BAP), snake venom phosphodiesterase (SVPD), and T2 RNase followed by cellulose tlc (solvent A), and autoradiography (Fig. 1). BAP digestion and isolation of the 2',5'-A_3 core and 2',5'-3'dA_3 core from reticulocyte lysates was accomplished by co-chromatography with chemically synthesized nucleotides. All ^{32}P in both nucleotides resided in the regions of their respective core trimer nucleotides. Additionally, alkaline hydrolysis was performed on the 2',5'-A_n core followed by tlc in solvent A. The products of hydrolysis were in agreement with the alkaline hydrolysis reported by Lengyel and coworkers[26]. The chemical synthesis of 2',5'-3'dA_3 core was as described[27].

Addition of 2',5'-[^{32}P]A_3 core to lymphocytes and lymphoblasts. The isolation and maintenance of human umbilical cord lymphocytes (HUCL) and peripheral blood lymphocytes (PBL) in a viable state throughout the period of the experiments were as described[14]. Four ml of HUCL (2 x 10^6 cells/ml, 80% viable as determined by Trypan blue exclusion) or C85-5C lymphoblasts (6 x 10^5 cells/ml, 99% viable) were added to sterilized

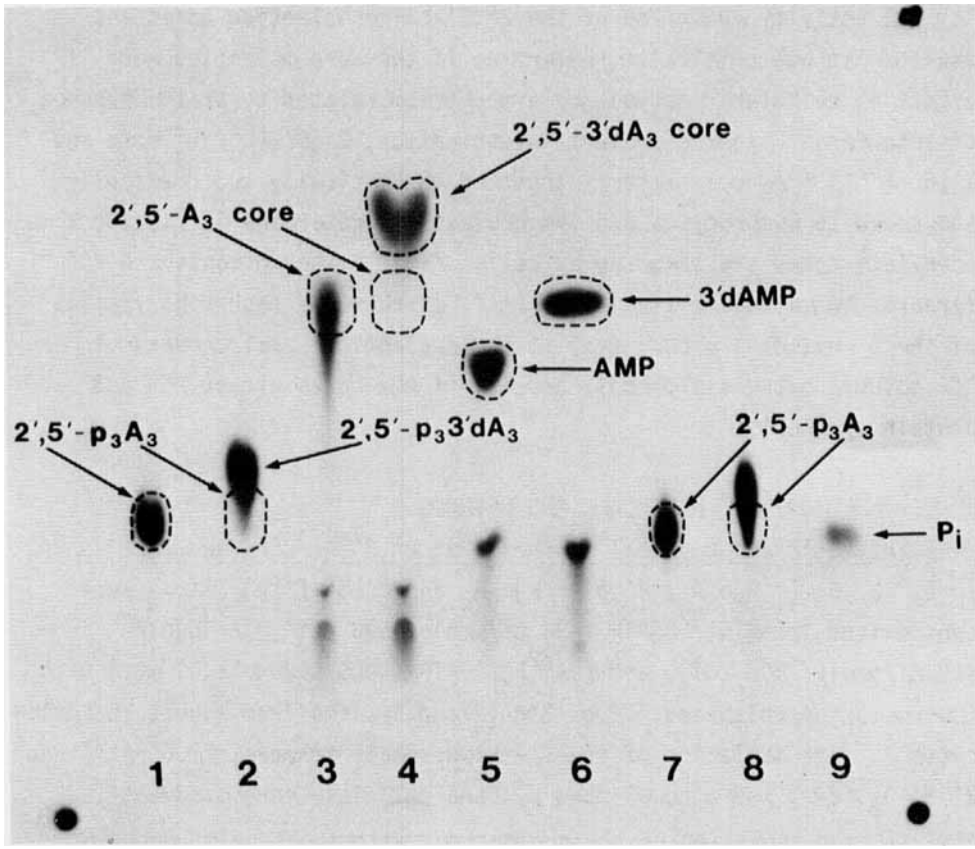

FIGURE 1. Cellulose thin layer chromatography (solvent A) of 2',5'-[^{32}P]p_3A_n and 2',5'-[^{32}P]$p_3$3'dA_n enzyme digests (autoradiogram). Lane 1: 2',5'-[^{32}P]p_3A_n and authentic 2',5'-p_3A_3. Lane 2: 2',5'-[^{32}P]$p_3$3'dA_3 and authentic 2',5'-p_3A_3. Lane 3: BAP digest of 2',5'-[^{32}P]p_3A_n and authentic 2',5'-A_3 core. Lane 4: BAP digest of 2',5'-[^{32}P]$p_3$3'dA_n and authentic 2',5'-A_3 core and 2',5'-3'dA_3 core. Lane 5: SVPD digest of 2',5'-[^{32}P]p_3A_n and authentic AMP. Lane 6: SVPD digest of 2',5'-[^{32}P]-$p_3$3'dA_n and authentic 3'dAMP. Lane 7: T2 RNase digest of 2',5'-[^{32}P]-p_3A_n and authentic 2',5'-p_3A_3. Lane 8: T2 RNase digest of 2',5'-[^{32}P]-$p_3$3'dA_n and authentic 2',5'-p_3A_3. Lane 9: [^{32}P]inorganic phosphate standard. The dotted circles indicate areas of uv absorbance corresponding to the standard when the chromatogram was visualized under short wave uv light. Dark areas indicate ^{32}P.

glass tubes (13 x 100 mm) and 2',5'-[^{32}P]A_3 core (7 μCi, 25 μM final concentration) was added. Incubations were done in a 5% CO_2-in-air incubator at 37°C for 12 hr. The cells were then washed and lysed by a modification of the method for cell-associated oligonucleotides of Knight et al.[28] Cells were centrifuged at 300 x g, 10 min, 25°C and washed three times with Hanks' buffer. Cells were lysed with 0.5%

Nonidet P-40 containing 90 mM KCl, 1.5 mM $Mg(OAc)_2$ and 10 mM HEPES·KOH (pH 7.5) at 0°C, 200 µl total volume. Trichloroacetic acid (TCA) (40%, 0°C) was added 5 min. after lysis to a final concentration of 5% and the mixture was centrifuged (800 x g, 20 min, 25°C). The supernatants were extracted with ether to remove the TCA and evaporated to dryness under a stream of air. Twenty microliters of glass distilled water was added. A 10 µl aliquot was applied to a PEI cellulose tlc (Brinkman, without fluorescent indicator) and developed in solvent B. The chromatograms were dried and subjected to autoradiography for 4 days. In addition, separation of nucleotides was accomplished by DEAE cellulose column chromatography[20].

Addition of 2',5'-[^{32}P]3'dA_3 core to C85-5C lymphoblasts. 2',5'-[^{32}P]3'dA_3 core (0.35 µCi; 25 µM) was added to C85-5C lymphoblasts (4 ml at 7 x 10^5 cells/ml) and incubated 6 hr, 37°C. Preparation of the TCA-soluble cytoplasmic extract was as described above.

Degradation of 2',5'-[^{3}H]p_3A_n in the presence of 2',5'-core oligonucleotides. The hydrolysis of 2',5'-[^{3}H]p_3A_n was assayed in the presence of increasing concentrations of either 2',5'-A_3 core of 2',5'-3'dA_3 core. Incubation mixtures contained 0.3 parts of HeLa cell extract, 1 µM 2',5'-[^{3}H]p_3A_n, 2.5 mM $Mg(OAc)_2$, 120 mM KCl, 1 mM DTT, 20 mM HEPES·KOH (pH 7.5), in a volume of 30 µl. Core 2',5'-oligonucleotides were added to the incubations at final concentrations of 0-200 µM. Samples were incubated 10 min, 30°C, heated to 95°C for 3 min, and processed as described previously[20]. The amount of 2',5'-[^{3}H]p_3A_n not hydrolyzed enzymatically was determined by DEAE cellulose chromatography as described[20].

Stability of oligonucleotides in lymphocyte medium. During the course of incubations of PBL (2 x 10^6 cells/ml, 0.13 ml) with 2',5'-[^{32}P]A_3 core (0.025 µCi, 25 µM), 10 µl samples of medium were withdrawn at 0, 0.5, 3, 6, and 17 hr, applied to a cellulose tlc and chromatographed with the appropriate standards in solvent A. The lanes of the developed chromatogram were cut into 1 cm^2 pieces and assayed for radioactivity in 5 ml of non-aqueous scintillation solution. A similar experiment was performed with 2',5'-[^{32}P]3'dA_3 core.

Effect of 2',5'-A_3 core and the 2',5'-3'dA_3 core on DNA synthesis. HUCL (2 x 10^6 cells/ml, 1 ml), C85-5C lymphoblasts (7 x 10^5 cells/ml, 1 ml) or EBV-infected HUCL (1 x 10^6 cells/ml, 1 ml) were treated with either 2',5'-A_3 or 2',5'-3'dA_3 core (25 µM final concentration in

medium) and incubated at 37°C. Duplicate tubes were pulsed with 10 μCi [^{3}H]thymidine (6.7 Ci/mmole) for 1 hr, 37°C. The cells were processed for incorporation of [^{3}H]thymidine as described previously[14].

Radiobinding assays. Radiobinding assays were performed as described[28]. 2',5'-p_3A_4[^{32}P]pCp was purchased from Amersham.

Analysis of 2',5'-3'dA_3 and related nucleotides by high performance liquid chromatography (HPLC). The HPLC system was comprised of a micro-Bondapak C_{18} column, two 6000A pumps, a solvent programmer (model 660), a U6K injector, and a fixed wavelength (254 nm) spectrophotometric detector (Waters Associates) linked to a dual-pen recorder (LKB, model 2210) and integrator (Hewlett Packard, model 3390A); solvent A: 50 mM ammonium phosphate, pH 6.0; solvent B: methanol:H_2O, 1/1, v/v . Linear gradient: 20% to 40% solvent B in 22.5 min; flow rate = 1 ml/min. Retention times of 3'dATP, 3'dAMP, 3'-deoxyinosine, 3'-deoxyadenosine (cordycepin) and 2',5'-3'dA_3 core were: 3.5, 5.5, 8.8, 14.2 and 18.8 minutes, respectively.

RESULTS

Stability of 2',5'-[^{32}P]A_3 core and 2',5'-[^{32}P]3'dA_3 core in lymphocyte medium. At 0-17 hr following the addition of the 2',5'-[^{32}P]A_3 or 2',5'-[^{32}P]3'dA_3 cores (25 μM) to culture medium, samples were removed, applied to cellulose tlc (solvent A) with marker 2',5'-A_3 core, 2',5'-3'dA_3 core, AMP, 3'dAMP and inorganic phosphate (adjacent lane) and analyzed for ^{32}P in the regions equivalent to: inorganic phosphate (R_f 0.28), AMP (R_f 0.54), 3'dAMP (R_f 0.65), 2',5'-A_3 core (R_f 0.63), and 2',5'-3'dA_3 core (R_f 0.79). Degradation of 2',5'-[^{32}P]A_3 core and 2',5'-[^{32}P]3'dA_3 core was calculated as the decrease in radioactivity co-chromatographing with authentic markers as a percentage of the total radioactivity compared to the zero time point. The half-life of 25 μM 2',5'-[^{32}P]A_3 core in the culture medium is approximately 3 hr. In contrast, the half-life of 25 μM 2',5'-[^{32}P]3'dA_3 core is about 17 hr (Fig. 2).

Metabolic fate of 2',5'-[^{32}P]A_3 core added to HUCL, C85-5C lymphoblasts, and EBV-infected HUCL. Because of the observed inhibition of transformation of EBV-infected lymphocytes by 2',5'-A_3 core and 2',5'-3'dA_3 core[14], the possibility that the 2',5'-A_3 cores might be internalized and rephosphorylated at the 5'-hydroxyl groups was investigated.

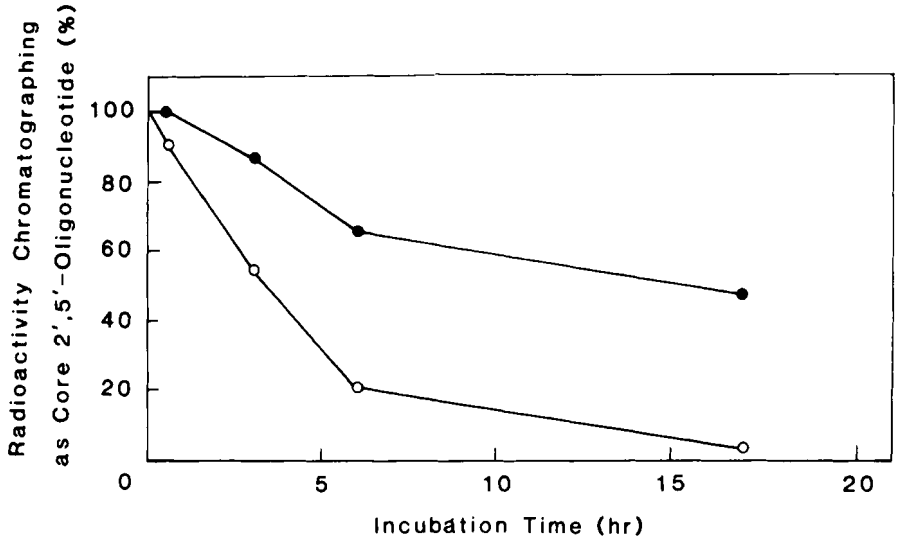

FIGURE 2. Stability of 2',5'-[^{32}P]A_3 core and 2',5'-[^{32}P]3'dA_3 core in lymphocyte medium. The stability of 2',5'-[^{32}P]A_3 core (25 μM, o—o) and 2',5'-[^{32}P]3'dA_3 core (25 μM, •—•) in lymphocyte medium was determined by cellulose tlc (solvent A) of samples withdrawn from the medium at times indicated.

When 2',5'-[^{32}P]A_3 core (7 μCi, 25 μM) was added to medium containing lymphoblasts and incubated for 12 hr at 37°C, 38,000 dpm (0.25%) were in the TCA-soluble cytoplasmic extract. When an aliquot of lymphoblast cell-free extract (9000 dpm) was applied to a DEAE-cellulose column and eluted with a linear 50-150 mM NaCl/50 mM Tris/HCl gradient in 7 M urea, no radioactivity was detected in the fractions equivalent to 2',5'-p_3A_3 (charge -6)(Fig. 3). Chromatography of an aliquot of the C85-5C lymphoblast cell-free extracts (9000 dpm) on PEI cellulose tlc (solvent B) revealed four distinct radioactive regions with R_f values of 0.41, 0.50, 0.60, and 0.71 for ATP, ADP, AMP, and inorganic phosphate. When a 10 μl aliquot of the cell-free extract (9000 dpm) was treated with BAP, followed by cellulose tlc (solvent A), there was no radioactivity in the region equivalent to 2',5'-A_3 core (R_f 0.71). All of the ^{32}P was detected as inorganic phosphate (R_f 0.33). These results show that the only ^{32}P-labeled compounds detected in the cell-free extracts are ATP, ADP, AMP and inorganic phosphate. The same results were obtained following the addition of 2',5'-[^{32}P]A_3 core to uninfected HUCL (data not shown). In a separate experiment, HUCL were infected with EBV[14]. 2',5'-[^{32}P]A_3 core was added simul-

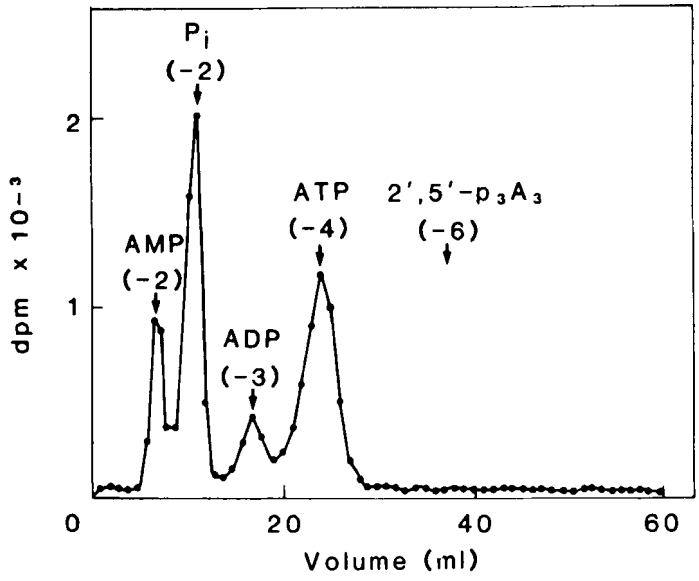

FIGURE 3. Chromatography of cell-free extracts from C85-5C lymphoblasts 12 hr after treatment with 2',5'-[^{32}P]A_3 core. The cytoplasmic TCA-soluble extract from 2',5'-[^{32}P]A_3 core-treated C85-5C lymphoblasts (9000 dpm) was applied to a DEAE cellulose column (Whatman DE-52, 0.5 x 17 cm)[20]. The nucleotides were displaced with a 50-150 mM linear gradient of NaCl (40 ml/40 ml), 50 mM Tris-HCl (pH 8.0) in 7 M urea; flow rate 4 ml/hr; 1 ml fractions. Arrows indicate displacement of charge markers. Inorganic phosphate was located by the displacement of [^{32}P]inorganic phosphate in a separate experiment.

taneously with EBV. Nine days after virus infection, HUCL extracts were chromatographed on PEI cellulose tlc (solvent B). All of the radioactivity was in the region equivalent to inorganic phosphate (R_f 0.71, 880 dpm).

Metabolic fate of 2',5'-[^{32}P]3'dA_3 core added to C85-5C lymphoblasts. When 2',5'-[^{32}P]3'dA_3 core (0.35 µCi, 25 µM) was added to medium containing C85-5C lymphoblasts, and incubated for 6 hr at 37°C, a total of 9000 dpm was found in the supernatant of the cells treated with TCA. This only represented 1.3% of the ^{32}P core added. Cellulose tlc (solvent A) showed three radioactive compounds. Of the 1.3% ^{32}P in the supernatant of the TCA-extracted cells, 80% was identified as the cordycepin trimer core (R_f 0.79), 4% as 3'dAMP (R_f 0.65), and 16% as inorganic phosphate (R_f 0.30). There was no 5'-rephosphorylated cordycepin trimer core. In the binding experiments, lymphocytes (2 x 10^6 cells/200 µl) were treated with 200 µM 2',5'-[^{3}H]3'dA_3 core and incu-

bated at 37°C. Samples were taken at 0, 6, 18 and 24 hr and extracted as described above. After 6 hr, only 0.55% of the tritium was found in the supernatant of the TCA-extracted cells. Of this amount, only 0.1% displaced the 2',5'-$p_3A_4[^{32}P]pCp$ analog in the binding assay (Fig. 4).

Determination of 5'-rephosphorylation of 2',5'-A_3 or 2',5'-3'dA_3 cores by radiobinding assay. To determine if there were 5'-rephosphorylation of the 2',5'-A_3 or 2',5'-3'dA_3 cores, the cores (200 µM) were added to lymphocytes (2 x 10^6 cells/200 µl). After 0, 6, 18 and 24 hr, 200 µl aliquots were removed, washed and extracted (see Materials and Methods). To determine if 5'-rephosphorylation of the cores occurred, assays were done measuring competition for the pCp analog using serial dilutions of the cell extracts compared to competition by non-radioactive 2',5'-p_3A_3 (0.2 - 1 nM), 2',5'-$p_3$3'dA_3 (0.2 - 1 nM), 2',5'-A_3 core (0.2 - 1 µM) and 2',5'-3'dA_3 core (0.2 - 1 µM). The assays were conducted with lysates from rabbit reticulocytes as the source of binding protein. 2',5'-p_3A_3 and 2',5'-$p_3$3'dA_3 (0.7 nM) com-

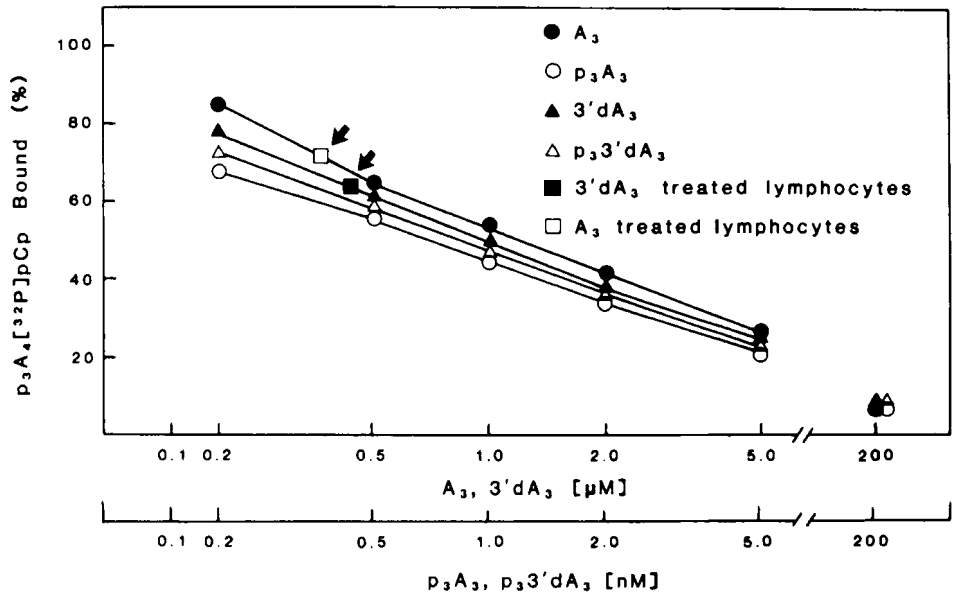

FIGURE 4. Radiobinding assays of lymphocyte extracts pretreated with 2',5'-A_3 core of 2',5'-3'dA_3 core. Competition for bound 2',5'-p_3A_4-$[^{32}P]pCp$ by lymphocytes pretreated with 2',5'-A_3 core (□) or 2',5'-3'dA_3 core (■) was compared with competition by non-radioactive 2',5'-p_3A_3 (o), 2',5'-A_3 core (●), 2',5'-$p_3$3'dA_3 (Δ), and 2',5'-3'dA_3 core (▲). Assays were performed as described [28]. Data represent an average of three experiments.

peted off 50% of the bound 2',5'-$p_3A_4[^{32}P]pCp$, whereas the lymphocyte extracts showed a 25-30% competition (Fig. 4). This technique does not eliminate the possibility that a small amount of 2',5'-oligocordycepin core trimer is bound to the cell surface.

To determine whether 2',5'-3'dA_3 core was associated with the cells, lymphocytes (2 x 10^6/200 µl, 0.36 µl total cellular volume) were treated with 15 µM 2',5'-[^{3}H]3'dA_3 core (10 µCi/µmole) at 37°C. After 10 min, 4 hr, and 18 hr, aliquots were removed and extracted as described in Materials and Methods. The 2',5'-3'dA_3 core and related nucleotides extracted from the lymphocytes were analyzed on HPLC. 3'dATP, 3'dAMP, 3'-deoxyinosine [3'dI], cordycepin [3'dA] and 2',5'-3'dA_3 core standards separated as shown in Fig. 5A. No radioactivity was associated with lymphocytes after 10 min. However, incubations for 4 hr or 18 hr showed either binding or uptake of 2',5'-[^{3}H]3'dA_3 core by lymphocytes. After 4 hr, the concentration of 2',5'-[^{3}H]3'dA_3 core was 44.5 µM, which is 2.7-fold greater than the 15 µM 2',5'-[^{3}H]3'dA_3 core in the medium (Fig. 5B). Radioactivity was also detected in the 3'dATP and 3'dAMP regions. After 18 hr, 24 µM 2',5'-[^{3}H]3'dA_3 core was detected in the TCA-soluble extract, which is 1.7-fold higher than the 15 µM 2',5'-[^{3}H]3'dA_3 core in the medium. The results presented suggest a

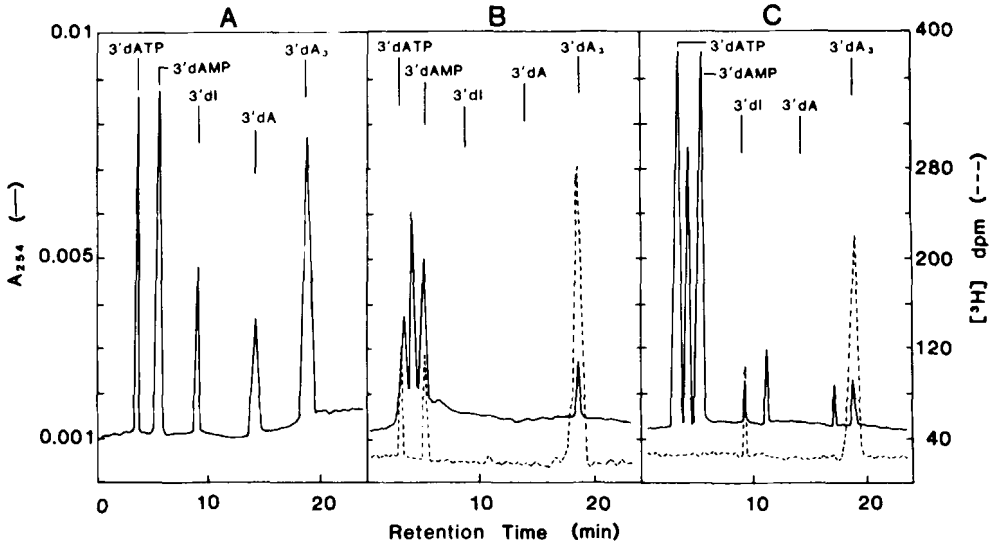

FIGURE 5. HPLC profile of 2',5'-[^{3}H]3'dA_3 core and metabolites bound to or taken up by lymphocytes following TCA extraction. A, standards; B, 4 hr; C, 18 hr.

relationship between the 2',5'-[^{3}H]3'dA$_3$ core in the TCA-soluble extracts (Fig. 5) and the half-life of the 2',5'-[^{32}P]3'dA$_3$ core in the medium (Fig. 2).

Degradation of 2',5'-[^{3}H]p$_3$A$_n$ in the presence of 2',5'-A$_3$ or 2',5'-3'dA$_3$ core. Because our original studies on the metabolic stability of the 2',5'-A molecule modified at the 2'-terminus were done with HeLa cells[20], we sought to determine whether there was competition between the 2',5'-A molecule and the cordycepin analog using cell-free extracts from HeLa cells. Experiments were done to determine the effect of increasing concentrations of unlabeled 2',5'-A$_3$ core or 2',5'-3'dA$_3$ core on the enzymatic hydrolysis of 2',5'-[^{3}H]p$_3$A$_n$ by 2',5'-phosphodiesterase from cell-free extracts of HeLa cells. Conditions were established that resulted in the presence of excess 2',5'-A$_3$ core in the assays (Fig. 6). The hydrolysis of 1 μM 2',5'-[^{3}H]p$_3$A$_n$ was monitored in the presence of increasing concentrations of 2',5'-A$_3$ core (0-200 μM). In the presence of 200 μM 2',5'-A$_3$ core, degradation of

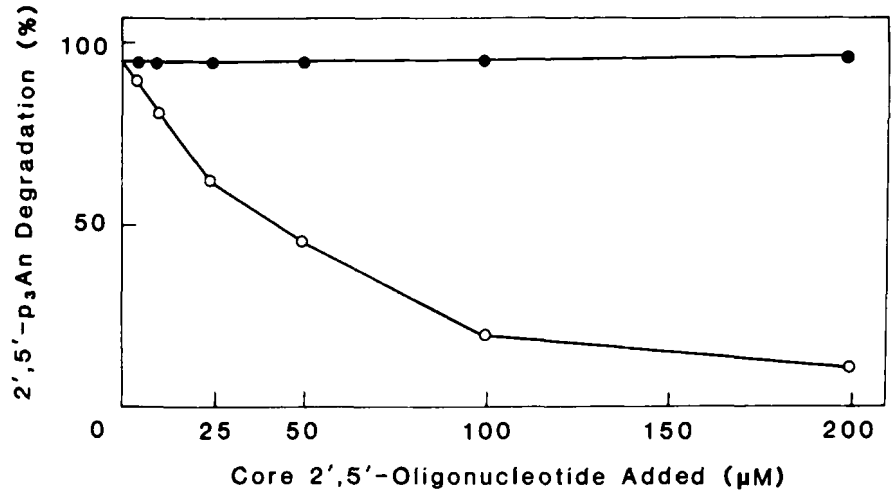

FIGURE 6. Degradation of 2',5'-[^{3}H]p$_3$A$_n$ in the presence of increasing concentrations of 2',5'-A$_3$ core or 2',5'-3'dA$_3$ core. 2',5'-[^{3}H]p$_3$A$_n$ and cores were added simultaneously. 2',5'-[^{3}H]p$_3$A$_n$ (1 μM) was incubated for 10 min with 0.3 parts of HeLa cell extract and the concentration of core 2',5'-oligonucleotide indicated on the abcissa (2',5'-A$_3$ core, o-o; 2',5'-3'dA$_3$, ●-●). The amount of undegraded 2',5'-[^{3}H]p$_3$A$_n$ was determined by DEAE cellulose chromatography[20]. With no added core 2',5'-oligonucleotide, about 95% of the 2',5'-[^{3}H]-p$_3$A$_n$ was degraded. Concentrations are expressed in AMP and 3'dAMP equivalents.

TABLE 1. DNA Synthesis in Lymphocytes and Lymphoblasts Treated with 2',5'-A_3 Core and 2',5'-3'dA_3 Core

Addition	Concentration (μM)	Incubation (hr)	Cell Type	dpm[b]	% of control[c]
none	—	12	HUCL	6,500	100
2',5'-A_3	25	12	HUCL	3,200	49.5
none	—	12	C85-5C	1,110,000	100
2',5'-A_3	25	12	C85-5C	515,000	44
none	—	216	EBV-infected HUCL	3,000	100
2',5'-A_3	25	216	EBV-infected HUCL	1,500	50
none	—	6	PBL	30,100	100
2',5'-3'dA_3	100	6	PBL	20,400	66

[a]DNA synthesis was determined by the method of Krishnan and Baglioni[29].
[b][^{3}H]Thymidine incorporation (10 μCi) into TCA-insoluble material, average of duplicate samples.
[c]Based on control assays, average of 4-7 assays.

1 μM 2',5'-[^{3}H]p_3A_n is reduced to 10%. In contrast, 0-200 μM 2',5'-3'dA_3 core did not compete for the degradation of 1 μM 2',5'-[^{3}H]p_3A_n.

Effect of 2',5'-A_3 core and 2',5'-3'dA_3 core on DNA synthesis. HUCL, PBL and C85-5C lymphoblasts were treated with either 2',5'-A_3 core or 2',5'-3'dA_3 core. DNA synthesis was measured following addition of 2',5'-oligonucleotides to the tissue culture medium (Table 1). 2',5'-A_3 core (25 μM) inhibited DNA synthesis approximately 50% in HUCL and C85-5C lymphoblasts. 2',5'-3'dA_3 core (25 μM) inhibited DNA synthesis 24% in PBL. Protein synthesis was not inhibited by either 2',5'-A_3 core or 2',5'-3'dA_3 core in HUCL, C85-5C lymphoblasts or EBV-infected HUCL (data not shown).

DISCUSSION

Because of the known biological activities of the 2',5'-oligoadenylate molecule in mammalian cells, we determined the metabolism of the 2',5'-A_3 core and the cordycepin analog in lymphocytes and lymphoblasts and the half-life in tissue culture medium. These studies are the first report on the enzymatic synthesis, characterization and

metabolic stability of ^{32}P and ^{3}H labeled 2',5'-adenylate and cordycepin trimer cores in lymphocytes, lymphoblasts, and tissue culture medium. 2',5'-[^{32}P]A_3 core is rapidly hydrolyzed by tissue culture medium ($t_{1/2}$ 3 hr), but the cordycepin trimer core is hydrolyzed much more slowly ($t_{1/2}$ 17 hr). These data agree with earlier reports on the hydrolysis of the 2',5'-adenylate molecule by 2',5'-phosphodiesterase[30,31]. There was no detectable 5'-rephosphorylation of the 2',5'-A_3 core or the 2',5'-3'dA_3 core as determined by ion exchange tlc chromatography of TCA-soluble cytoplasmic extracts of lymphoblasts. Furthermore, by the use of the more sensitive radiobinding assay, supernatants of TCA extracts of lymphocytes treated with 2',5'-A_3 or 2',5'-3'dA_3 cores displaced the pCp analog. The displacement observed could be due to cores at a concentration of 0.35-0.45 μM or to rephosphorylated oligonucleotides at a concentration of 0.35-0.45 nM (Fig. 4). On the basis of this displacement data, the maximum concentration of 5'-rephosphorylated cordycepin trimer possible would be 0.45 nM. This concentration of the rephosphorylated cordycepin trimer analog would not be sufficient to explain the inhibition of protein synthesis by activation of the 2',5'-A dependent endonuclease. We interpret these data to mean that the action of the cores is by a mechanism(s) independent of the interferon/2',5'-oligoadenylate system[5,6,8-16,32]. Evidence to support this hypothesis is our report that 2',5'-A_3 core and the cordycepin core analog inhibit the formation of the EBV-associated nuclear antigen[18].

The rapid hydrolysis of the 2',5'-A_3 core ($t_{1/2}$ 3 hr) in tissue culture medium and the identification of ATP, ADP, AMP and inorganic phosphate in the supernatants of the TCA-treated cells could be explained by the degradation of 2',5'-A_3 core by phosphodiesterases in the medium, and subsequent uptake of adenosine by the cells and conversion to the nucleotides. Similarly the [^{32}P]- or [^{3}H]2',5'-cordycepin trimer core ($t_{1/2}$ 17 hr in tissue culture medium) could be hydrolyzed to cordycepin and resynthesized in the cell to the cordycepin nucleotides. However, when the supernatants from the TCA-treated cells were analyzed for 2',5'-A_3 core and 2',5'-3'dA_3 core by either radiobinding assay or HPLC, 2',5'-A_3 core and 2',5'-3'dA_3 core were detected. It is not clear if this 2',5'-A_3 core and 2',5'-3'dA_3 core is due to binding and/or uptake by the lymphocytes. Of interest is the observation that

Glazer and coworkers that 2',5'-3'dA_3 core is rapidly hydrolyzed to cordycepin in tissue culture medium[33]. Their suggestion is that cordydepin trimer core acts as a prodrug of cordycepin. However, this observation does not appear to apply to lymphocytes. There appear to be several possible explanations for the antimitogenic effect of cordycepin trimer core in different cell systems. In lymphocytes, it is possible that cordycepin trimer core binds to the cell surface and is internalized, is bound to specific receptors and not internalized, or enters the cell without specific receptors. In contrast, Chapekar and Glazer[33] and Eppstein et al.[34] provide evidence that cordycepin and xyloadenosine cores act as prodrugs. Finally, it is known that the 2',5'-phosphodiester bond is markedly different than the 2',5'-phosphodiester bond. The 2',5'-linkage is similar to Z DNA with the sugar phosphate backbone oriented inward towards the bases, whereas in the 3',5'-phosphodiester linkage the sugar phosphate backbone is oriented away from the bases[35,36]. The recent report by Doornbos et al.[37] on the conformational analysis of cordycepin trimer core via CD and NMR provides evidence that base stacking of the 2',5'-oligonucleotides differs from that of the 3',5'-oligonucleotides. Such conformational differences may also explain the mechanism(s) of action of the 2',5'-adenylate and analogs.

Acknowledgements

This research was supported in part by research grants from the National Institutes of Health, GM-26134 (RJS), CA-12227 (EEH) and CA-23999 (EEH). The authors wish to thank Nancy Lee Reichenbach for her technical and editorial assistance.

REFERENCES

1. Pestka, S., ed. (1981) Methods Enzymol. 78A,78B, The Interferons, Academic Press, New York.
2. Torrence, P. F. (1982) Molec. Aspects Med. 5, 129-171.
3. Haugh, M. C., Cayley, P. J., Serafinowska, H. T., Norman, D. G., Reese, C. B., and Kerr, I. M. (1983) Eur. J. Biochem. 132, 77-84.
4. Goswami, B. B., Crea, R., van Boom, J. H., and Sharma, O. K. (1982) J. Biol. Chem. 257, 6867-6870.
5. Williams, B. R. G., and Kerr, I. M. (1978) Nature 278, 88-89.
6. Drocourt, J.-L., Dieffenbach, C. W., Ts'o, P. O. P., Justesen, J., and Thang, M. N. (1982) Nucleic Acids Res. 10, 2163-2174.

7. Baglioni, C., D'Alessandro, S. B., Nilsen, T. W., denHartog, J. A. J., Crea, R., and vanBoom, J. H. (1981) J. Biol. Chem. 256, 3253-3257.

8. Eppstein, D. A., Marsh, Y. V., Schryver, B. B., Larsen, M. A., Barnett, J. W., Verheyden, J. P. H., and Prisbe, E. J. (1982) J. Biol. Chem. 257, 13390-13397.

9. Eppstein, D. A., Barnett, J. W., Marsh, Y. V., Gosselin, G., and Imbach, J.-L. (1983) Nature 302, 723-724.

10. Kimchi, A., Shure, H., and Revel, M. (1979) Nature 282, 849-852.

11. Devash, Y., Biggs, S., and Sela, I. (1982) Science 216, 1415-1416.

12. Bugany, H., Bosch, J. V., Ferdinand, F. J., and Friis, R. R. (1983) Virology 127, 290-298.

13. Jurovčík, M., and Smrt, J. (1981) FEBS Lett. 133, 178-180.

14. Doetsch, P. W., Suhadolnik, R. J., Sawada, Y., Mosca, J. D., Flick, M. B., Reichenbach, N. L., Dang, A. Q., Wu, J. M., Charubala, R., Pfleiderer, W., and Henderson, E. E. (1981) Proc. Natl. Acad. Sci. USA 78, 6699-6703.

15. Suhadolnik, R. J., Wu, J. M., Doetsch, P. W., and Henderson, E. E., patent pending.

16. Leanderson, T., Nordfelth, R., and Lundgren, E. (1982) Biochem. Biophys. Res. Commun. 107, 511-517.

17. Imai, J., Johnston, M. J., and Torrence, P. F. (1982) J. Biol. Chem. 257, 12739-12745.

18. Henderson, E. E., Doetsch, P. W., Charubala, R., Pfleiderer, W., and Suhadolnik, R. J. (1982) Virology 122, 198-201.

19. Cory, J. G., Suhadolnik, R. J., Resnick, B., and Rich, M. A. (1965) Biochim. Biophys. Acta 103, 646-653.

20. Doetsch, P. W., Wu, J. M., Sawada, Y., and Suhadolnik, R. J. (1981) Nature 291, 355-358.

21. Black, P. L., Suhadolnik, R. J., and Henderson, E. E. (1983) Fed. Proc. 42, 678 (abstr.).

22. Reichman, M., Devash, Y., Suhadolnik, R. J. and Sela, I. (1983) Virology 128, 240-244.

23. Willis, D. H., Pfleiderer, W., Charubala, R., and Suhadolnik, R. J. (1983) Fed. Proc. 42, 1833 (abstr.).

24. Suhadolnik, R. J., Devash, Y., Reichenbach, N. L., Flick, M. B., and Wu, J. M. (1983) Biochem. Biophys. Res. Commun. 111, 205-212.

25. Lee, C., and Suhadolnik, R. J. (1983) FEBS Lett. 157, 205-209.

26. Samanta, H., Dougherty, J. P., and Lengyel, P. (1980) J. Biol. Chem. 255, 9807-9813.

27. Charubala, R., and Pfleiderer, W. (1980) Tetrahedron Lett. 21, 4077-4080.

28. Knight, M., Wreschner, D. H., Silverman, R. H., and Kerr, I. M. (1981) Methods Enzymol. 79, 216-227.

29. Krishnan, I., and Baglioni, C. (1980) Proc. Natl. Acad. Sci. USA 77, 6506-6510.

30. Minks, M. A., Benvin, S., Maroney, P. A., and Baglioni, C. (1979) J. Biol. Chem. 254, 5058-5064.

31. Eppstein, D. A., Peterson, T. C., and Samuel, C. E. (1979) Virology 98, 9-19.

32. Eppstein, D. A., Schryver, B. B., Marsh, Y. V., Larsen, M. A., and Kurahara, C. G. (1983) J. Interferon Res., in press.

33. Chapekar, M. S., and Glazer, R. I. (1983) Biochem. Biophys. Res. Commun., in press.

34. Eppstein, D. A., Marsh, Y. V., and Schryver, B. B. (1983) Virology, in press.

35. Kondo, N. S., Holmes, H. M., Stempel, L. M., and Ts'o, P. O. P. (1970) Biochemistry 9, 3479-3498.

36. Parthasarathy, R., Malek, M., and Fridey, S. M. (1982) Proc. Natl. Acad. Sci. USA 79, 7292-7296.

37. Doornbos, J., Charubala, R., Pfleiderer, W., and Altona, C. (1983) Nucleic Acids Res. 11, 4569-4582.

Received July 12, 1983