Studies on cAMP phosphodiesterase activity during infection of vero cells with herpes simplex type 1.

Susan Teresa. Wojtak

University of Windsor

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STUDIES ON CAMP PHOSPHODIESTERASE ACTIVITY
DURING INFECTION OF VERO CELLS WITH
HERPES SIMPLEX TYPE 1

by

Susan Teresa Wojtak

A Thesis
submitted to the
Faculty of Graduate Studies and Research
through the Department of
Biological Sciences in Partial Fulfillment
of the requirements for the Degree
of Master of Science
at the University of Windsor

Windsor, Ontario, Canada

1987
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ISBN 0-315-43782-0
ABSTRACT

STUDIES ON cAMP PHOSPHODIESTERASE ACTIVITY DURING INFECTION OF VERO CELLS WITH HERPES SIMPLEX TYPE 1

by

Susan Teresa Wojtak

The cAMP phosphodiesterase (PDE) activity of VERO cells during infection with herpes simplex type 1 was investigated using a fluorescent assay based on the hydrolysis of 2'-0-anthraniolyl cAMP, a fluorescent analogue of cAMP. At low multiplicities of infection, PDE activity of crude extracts from infected cells was lower than in mock-infected control extracts when examined during the first 4 hours of infection. The activity of mock-infected controls fluctuated within this time interval.

At 4 hours postinfection, extracts from cultures infected with a high virus multiplicity showed higher levels of PDE activity than controls. The antiviral Virazole, at a concentration of 500 ug/mL inhibited the PDE activity changes resulting from low but not by high multiplicities of
infection. Examination of virus production over a 24-hour growth period indicated that Virazole repressed multiplication of HSV-1 only in cells infected with a low inoculum level.

By the fluorescent assay technique, PDE activity was also detectable in a strain of human embryonic lung fibroblasts.
DEDICATION

To my Mum, this odd-looking grandchild.

To my brothers and sisters, thanks for their friendship and support.
ACKNOWLEDGEMENTS

I would like to thank Dr. Leslie R. Sabina, my advisor during the past several years of my undergraduate and graduate studies, for his assistance and for introducing me to the field of virology.

For taking the time to review this manuscript, I would like to thank Dr. D. Thomas of the Department of Biological Sciences and Dr. N. Taylor of the Department of Chemistry and Biochemistry.

The patience of Dr. Nadarajah Karuppiah in explaining the chromatographic techniques used in the experimental work is very much appreciated. My thanks, also, to Dr. M. J. Dufresne for the use of the spectrofluorometer.

I am indebted to Mrs. Dolorès Reid for her superb typing of the manuscript.
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<tr>
<td>AMP</td>
<td>adenosine 5′-monophosphate</td>
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<tr>
<td>ANT</td>
<td>2′-0-anthraniloyl</td>
</tr>
<tr>
<td>CaM</td>
<td>calmodulin</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic 3′:5′-adenosine monophosphate</td>
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<tr>
<td>CPE</td>
<td>cytopathic effect</td>
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<tr>
<td>db-cAMP</td>
<td>dibutyryl-cAMP</td>
</tr>
<tr>
<td>dd</td>
<td>deionized-distilled</td>
</tr>
<tr>
<td>DEAE</td>
<td>diethylaminoethyl</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>(E)MEM</td>
<td>minimum essential medium with Earle's salts</td>
</tr>
<tr>
<td>FCS</td>
<td>heat-inactivated fetal calf serum</td>
</tr>
<tr>
<td>FL</td>
<td>Friend erythroleukemia cells</td>
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<tr>
<td>GMP</td>
<td>guanosine 5′-monophosphate</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>HEL</td>
<td>human embryonic lung cells</td>
</tr>
<tr>
<td>HSV-1</td>
<td>herpes simplex type 1</td>
</tr>
<tr>
<td>HSV-2</td>
<td>herpes simplex type 2</td>
</tr>
<tr>
<td>IMP</td>
<td>inosine 5′-monophosphate</td>
</tr>
<tr>
<td>IUDr</td>
<td>5′-iododeoxyuridine</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
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<td>mRNA</td>
<td>messenger RNA</td>
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xi
<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PDE</td>
<td>cAMP phosphodiesterase</td>
</tr>
<tr>
<td>PFU</td>
<td>plaque-forming units</td>
</tr>
<tr>
<td>PGE</td>
<td>prostaglandin E₂</td>
</tr>
<tr>
<td>p.i.</td>
<td>postinfection</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>TCID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>50% tissue culture infective dose</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>Tric-HCL</td>
<td>Tris (hydroxymethyl) aminomethane hydrochloride</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>VERO</td>
<td>African green monkey kidney cells</td>
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INTRODUCTION

The herpes simplex viruses types 1 and 2 (HSV-1 and HSV-2) are members of the Herpesviridae family of enveloped double-stranded DNA viruses. They have been further classified into the subfamily alpha herpesvirinae whose major characteristics are a rapid spread in cell culture, the efficient destruction of infected cells, the ability to establish latent infections primarily in nerve ganglia, and a shorter reproductive cycle than is present in the remaining two subfamilies, the beta- and gamma herpesvirinae (Roizman and Battserson, 1985).

Infection of cell cultures with HSV-1 and HSV-2 causes marked changes in the pattern of host macromolecular synthesis. Inhibition of the synthesis of host cell DNA, RNA, and protein within the first few hours following infection has been observed in HeLa, Friend erythroleukemia (FL), and VERO cells (Roizman, Borman, and Rousta, 1965; Nishioka and Silverstein, 1977, Fenwick and Walker, 1978). The shutdown of host protein synthesis has been divided into two stages, primary and secondary shutoff, with the latter stage requiring the expression of viral genes (Nishioka and Silverstein, 1978; Read and Frenkel, 1983; Fenwick and McMenamin, 1984; Roizman and Batterson, 1985).

The first stage of inhibition of host protein synthesis is believed to be mediated by a component of the
infecting virus particle; the effects are manifested even under conditions where viral gene expression is prevented. In studies on VERO cells using HSV-2, Fenwick and Walker (1978) found a decline in the rate of protein synthesis within 30 minutes of infection. By two to three hours, most host polypeptides were no longer synthesized while production of new viral polypeptides had begun. The decline in host protein synthesis was also induced by infection with virus irradiated by ultraviolet light although synthesis of new viral polypeptides was not detected.

An early study by Sydiskis and Roizman (1966) showed a correlation between the timing of the decline in protein synthesis in infected HEp-2 cells and the breakdown of cytoplasmic polysomes. Nishioka and Silverstein (1978) proposed the role of the virion-associated component to be the disaggregation of polysomes. Similar to the results of Fenwick and Walker with HSV-2, Nishioka and Silverstein detected a decrease in protein synthesis in FL cells infected with untreated and UV-irradiated HSV-1. Dissociation of polyribosomes to monoribosomes also occurred following infection with either the normal or irradiated virus. No breakdown of polysomes was found, however, after infection with heat-treated virus particles. They concluded that the dissociation of polyribosomes requires a heat-labile virion-associated factor.

The second stage of shutdown, also termed delayed, secondary, or expression-dependent shutoff (Fenwick and McMenamin, 1984) requires the de novo synthesis of viral
proteins and has been characterized by the degradation of cellular messenger RNA (mRNA) (Nishioka and Silverstein, 1977). Nishioka and Silverstein (1978) reported that at four hours following infection, the cytoplasm of infected FL cells contained 50% fewer globin mRNA transcripts than did the cytoplasm of mock-infected control cells. No degradation of mRNA occurred when irradiated virus was used nor when synthesis of viral proteins was prevented by the presence of cycloheximide. As well, an increase in the multiplicity of infection (MOI) from 10 to 100 plaque-forming units (PFU)/cell did not enhance mRNA degradation. They suggested a difference should be evident if a structural component of the virion were responsible.

In contrast to these findings, the work of Schek and Bachenheimer (1985) and Strom and Frenkel (1987) indicated that a virion-associated component appeared to cause mRNA degradation in VERO cells. Loss of actin, histone, and tubulin mRNA was not prevented by treatment of the infected cells with actinomycin D which inhibits viral gene expression. Schek and Bachenheimer also noted that a stepwise increase from 0.2 to 4 PFU/cell caused progressively greater degradation of mRNA. For continued and maximal shutoff at later stages of the infection, expression of viral genes was required in VERO cells (Read and Frenkel, 1983).

The significant loss of host protein synthesis following herpesvirus infection seems to be the result of the disaggregation of polysomes and the degradation of mRNA.
transcripts with the requirement for viral gene expression perhaps being influenced by cell type and MOI.

Development of antivirals effective in repressing reproduction of herpes viruses in vivo has generally focused on nucleoside analogues which inhibit viral DNA replication. A number of these drugs are fairly toxic however and some, such as the thymidine analogue 5'-iododeoxyuridine, become incorporated into host cell DNA (Prusoff et al., 1983).

Virazole (1-B-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), also called Ribavirin, is a water-soluble, stable nucleoside (Sidwell, et al., 1972). It has been reported to have effective antiviral activity in cell culture against a large number of DNA and RNA viruses including cytomegalovirus, HSV-1 and HSV-2, vaccinia, influenza, measles, and vesicular stomatitis virus (VSV) and viruses exposed to it have failed to develop drug resistance (Huffman, et al., 1973).

Studies by Streeter et al. (1973) with measles virus in VERO cells showed that the inhibitory activity of Virazole on measles replication could be substantially reversed by guanosine and xanthosine, and partially by inosine while other nucleosides had no effect. Further investigation indicated that the possible mechanism of action may be through the guanosine monophosphate (GMP) synthetic pathway. Virazole 5'-monophosphate but not Virazole itself was found to be a competitive inhibitor of IMP dehydrogenase which converts inosine monophosphate (IMP) to xanthosine monophosphate, a precursor of GMP. Livers from mice
injected with radiolabelled Virazole indicated the formation of the phosphorylated form of the drug in vivo.

Inhibition of IMP dehydrogenase would reduce the levels of GTP and deoxy-GTP resulting in a decrease in nucleic acid synthesis and GTP-dependent processes such as peptide elongation and translocation reactions. The triphosphorylated form of Virazole can inhibit the capping of viral mRNA by competing with GTP for the viral mRNA guanyl transferase (when present) which transfers the GMP from GTP to acceptor mRNA (Prusoff, et al., 1983).

In in vivo tests, Virazole has prevented tissue damage in eyes and skin of rabbits infected with herpes simplex and has lessened the severity of respiratory infections in mice and ferrets (Sidwell, et al., 1972). It has a relative lack of toxicity in culture for most normal and virus-infected cells, however, the compound must remain in contact with the cells as its removal from the medium reverses its inhibitory effects (Huffman, et al., 1973; Powers, Peavy, and Knight, 1982). Toxicity has been noted with certain cell types, most notably the cells of the immune system. In vitro, Virazole had an antiproliferative effect on murine lymphocytes and interfered with suppressor T cell functions (Powers, Peavy, and Knight, 1982). Work by Potter et al. (1976) on guinea pigs indicated the drug caused an inhibition in the serum antibody response to influenza but lymphocyte proliferation was not repressed. Interestingly, Virazole did slow the growth of transplanted, adenovirus 12 tumours in mice.
Most of the investigations on the association of cyclic 3′:5′ adenosine monophosphate (cAMP) and viruses in cell culture have centred on the oncogenic viruses. These studies have yielded a variety of results, perhaps a consequence of the different viruses and cell lines used.

Latent Epstein-Barr virus (EBV) can be induced by treatment of cells with 5′-iododeoxyuridine (IUdR) (Zimmerman, Glaser, and Rapp, 1973). Treatment of Burkitt lymphoblastoid cell–human cell hybrids with IUdR and dibutyryl-cAMP (db-cAMP) had a synergistic effect. Higher levels of EBV antigens and viral particles resulted than from either compound alone. Db-cAMP is a more membrane-soluble derivative of cAMP and also increases intracellular levels of cAMP (Trofatter and Daniels, 1980; Lehninger, 1981). Addition of cAMP increased EBV antigen levels but not as effectively as the dibutyryl derivative. Tihon and Green (1973) reported the treatment of Chinese hamster ovary cells with 1–2 mM db-cAMP altered the morphology and growth characteristics of the transformed line to that of normal fibroblasts. Db-cAMP also induced the formation of the 7OS RNA, RNA-dependent DNA polymerase, and the 100 nm spherical particles associated with an RNA tumour virus.

Smith, Defendi, and Wigglesworth (1973), however, noted increased transformation of BHK21 cells by polyoma virus in the presence of db-cAMP. Above the optimal level of 0.6 mM, though, transformation was not enhanced. Time of addition was a factor too, with an increase in transformation occurring only if db-cAMP was used while the virus
was stimulating host cell DNA synthesis, a necessary step for the integration of the viral genome into host DNA.

Research by Zimmerman and Raska (1972) showed that the addition of db-cAMP to BHK21 cells prevented the stimulation of host DNA synthesis by the oncogenic virus adenovirus-12 and reduced the synthesis of a virus-encoded antigen. If db-cAMP was added after viral transcription had taken place, less inhibition resulted. They proposed that one of the effects of the adenovirus infection may be the reduction of intracellular cAMP concentration due either to inactivation of adenyl cyclase or to a virus-induced nucleotidase.

With regard to productive infections, large decreases in cAMP levels have been noted in HEp-2 cells and human fibroblasts following HSV-1 infection (Stanwick, Anderson, and Nahmias, 1977; Trofatter and Daniels, 1980). The work of Stanwick et al. indicated that theophylline, an inhibitor of cAMP phosphodiesterase, significantly reduced viral yields and that virus multiplication in lung fibroblasts was also inhibited by db-cAMP. Insulin and db-GMP which lower cAMP levels, increased viral yield. Enhancement or reduction of virus titre by the drugs could only be effected if they were added within three hours of virus adsorption.

Prostaglandins which may exacerbate development of herpetic lesions in vivo, increase levels of cAMP (Trofatter and Daniels; 1980). Studies by Trofatter and Daniels have shown that while the phosphodiesterase inhibitors theophylline and 1-methyl-3-isobutylxanthine hindered the growth and invasiveness of HSV-1 in skin fibroblasts,
compounds which increase cAMP levels, db-cAMP and prostaglandin E$_2$ (PGE$_2$), had no effect. They did reduce the responsiveness of the cells to interferon, though, as did the xanthine derivative. They suggested that PGE$_2$ and other cAMP-elevating reagents could enhance growth of the virus by inhibiting both the action and production of interferon.

An early report was made by Sutherland and Rall in 1958 on the enzymatic hydrolysis of cAMP by a partially purified enzyme from bovine brain, heart, and liver. The enzyme which was activated by magnesium ions and inhibited by caffeine converted the cyclic nucleotide to 5'-AMP. Further studies on this phosphodiesterase by Butcher and Sutherland (1962) determined that it was present in soluble and particulate fractions of tissue homogenates and that theophylline, also, was inhibitory.

Later investigations indicated that many tissues contained more than one type of PDE. Reviews by Wells and Hardman (1977) and Manalan and Klee (1984) list three forms. The first is a cAMP-low affinity or high Km form which is stimulated by the heat-stable activator protein, calmodulin. The activity of the second form is stimulated by low concentrations of cGMP and the third, possibly a membrane-bound form, is responsive to changes in the levels of certain hormones.

Assay procedures for cAMP phosphodiesterase activity generally rely on the use of a radiolabelled substrate which is separated from the enzyme reaction products by anion-exchange chromatography (Wells and Hardman, 1977; Thompson
et al., 1979). Another commonly used method involves the measurement of inorganic phosphate released in a coupled reaction using 5'-nucleotidase in the second step (Butcher, 1974).

This investigation was undertaken to determine
i) if cAMP phosphodiesterase activity could be detected in crude homogenates of cultured cells using a recently developed fluorescent assay,
ii) the consequences of infection with HSV-1 on PDE activity in VERO cells,
iii) the effects of Virazole on the enzyme activity of infected and uninfected cells.
MATERIALS AND METHODS

Chemicals

Isatoic anhydride was purchased from Aldrich Chemical Co., Milwaukee, Wisconsin; calmidazolium, porcine brain calmodulin, and bovine heart cyclic nucleotide phosphodiesterase (calmodulin-deficient) from Boehringer, Mannheim, West Germany; Virazole (1-B-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) from ICN Pharmaceuticals Inc., Cleveland, Ohio; Sephadex LH-20 and DEAE-Sephadex A-25 from Pharmacia, Uppsala, Sweden; adenosine 5'-monophosphate (AMP), adenosine 3':5'-cyclic monophosphate (cAMP), leupeptin, pepstatin, soybean trypsin inhibitor and zirconyl chloride (ZrOCl₂·8H₂O) from Sigma Chemical Co., St. Louis, Missouri.

Cell Cultures

African green monkey kidney cells (VERO), a heteroploid continuous cell line, were supplied by the American Type Culture Collection, Rockville, Md. A diploid line of human embryonic lung cells (HEL) was a gift from D. Millinoff, University of Texas, Galveston. Growth medium used for both cell lines was minimum essential medium containing Earle's salts, (E)MEM, (Gibco Laboratories, Grand
Island, N.Y.) supplemented with 10% fetal calf serum (FCS) (Gibco), 0.105% NaHCO₃, and penicillin and streptomycin (Flow Laboratories, McLean, Va.) at 50 I.U./mL and 50 µg/mL, respectively.

For routine passaging of cells, Corning and Nunc type 75 cm² tissue culture flasks were used. Falcon roller bottles with 850 cm² surface areas were each seeded from either 3 flasks of confluent 5-day VERO cell stocks or 4 flasks of 11-day old HEL cells. At this inoculum level, cell layer confluency was achieved in 2 days by VERO cells and in 4 days by HEL. cAMP phosphodiesterase assays were carried out with 4-day old roller bottle cultures.

Nuclei Counts

Because of the large populations present in roller bottles, detachment of cells from the vessel surface for cell counts leads to extensive clumping making separation and enumeration of cells difficult. Therefore, to estimate cell number, a modification of the method of Parker (1961) for isolating nuclei was used.

After aspirating off growth medium from a 4-day old roller bottle culture, the cell layer was washed twice with phosphate-buffered saline (PBS) then incubated at 37°C for 30 minutes with rotation in an overlay of 25 mL of 0.02% Difco Bacto-crystal violet in 0.4 M citric acid. Cells were then mopped into the dye solution, the suspension divided in half and centrifuged into pellets at 1500 rpm for 15 min in
a Sorvall SPLX angle rotor. Each pellet was resuspended up to a volume of 3 mL in fresh dye solution; portions were diluted further for counting in a hemocytometer. Nuclei were found to be separate and stained faint blue. Typical nuclei counts for VERO cultures were about $1.8 \times 10^8$ and for HEL, $5.2 \times 10^6$ roller bottle.

For tightly sheeted cultures of 75 cm$^2$ flasks, whole cell and nuclei counts gave similar population estimates.

Virus

Herpes simplex virus type I (HSV-1) (Mayo 1814 strain) was donated by Dr. D.A. Kennedy, Laboratory Control Disease Centre, Ottawa. For preparation of virus stock, growth medium was removed from 2-day old roller bottle cultures of VERO cells. Fifteen mL of virus inoculum was added and allowed to adsorb to the cell layer at 37°C and a bottle rotation speed of one-half turn per minute. After a 1 hour adsorption period, i.e. at 1 hour postinfection (p.i.), virus growth medium was added up to 100 mL. The medium consisted of (E)MEM with 0.105% NaHCO$_3$, 2% FCS, penicillin (50 I.U./mL) and streptomycin, (50 ug/mL). For harvesting of the virus, at 18-24 hours p.i., cells were mopped into the medium, dimethyl sulfoxide (DMSO) was added to a final concentration of 0.5%, the cell suspension frozen at -60°C, thawed, and sonicated for 7 minutes in a Sonicor to release intracellular virus. The viral fluid was then centrifuged to remove cell debris and the supernatant stored at -60°C.
Virus titres in terms of 50% tissue culture infective dose (TCID\textsubscript{50}) were determined by the method of Spearman and Karber (Finney, 1964). Titration results were recorded at 72 hr. p.i. of confluent VERO cells grown in 96-well Nunc microtitre plates and are based on an inoculum level of 50 
ulL viral fluid/well.

Virus Growth Curve

Confluent cultures of VERO cells were infected with virus at a multiplicity of 1.75 TCID\textsubscript{50}/cell. After a 1 hour adsorption period, virus inoculum was removed and replaced with an overlay medium of (E)MEM, NaHCO\textsubscript{3}, and antibiotics. For one-half of the cultures, the overlay was supplemented with 500 ug/mL virazole. At various times p.i., duplicate cultures were removed from incubation, frozen, and held at -20°C until harvesting of the virus for titration. The above procedure was also done using a 10-fold greater multiplicity of virus.

Preparation Of Crude Enzyme Extract

Growth medium was aspirated off two 4-day old roller bottle cultures. The cultures were washed twice in 50 mL of cold PBS, mopped into 20 mL PBS and centrifuged at 5000 rpm for 10 minutes in an RC2-B refrigerated centrifuge using an SS-34 rotor. Cell pellets were resuspended in 5 mL PBS, pooled and recentrifuged at 5000 rpm. The cell pellet was
transferred in 2.5 mL PBS to a 3-mL capacity polyallomer centrifuge tube for centrifugation at 6500 rpm for 15 min. The supernatant was carefully poured off and the pellet resuspended gently in 760 μL of a hypotonic homogenizing solution: ice-cold deionized-distilled (dd) H₂O (pH 7 at 27°C) containing 15 mM 2-mercaptoethanol, 2.5 μg/mL leupeptin, 0.75 μg/mL pepstatin and 100 μg/mL soybean trypsin inhibitor. After 10 min incubation on ice, the cell suspension was homogenized at 30% power with 3 pulses, 10 seconds each, using a Janke and Kunkel Ika-Werk ultra-turrax tissumizer with a Tekmar microprobe (model SDT 080EN) and tissumizer control module. Samples of homogenate observed by light microscope were free of whole cells. To stabilize the homogenate, 40 μL of cold buffer containing 20 mM magnesium acetate and 800 mM Tris-HCl (pH 7.5) were then added. Cell debris was pelleted out at 6500 rpm for 15 min and the supernatant (crude enzyme extract) was removed and held on ice until tested for enzymatic activity.

For virus and mock-infected cultures, following the removal of growth medium, 25 mL of virus stock or control medium were added to each of 2 roller bottles. Control medium consisted of virus growth medium supplemented with 0.5% DMSO. After a 1 hour incubation, the inoculum was aspirated off and replaced with 100 mL of overlay medium: (E)MEM with NaHCO₃. At different times, overlay medium was removed, cell layers were washed either once with PBS or twice for cultures not incubated with overlay medium (zero and 1 hour samples). Preparation of crude extract was then
carried out as described previously.

Fluorescent Analogues

Synthesis and purification of 2'-0-anthraniloyl AMP (ANT-AMP) and 2'-0-anthraniloyl cAMP (ANT-cAMP) were done according to the method of Hiratsuka (1982). Purity of the analogues was checked by thin layer chromatography (TLC) using Baker-Flex silica gel plates.

PDE Assay

For the assay of 3':5'-cyclic AMP phosphodiesterase (PDE) activity, the method of Karuppiah and Nutus (1985) was employed. The enzyme reaction mixture contained in a final volume of 200 μL: 20 μL enzyme assay buffer (400 mM Tris-HCl, 10 mM magnesium acetate, 40 mM imidazole, pH 7.5), 10 μL of 1 μg/μL calmodulin, 10 μL of 200 mM CaCl₂ and 150 μL crude enzyme extract. Positive control consisted of 25 μL of Boehringer purified PDE with 125 μL dd H₂O. For the negative control, 150 μL dd H₂O was used unless otherwise noted. The reaction mixture was incubated at 30°C for 5 min prior to the addition of 10 μL of 6 mM ANT-cAMP, for a final substrate concentration of 300 mM. The reaction was stopped after a further 10 min by boiling 2 min. Assay tubes were cooled on ice, centrifuged at 5000 rpm for 15 min and the supernatant was added to 100 μL of 100 mM ZrOCl₂ in 150 mM sodium citrate (pH 7.0) for a 10 min incubation at room
temperature (N. Karuppiah, personal communication). The mixture was layered onto a 0.45 mL resin volume bed (0.9 cm x 0.7 cm) of DEAE-Sephadex A-25 (in a 6 mL-capacity Biorad Econocolumn) equilibrated with a buffer of 10 mM sodium citrate containing 2 mM ZnOCl₂ (pH 7.0). The column bed was washed with equilibrating buffer until the wash fluid was free of fluorescent material (approx. 25 mL of buffer were required).

For collection of eluant, a 20 G needle (Yale) with a tubing-sheathed shaft was fitted over the end of the Econocolumn for insertion into the drop former of the fraction collector (Isco Cygnet). ANT-AMP was eluted with 10 mM sodium citrate containing 2 M NaCl (pH 7.0); 47 drops (approx. 2.5 mL) of eluant were collected. With greater elution volumes (3-5 mL), the product was diluted giving lower fluorescence readings.

Fluorescent activity was measured with a Turner model 430 spectrofluorometer. Excitation and emission wavelengths were 330 and 425 nm, respectively. Two readings were taken for each sample of eluant at both the minimum and 3x sensitivity settings.

Protein Assay

Protein concentrations were determined by the method of Bradford (1976) using the Biorad protein assay dye reagent with bovine serum albumin as the standard.
RESULTS

ANT-AMP Standard Curve

The fluorescent assay for cyclic nucleotide phosphodiesterases developed by Karuppiiah and Mutus (1985) uses a fluorescent analogue of cAMP, 2'-0-anthraniloyl cAMP (ANT-cAMP) as the enzyme substrate. The fluorescent product of the reaction, 2'-0-anthraniloyl AMP (ANT-AMP) can be separated from the substrate using a DEAE-Sephadex anion-exchange column; ANT-AMP binds to the column and the ANT-cAMP (shown in Fig. 1) washes through. The ANT-AMP is then eluted, the fluorescence activity measured and compared with an ANT-AMP standard curve prepared by measuring the fluorescence of known amounts of synthesized ANT-AMP, which were applied to columns in the same manner.

In producing the standard curve shown in Fig. 2, a 4:1 mixture of ANT-cAMP to ANT-AMP was used as described by Karuppiiah and Mutus to mimic the enzymatic reaction. At the maximum concentration used (40 nmol), ANT-cAMP applied alone to the column gave an eluant with zero fluorescence.

The original method used a flow spectrofluorometer to monitor fluorescence as material eluted. For these studies, a fixed volume of 2.5 mL eluant was used with test samples. The standard curve shows linearity using this eluant volume.

Table 1 gives the comparison of the \( R_F \) values for the
Fig. 1. Structural formula of 2'-0-anthraniloyl cAMP. The illustration shows the fluorescent anthraniloyl moiety connected to the ribose sugar of cyclic adenosine 5'-monophosphate. (A) denotes the purine adenine.
Fig. 2. ANT-AMP standard curve. The curve was obtained with a 4:1 mixture of ANT-cAMP and ANT-AMP. This mixture of volume 200 uL was incubated with 100 uL of 100 mM ZrOCl₂ in 150 mM sodium citrate (pH 7.0) prior to application to a DEAE-Sephadex column. Varying amounts of ANT-AMP (0.625-10.0 nmol) were used and were based on ANT-AMP concentrations prepared using a molar absorption coefficient of 20,000 M⁻¹ cm⁻¹ at a wavelength of 252 nm. ANT-AMP was eluted in 47 drops (2.5 mL) of 10 mM sodium citrate containing 2 M NaCl (pH 7.0). Each data point represents the mean of three determinations with different columns. Fluorescence was measured at a 3x sensitivity setting. Excitation and emission wavelengths were 330 nm and 425 nm, respectively.
Fig. 2

FLUORESCENCE (425 nm)

ANT-AMP (nmol)
Table 1. $R_F$ values for ANT-cAMP and ANT-AMP. Values were derived using thin-layer chromatography in a solvent mixture of 1-propanol:NH$_4$OH:H$_2$O (6:3:1).

<table>
<thead>
<tr>
<th></th>
<th>ANT-cAMP</th>
<th>ANT-AMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental value</td>
<td>0.74</td>
<td>0.53</td>
</tr>
<tr>
<td>Published value</td>
<td>0.74</td>
<td>0.52</td>
</tr>
</tbody>
</table>

1 Results of Hiratsuka, (1982).
ANT-CAMP used in the PDE assay and the ANT-AMP used for the standard curve with published $R_f$ values (Hiratsuka, 1982).

**PDE Activity of VERO Cell Crude Extract**

For this particular study, the crude extracts from three pairs of 4-day old VERO roller bottle cultures were pooled. To test their effects on extract enzyme activity, various drugs were added to the enzyme reaction mixture (Table 2). In order to maintain the same assay volume and allow for the use of equivalent aliquots of crude extract, the drugs were substituted for CaCl$_2$ and calmodulin (CaM). Omission of CaM alone (Sample A) resulted in a drop in activity. Activity was further reduced when calcium was also omitted (Sample B). Samples A and B show there is a requirement for calcium and CaM by a component of the reaction.

The drug calmidazolium is an inhibitor of calmodulin-dependent PDE and other CaM-dependent enzymes (Gietzen, Wüthrich, and Bader, 1981; Van Belle, 1981). Addition of calmidazolium did not cause a further reduction in activity at the concentration used (Sample C). This could indicate that a reduction to the basal level activity of a CaM-dependent PDE was achieved effectively by omitting calcium and CaM.

The higher fluorescence readings occurring when a low level of DMSO is present (refer to Samples B, C, D) are probably not due to any direct effect on the fluorescent
Table 2. Effect of calmidazolium, DMSO, and Virazole on PDE activity of VERO cell crude extract.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ca++</th>
<th>CaM</th>
<th>DMSO</th>
<th>Calmidazolium</th>
<th>Virazole</th>
<th>Fluorescent units/150 uL</th>
<th>ANT-AMP (nmol)/150 uL</th>
<th>% of control-a</th>
</tr>
</thead>
<tbody>
<tr>
<td>control-a</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>74.88</td>
<td>4.4</td>
<td>100</td>
</tr>
<tr>
<td>control-b</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>66.47</td>
<td>3.9</td>
<td>88.6</td>
</tr>
<tr>
<td>A</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>55.38</td>
<td>3.2</td>
<td>72.7</td>
</tr>
<tr>
<td>B</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.5%</td>
<td>1 uM</td>
<td>59.63</td>
<td>3.5</td>
<td>79.5</td>
</tr>
<tr>
<td>C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.5%</td>
<td>-</td>
<td>59.13</td>
<td>3.4</td>
<td>77.3</td>
</tr>
<tr>
<td>D</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10.0%</td>
<td>-</td>
<td>44.50</td>
<td>2.6</td>
<td>59.1</td>
</tr>
<tr>
<td>E</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2 mg/mL</td>
<td>61.88</td>
<td>3.6</td>
<td>81.8</td>
</tr>
</tbody>
</table>

1. Ten uL of 200 mM CaCl, and 10 uL of 10 ug/uL calmodulin (CaM) were used as in the standard PDE assay procedure. Where one or both were omitted, they were replaced by 10 uL H2O (A) or 20 uL of drug. Final drug concentrations are shown.

2. Eluant fluorescence readings obtained when 150 uL of crude extract were used in the enzyme reaction mixture.

3. Based on fluorescence readings and derived from the ANT-AMP standard curve.

4. Based on ANT-AMP values.

5. Negative control - the sample was boiled 1 min prior to the addition of substrate and then boiled another minute.

6. Calmidazolium was solubilized in DMSO.
product, ANT-AMP. When a final concentration of 2% DMSO was mixed with 0.625 nmol ANT-AMP, fluorescence was not enhanced. A fluorescence reading of 11.0 was obtained in comparison to the reading of 11.8 for 0.625 nmol ANT-AMP in the standard curve (Fig. 2). Chang and Simon (1968) observed an increase in the activity of some enzymes in vitro in the presence of certain concentrations of DMSO, though other enzymes were inhibited particularly at high DMSO concentrations. Under the conditions used in this study, a final DMSO concentration of 10% resulted in a 12% drop in production of ANT-AMP (refer to Samples B, E).

PDE Activity during the Early Stages of HSV-1 Infection

To determine if a productive infection with HSV-1 would cause detectable changes in PDE activity, the activity in extracts from mock-infected cultures and cultures infected at a low multiplicity of virus were compared (Fig. 3). Cultures were examined during the early stages of infection before there were any visible cytopathic effects (CPE) resulting from viral infection.

Cell growth medium was aspirated off duplicate cultures and 25 mL viral fluid or control medium were added to each. After 1 hour, the inoculum was removed and replaced with 100 mL fresh serumless medium. The medium changes required in the experiment seemed to affect PDE activity in mock-infected controls. This may result from the low level of DMSO present or the serum levels changing from 10% in the cell growth medium to 2% in the inoculum and
Fig. 3. cAMP phosphodiesterase activity in HSV-1 infected and uninfected VERO cells. At the indicated times post-infection, crude extracts were prepared from mock-infected cultures (●) or cultures infected with approximately 2 TCID₅₀/cell (▲). Control PDE activity is the total activity present in cultures harvested at zero time.
then to a complete lack of serum in the final overlay. Changes in serum concentration have been found to cause alterations in PDE activity (Strada and Thompson, 1978).

The initial rise in activity in mock-infected cultures at 1 hour p.i. was not evident in infected samples; a decrease in activity had already begun. The drop to a low point at two hours followed by an increase in activity parallels the pattern seen in the mock-infected controls but the actual activities seem to be much lower.

Although the PDE activity probably returned to normal levels in control cultures somewhere between 4 and 5 hours p.i., the formation of small polykaryocytes began during this interval in infected cells. Further tests were therefore done at 4 hours p.i. when control cultures had activity near that of the zero hour control.

The percentages used to generate the curves shown in Fig. 3 and Fig. 5 were determined using the activities calculated for the total extracts (see Table 3). Extract volumes for samples harvested at different times post-infection were generally larger than the zero hour control sample, especially with extracts from infected cultures. Cell swelling following infection has been noted with the paramyxovirus Sendai which is also capable of inducing cell fusion and formation of polykaryocytes (Bashford, Micklem, and Pasternak, 1985). The swelling is believed to be a consequence of permeability changes in the membrane.

Because of the variations in cell extract volume and the drop in protein concentration in infected samples, it
<table>
<thead>
<tr>
<th>Sample</th>
<th>Fluorescent units (nmol)</th>
<th>ANT-AMP&lt;sup&gt;2&lt;/sup&gt; (nmol)</th>
<th>Total extract volume (μL)</th>
<th>ANT-AMP&lt;sup&gt;3&lt;/sup&gt; total extract % of&lt;sup&gt;4&lt;/sup&gt;</th>
<th>Protein mg/mL</th>
<th>ANT-AMP&lt;sup&gt;5&lt;/sup&gt; (nmol) mg protein % of&lt;sup&gt;5&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>150 μL</td>
<td>150 μL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VERO</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 hr</td>
<td>83.6</td>
<td>4.9</td>
<td>670</td>
<td>21.9</td>
<td>7.6</td>
<td>32.7</td>
</tr>
<tr>
<td>4 hr uninfected</td>
<td>56.5</td>
<td>3.3</td>
<td>820</td>
<td>18.0</td>
<td>8.3</td>
<td>22.0</td>
</tr>
<tr>
<td>4 hr infected</td>
<td>38.6</td>
<td>2.25</td>
<td>870</td>
<td>13.1</td>
<td>6.67</td>
<td>15.0</td>
</tr>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>0 hr</td>
<td>6.3</td>
<td>0.35</td>
<td>690</td>
<td>1.6</td>
<td>0.95</td>
<td>2.3</td>
</tr>
<tr>
<td>Boehringer PDE</td>
<td>(90.0)&lt;sup&gt;6&lt;/sup&gt;</td>
<td>(5.7)</td>
<td>N/A&lt;sup&gt;7&lt;/sup&gt;</td>
<td></td>
<td>7.5</td>
<td>228</td>
</tr>
</tbody>
</table>

<sup>1</sup> Eluant fluorescence readings obtained when 150 μL of crude extract were used in the enzyme reaction mixture.
<sup>2</sup> Based on fluorescence readings and derived from the ANT-AMP standard curve.
<sup>3</sup> Calculated values: ANT-AMP(nmol)/150 μL x total extract (μL).
<sup>4</sup> Based on ANT-AMP(nmol)/total extract.
<sup>5</sup> Based on ANT-AMP(nmol)/mg protein.
<sup>6</sup> Values for Boehringer purified PDE are based on 25 μL of an enzyme solution of 1 U/mL.
<sup>7</sup> Not applicable.
was felt that a better comparison of PDE levels could be made by examining differences based on the calculated activity for the total extract (nmol ANT-AMP/total volume). Although different percentages result when calculations are based on nmol/mg protein, the same trend is evident for the VERO cell line samples (Table 3).

PDE Activity Following Infection with Heat-Inactivated Virus

To determine if a productive virus infection was required to cause the large drop in activity found in infected samples harvested at 4 hours p.i., virus inoculum was heat-inactivated prior to addition to the cell layer. The inoculum was heated at 56°C for 10 minutes (Nishicka and Silverstein, 1978) which caused a drop in titre from $10^{6.5}$ to $10^{2.75}$ TCID$_{50}$/50 uL.

When the same volume of inoculum was used as for the 4-hour infected sample shown in Table 3, no decrease in PDE activity was found (data not shown). In fact, the PDE activity was 10% higher than the zero hour control. The reason for the increase is not known.

Effect of Virazole on PDE Activity

As mentioned in the Introduction, the broad-spectrum antiviral drug Virazole (Fig. 4) inhibits synthesis of viral DNA and RNA. If the expression of viral genes were required for the reduction in PDE activity, then Virazole should
Fig. 4. Structural formula of Virazole (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide).
prevent it. When 500 ug/mL Virazole was added at 1, 2, or 3 hours p.i. to cultures inoculated with a low MOI of virus, the reduction in PDE activity at 4 hours p.i. was not seen (Fig. 5).

To establish that Virazole itself was not directly enhancing PDE activity, the same drug concentration was added to mock-infected cultures at 1 hour p.i. No differences in PDE activity were found between drug-treated and non-treated mock-infected cultures at 4 hours p.i. (Fig. 5). At a 4-fold greater concentration of 2 mg/mL, a slight increase in activity seemed to be present when Virazole was added directly to crude extract (Table 2, refer to Samples B, F).

It was thought that using a higher MOI of virus would cause a much larger decrease in PDE activity, but this was not the case (Fig. 5.) The activity in the total extract from cells infected at a high multiplicity was greater than the zero hour control value. Addition of Virazole had no effect. Whether or not Virazole was present, at 4 hours p.i. cultures inoculated with a high MOI had visible changes in morphology. Separation of individual cells from their neighbours had begun and they had taken on a more rounded appearance. The extract volumes were also not as high in comparison to the zero hour controls as were those from samples infected with lower multiplicities (data not shown).

When the homogenate pellet from a zero hour control sample was resuspended and rehomogenized, the resulting extract contained roughly 40% of the activity present in the initial extract. It is possible that the increase in
Fig. 5. Effect of Virazole addition at various times post-infection. Cultures were inoculated at a multiplicity of approximately 2 (○) or 20 (●) TCID$_{50}$/cell. At 1 hour p.i., virus was removed and replaced with 100 mL overlay medium. Virazole at a final concentration of 500-μg/mL was added at 1, 2 or 3 hours p.i. to cultures infected at low and high multiplicities. Points on the vertical axis, that is, those preceding the broken lines, indicate cultures not treated with drug. (▲) and (■) respectively, denote uninfected cultures not treated or treated with Virazole at 1 hour p.i. All infected and uninfected cultures were incubated until 4 hours p.i. at which time the extracts were harvested and assayed for PDE activity. Values given are percentages of zero hour control PDE activity.
activity resulting from the high MOI could be due to easier release of enzyme from membranes damaged by the physical entry of so many viral particles.

The protein concentration of the inoculums used to obtain a high or low MOI were compared in case concentration differences in protein might somehow be responsible for the different effects noted in PDE activity. The protein concentrations of the virus stock used for the high MOI and the inoculum diluted with virus-growth medium at a low MOI were similar.

The envelopes of the herpes simplex viruses are obtained from cell membrane systems. The possibility existed that the high level of PDE present after a high MOI could be caused by carry-over of particulate enzyme in the virus particles. To investigate this possibility, 150 μL of undiluted viral fluid was used in the enzyme reaction mixture. This volume would contain about 1.7x10^7 TCID₅₀. At a 3x sensitivity setting, a fluorescence reading of 1.75 was observed which corresponds to the formation of about 1.1 nmol ANT-AMP. This particular trial was carried out only once. If the result was valid, then it is conceivable that the number of virus particles present in 25 mL of the viral fluid could contain enough activity to contribute to cellular PDE levels once the virus had entered the cells.

Effect of Virazole on HSV-1 Growth Curves

The effect of Virazole on HSV-1 multiplication was
examined over a 24-hour growth period (Fig. 6). Cells were infected at either 1.75 TCID₅₀/cell (Panel A) or 17.5 TCID₅₀/cell (Panel B).

During the eclipse period following adsorption and penetration of the virus, synthesis of viral components takes place. Assembly of new infectious particles begins between 4 and 8 hours p.i. Panel A shows that the increase in virus titre was linear up to about 12 hours. At all points, virus yield lagged behind in Virazole-treated cultures. By 24 hours, the titre was 100-fold lower in the presence of drug. When cultures were infected at a high multiplicity however, 24-hour titres were close for Virazole-treated and untreated cultures. Studies on Virazole by Sidwell et al. (1973) suggest that the replication of several viruses is more refractory to drug treatment in VERO cells than in other cell lines.

The inability of this concentration of Virazole to repress replication following a high MOI seems to reflect the results found with PDE activity. Virazole inhibited only those alterations in PDE activity caused by a low virus inoculum.

Comparison of Treatment with Virazole and Calmidazolium on HSV-1 Titres

The work by Sidwell et al. showed that high concentrations of Virazole (≥500 ug/mL) caused a decrease in cell viability, particularly after 48 hours. For this
Fig. 6. Effect of Virazole on HSV-1 growth curves. VERO cells were infected at a multiplicity of 1.75 (A) or 17.5 (B) TCID<sub>50</sub>/cell and incubated in the presence (▲) or absence (●) of 500 μg/mL Virazole. Duplicate cultures were removed from incubation at the indicated times and frozen until the virus was harvested for titrating. Titration results were recorded at 72 hours p.i.
Fig. 6

Titre of virus (TCID<sub>50</sub>/50 μL) vs. Time (hours)

A

B

10<sup>0</sup> 10<sup>1</sup> 10<sup>2</sup> 10<sup>3</sup> 10<sup>4</sup> 10<sup>5</sup> 10<sup>6</sup>
experiment, the results of which are shown in Table 4, the 
Virazole concentration was reduced to 250 μg/mL.
Fifty μL aliquots of successive dilutions of virus were 
added to preformed cell monolayers in 96-well microtitre 
plates. After 30 min incubation, 50 μL of control medium or 
medium supplemented with Virazole were added to each well.
The titres were determined at 72 hours p.i. Virus titre was 
reduced in the presence of the drug and no cytotoxicity was 
evident.

Calmidazolium was tested in the same manner at a final 
concentration of 1 μM. It also seems to have had an 
inhibitory effect on virus multiplication. Higher 
concentration of calmidazolium were not examined for their 
effects because a drug level of 2-2.5 μM was toxic to the 
cells by 72 hours. Mild damage by DMSO was seen at a 
concentration of 1.25%.

PDE Activity in HEL Cells

Unlike the heteroploid VERO cell line, the diploid HEL 
cells exhibit contact inhibition, the gradual decline in the 
rate of cell division as cell-to-cell contact is made. 
Cells responsive to contact show large increases in cAMP 
levels as the population becomes stationary (Heidrich and 
Ryan, 1971). Heidrich and Ryan found that PDE activities 
also increased as cells reached confluency.

Table 2 shows the PDE activity in HEL cells examined 
at confluency. Compared with the results for VERO cells,
Table 4. Effect of calmidazolium and Virazole on the titre of HSV-1.  

<table>
<thead>
<tr>
<th>Sample</th>
<th>Titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus control</td>
<td>$10^{5.25}$</td>
</tr>
<tr>
<td>Virus + 0.5% DMSO</td>
<td>$10^{5.25}$</td>
</tr>
<tr>
<td>Virus + 1 μM calmidazolium</td>
<td>$10^{4.75}$</td>
</tr>
<tr>
<td>Virus + 250 μg/mL Virazole</td>
<td>$10^{4.5}$</td>
</tr>
</tbody>
</table>

1. VERO monolayers in 96-well microtitre plates were inoculated with 50 μL viral fluid/well. After 30 min incubation, 50 μL medium containing (E)MEM, NaHCO$_3$, and antibiotics were added to control virus wells. For test samples, medium was supplemented with drug as indicated. Final concentrations of drug are shown. Readings were taken at 72 hr p.i.

2. Calmidazolium was solubilized in a final concentration of 0.5% DMSO.

3. Virazole was solubilized in H$_2$O.
very low levels of PDE were present in the total extract volume as determined by production of ANT-AMP. This is not unexpected in view of the roughly 80-fold smaller cell population. On the basis of nmol ANT-AMP produced per mg protein however, the activity is about half that present in VERO cells.

For comparison purposes, Table 3 also shows the activity achieved by Boehringer calmodulin-dependent PDE under the assay conditions used. It should be noted that the activity of the purified enzyme varied between batches and especially upon storage so other results may differ from that shown. It was useful though to include the commercial preparation as a positive control when running the experiments.
DISCUSSION

Alterations in the activities of several enzymes of cultured cells have been observed following infection with polio (Kovacs, 1958), respiratory syncytial virus (Yabrov, Golubev, and Skordintsev, 1964) and the bovine herpes virus, infection bovine rhinotracheitis (Sabina and Young, 1958). This investigation examined the effects of herpes simplex virus on cAMP phosphodiesterase, one of the regulatory enzymes of cAMP.

Lower levels of cAMP following infection with HSV-1 have been reported and agents which raise cAMP levels or inhibit PDE activity in vitro apparently decrease viral yield (Stanwick, Anderson, and Nahmias, 1977; Trofatter and Daniels, 1980). In the present study, no increase in PDE activity was observed in VERO cells infected with HSV-1 at a low multiplicity. A decrease in PDE activity was already detectable by 1 hour postinfection (Fig. 3). Although the presence of some form of PDE inhibition cannot be ruled out, the timing of the decrease coincides with the rapid shutdown of host protein synthesis resulting from virally-induced polysome disaggregation and mRNA degradation (see Introduction). Also, the decrease in activity present at 4 hours p.i. was not found if cultures were exposed to the antiviral Virazole (Fig. 5). This would suggest that expression of viral genes was required to induce the decrease.

There are several possible explanations for the increase in PDE activity found by 4 hours p.i. in cells
infected at a high virus-to-cell ratio (Fig. 5). Both the first and second explanations describe circumstances where Virazole could have no influence. The third might indicate a situation in which the inoculum level was too high for Virazole to repress gene expression adequately.

The first explanation is a more efficient release of enzyme from heavily damaged cells perhaps with some activity contributed by the membrane-associated PDE described by Appleman and Terasaki (1975) and Kakiuchi et al. (1978).

A second possibility is the transport of enzyme within the virus particles themselves. Very low activity was detected when 150 uL of viral fluid was used in the PDE assay. This could be due to the presence of cellular particulate enzyme in viral envelopes although Roizman and Batterson (1985) state that herpes simplex virions do not contain cellular proteins. Because the viral fluid to be used as inoculum was harvested using only a low-speed centrifugation step, the activity found in the fluid might also be attributable to any particulate enzyme in cellular material not pelleted.

The third highly speculative explanation stems from observations made during the growth curve experiments. Cultures infected with a low multiplicity of HSV-1 formed extensive networks of large polykaryocytes, multinucleate masses formed by the fusion of several to hundreds of cells. With cells infected at a high multiplicity a few small polykaryocytes were formed, but the most prevalent type of cytopathic effect was the separation, rounding and darkening
of individual cells. The beginnings of this latter type of morphological change were already apparent by 4 hours p.i. The two types of CPE's generated by the different multiplicities were similarly described in an early paper by Roizman (1962). It has since been reported that extensive formation of polykaryocytes is caused by spontaneously-arising HSV-1 mutants (Kousoulas, Person, and Holland, 1978; Roizman and Batterson, 1985).

Because high or low virus multiplicities from the same virus stock would cause rounding or fusion, respectively, then both a wild-type virus and fusion-inducing mutant must have been present. It is possible that the induction of a rise in cellular PDE levels is characteristic of wild-type virus but is detectable only when virions are present in high numbers. Cellular changes inducible by the mutants could be favourably expressed when virus numbers are small. This is conceivable in light of evidence that the 37°C used for optimal VERO cell growth in this work is within the optimal temperature range for the molecular events required for cell fusion, but above the optimum for virus growth (Kousoulas, Person, and Holland, 1978).

The assay technique for cAMP phosphodiesterase recently developed by Karuppiah and Mutus and chosen for these studies measures the formation of 2'-0-anthraniloyl AMP (ANT-AMP), a fluorescent analogue of AMP. Separation from the fluorescent substrate, 2'-0-anthraniloyl cAMP is achieved on an anion-exchange column to which, in the appropriate buffer, only the ANT-AMP binds. Although some
of their preliminary studies were done using crude extracts of tissue homogenates, in the majority, a purified calmodulin-dependent form of the enzyme was used.

Changes in enzyme activity seem to be detectable with cellular crude extracts as this present study indicates, but it is recognized that the use of a crude extract may lead to underestimates of total PDE activity. For example, if the column binding mechanism originally proposed by Karuppiah and Mutus is correct, then hydrolysis of the 5'-mono-phosphate by a 5'-nucleotidase would prevent binding of ANT-AMP to the anion-exchange column. Interference by other endogenous factors is an inherent difficulty with the use of crude extracts and has been cited as a problem with other techniques for studying cAMP phosphodiesterase activity (Thompson et al., 1979).
LITERATURE CITED


VITA AUCTORIS

Susan Teresa Wojtak

Born:
April 19, 1961. St. Lawrence, Newfoundland.

Daughter of Winifred and Karol Wojtak.
Sister of Andrew, Michael, Anne and Andrea.

Education:
De Salaberry Elementary School,
Chambly, Quebec.

Essex District High School,
Essex, Ontario.

University of Windsor, Windsor, Ontario.
Honours Bachelor of Science in Biology, 1984.

Awards:
University of Windsor, Entrance Scholarship, 1980-84.
NSERC Summer Research Scholarship, 1983.
NSERC Postgraduate Scholarship, 1984-86.