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STUDIES ON VIRUS-INDUCED CELL FUSION

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ABSTRACT

The cell fusion process has been characterized as regards macromolecular synthesis and post-macromolecular synthesis events. Studies with actinomycin D revealed that the critical period for RNA synthesis associated with cell fusion was 2-4 hr after infection, studies with cycloheximide showed that the critical period for protein synthesis was 3-5.5 hr after infection, and studies with 2-deoxyglucose showed that the critical period for glycosylation was 5-7 hr after infection. NH_4Cl and 2-adamantanone block events that occur subsequently (5.5-8 hr after infection). A temperature-sensitive step in fusion occurs from 4-6 hr after infection. Phosphonoacetic acid, a specific inhibitor of viral DNA synthesis, inhibits fusion only at a low MOI. Even in this case it decreases the rate but not the amount of fusion (if the kinetics curves are extended to sufficiently long times). There is no effect of phosphonoacetic acid at MOIs greater than 10 PFU/cell. Presumably the transcription and translation of 10 viral genomes/cell are required to achieve the maximum rate of fusion. 2-adamantanone partitions efficiently into cellular membranes and increases their fluidity. The effect of 2-adamantanone on cell fusion and on virus growth as well as its effect on the physical state of membranes has been studied extensively during the past year. Although 2-adamantanone does not inactivate mature virions, it does inhibit virus growth almost completely at the same concentration (5 mM). The molecular event sensitive to 2-adamantanone occurs late in viral infection and is at least partially reversible.

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PROGRESS REPORT

We are studying the mechanism of cell fusion induced by fusion-causing mutants of Herpes Simplex Virus Type 1 (HSV-1). We have isolated a number of these mutants which, unlike the wild type strain KOS, cause extensive cell fusion during an otherwise normal infection. These mutants are designated syn because of their ability to cause the formation of syncytia, cells containing many nuclei, during infection. The mutation is not conditional-lethal, but rather should be thought of as a plaque morphology mutation. A year ago the progress report included a summary of our work on the inhibition of cell fusion by ammonium ions, a study of the neutral glycolipids in mock infected, syn mutant infected and wild type infected cells, and a fluorescent enhancement assay of cell fusion. Manuscripts for these studies have been accepted for publication and are included as Appendices 1-3. Appendices 4-6 are other publications related to the research in this progress report and renewal proposal.

A year ago we proposed to study the kinetics of cell fusion using a variety of experimental conditions, to determine the effects of membrane-perturbing molecules such as 2-adamantanone on viral functions involving membranes and on the physical state of the membranes, to examine the fusion process using electron microscopy, and to measure the glycosidase and glycosyl transferase specific activities in mock infected, wild type infected and syn mutant infected cells. Considerable progress has been achieved on the first two projects and the last two have not yet been initiated.

With regard to the kinetics of cell fusion studied under different conditions, we have used a variety of metabolic inhibitors to carefully characterize the fusion process as regards macromolecular synthesis and post-macromolecular synthesis events. In these studies an inhibitor, for example actinomycin D which inhibits viral RNA synthesis, is added to parallel cultures at different times after infection with a syn mutant and is left in the culture thereafter. At later times the monolayers are harvested using a trypsin-EDTA solution and the number of single, or unfused, cells remaining is determined using a Coulter counter. This provides an operational definition of fusion: cells are considered to be fused if they are not separated into single cells by the harvesting procedure. This assay has been described previously and yields an exponential decrease in the number of single cells after the onset of fusion (Person, Knowles, Read, Warner and Bond, J. Virol. 17:183-190, 1976). The slopes of the fusion curves were determined in the presence and absence of a drug and the ratio of the slopes $\times 100$ is called percent fusion. The percent inhibition of fusion is taken as 100 minus the percent fusion.

Fusion kinetics curves are shown in Appendix 7, Fig. 1, for the addition of cycloheximide to infected cultures at 2, 4 and 5.5 hr after virus adsorption, as well as for an untreated control culture. When data such as these are expressed as percent inhibition vs. the time after virus adsorption a curve for the various metabolic inhibitors used is generated (Appendix 7; Fig. 2). Each point on these curves is derived from a ratio of the slopes of fusion kinetics curves for treated and untreated cultures. Data are shown for the inhibitor

of RNA synthesis (actinomycin D), protein synthesis (cycloheximide), and glycosylation (2-deoxyglucose). In addition there seems to be a temperature-sensitive step involved in fusion because a temperature shift to 41°C early in infection is inhibitory. Curves for NH_4^+ ions and the membrane perturber 2-adamantanone are also shown.

In general, each metabolic inhibitor shows a characteristic profile well separated from that of other inhibitors by at least 1-1.5 hr. For actinomycin D cell fusion is completely inhibited if the drug is added from 0-2 hr after infection. If it is added 4 hr after infection the rate of cell fusion is unaffected. Therefore, RNA synthesis required for cell fusion appears to occur between 2-4 hr after infection. Protein synthesis related to cell fusion occurs from about 3-5.5 hr after infection (cycloheximide), and glycosylation essential to cell fusion occurs from 5-7 hr after infection (2-deoxyglucose). NH_4Cl and 2-adamantanone block events that occur subsequent to these (5.5-8 hr after infection). A temperature-sensitive step in fusion occurs from 4-6 hr after infection. The order of events - RNA synthesis, protein synthesis and glycosylation - is in agreement with the observed order of events for the synthesis of glycoproteins or glycolipids.

Phosphonoacetic acid, a specific inhibitor of viral DNA synthesis, inhibits fusion only at a low MOI. Even in this case it decreases the rate but not the amount of fusion (if the kinetics curves are extended to sufficiently long times). There is no effect of phosphonoacetic acid at MOIs greater than 10 PFU per cell. Presumably the transcription and translation of 10 viral genomes per cell are required to achieve the maximum rate of fusion.

The latest time at which an agent causes 100% inhibition of fusion and the earliest time at which it causes 0% inhibition of fusion when added to parallel cultures determine a time interval over which the agent exerts its effect on fusion. If the agent is cycloheximide the time interval represents the interval at which protein synthesis is required for fusion. This is diagrammed below where the horizontal line represents the time after virus infection.

2-adamantanone partitions efficiently into cellular membranes and increases their fluidity. The effect of 2-adamantanone on cell fusion and on virus growth as well as its effect on the physical state of membranes has been studied extensively during the past year. Although 2-adamantanone does not inactivate mature virions, it does inhibit virus growth almost completely at the same concentration (5 mM). The molecular event sensitive to 2-adamantanone occurs late in viral infection and is at least partially reversible, as shown in the figures of Appendix 8. An examination of viral DNA synthesis using equilibrium

sedimentation shows that DNA synthesis is not specifically affected by the membrane perturber. All of the proteins seem to be made in normal amounts but a small effect is observed on the major glycoproteins (analyzed on SDS polyacrylamide gel electrophoresis). Therefore, we think that a step in the translocation of viral protein to the nucleus or a step in virus assembly may be the critical step blocked by 2-adamantanone. An examination of proteins and glycoproteins in nuclei is underway and an examination of the nature of virions accumulating in the nucleus is planned.

Reversibility means that removal of 2-adamantanone late in infection is accompanied by an increase in infectious virus particles (Appendix 8, Fig. 2). Reversibility occurs in the presence of cycloheximide or 2-deoxyglucose, implying that the sensitive step is after protein synthesis and glycosylation of glycoproteins. In preliminary experiments, an inhibitor of chymotrypsin-like protease activity has been found to partially inhibit the reversal. It is known that at least one peptide occurs as a precursor in immature virus particles and that a cleavage product of this peptide is found in mature virions. It is possible that 2-adamantanone blocks this cleavage and thereby prevents the formation of infectious virions. This result has not been confirmed by a study of protein profiles using SDS gel electrophoresis. Studies using the spin label 5N10 show that model membranes and mammalian cell membranes have an increased fluidity in the presence of 2-adamantanone.

PUBLICATIONS (published or accepted for publication):

1. R. W. Knowles and S. Person. 1976. Effects of 2-deoxyglucose, glucosamine, and mannose on cell fusion and the glycoproteins of Herpes Simplex Virus. J. Virol. 18:644-651.
2. W. Snipes, S. Person, G. Keller, W. Taylor and A. Keith. 1977. Inactivation of lipid-containing viruses by long-chain alcohols. Antimicrob. Agents Chemother. 11:98-104.
3. T. C. Holland and S. Person. Ammonium chloride inhibits cell fusion induced by syn mutants of Herpes Simplex Virus Type I. J. Virol., in press.
4. P. M. Keller, S. Person and W. Snipes. A fluorescence enhancement assay of cell fusion. J. Cell Sci., in press.
5. M. A. Ruhlrig and S. Person. Alterations of neutral glycolipids in cells infected with syncytia-producing mutants of Herpes Simplex Virus Type 1. J. Virol., in press.
6. E. C. Pollard, S. Person, M. Rader and D. J. Fluke. Relation of ultraviolet light mutagenesis to a radiation-damage inducible system in Escherichia coli. Radiat. Res., in press.

Manuscripts in preparation:

1. W. Snipes, P. Wanda, G. Keller, S. Person, T. Holland, A. Keith and P. Keller. Effects of butylated hydroxytoluene on Herpes Simplex Virus in vitro.
2. R. W. Knowles, S. Person, G. S. Read, T. C. Holland and P. M. Keller. A correlation between the inhibition of cell fusion and the appearance of surface glycoproteins in herpes simplex virus infected cells.
3. G. S. Read, S. Person and P. M. Keller. A genetic analysis of cell fusion by herpes simplex virus type 1.
4. S. Person, K. G. Kousoulas and T. C. Holland. Effects of metabolic inhibitors on cell fusion induced by syn mutants of herpes simplex virus type 1.
5. S. Person. Consequences of tritium decay: Transmutation effects for mutagenesis, DNA crosslinks and DNA single strand breaks.

Papers presented at meetings:

1. Knowles, R. W., S. Person, G. S. Read, T. Holland & P. Keller. A Correlation between the Inhibition of Cell Fusion and the Appearance of Surface Glycoproteins in HSV-1 Infected Cells. Herpes Virus Meetings at Cold Spring Harbor, NY, September, 1976.
2. Person, S. Cell Fusion Induced by Syncytia-Producing Mutants of Herpes Simplex Virus Type I. Seminar at National Heart & Lung Institute, NIH, June, 1976.
3. Read, G. S. & S. Person. The Genetics of Cell Fusion Produced by Syncytial Mutants of Herpes Simplex Virus Type I. Genetics Symposium, The Pennsylvania State University, June, 1976.
4. Read, G. S. & S. Person. The Genetics of Cell Fusion Produced by Syncytial Mutants of Herpes Simplex Virus Type I. Cold Spring Harbor, NY, September, 1976.
5. Keller, P., S. Person & W. Snipes. A Fluorescence Enhancement Assay of Cell Fusion. Biophysical Society Meeting, New Orleans, February, 1977.
6. Wanda, P., T. Holland, P. Keller, S. Person, A. Keith & W. Snipes. Effects of Butylated Hydroxytoluene (BHT) on Herpes Simplex Virus. Biophysical Society, February, 1977.
7. Murphy, R. & S. Person. Is Herpes Simplex Virus-Induced Cell Fusion Caused by Activation of Cell Genes? Undergraduate Meetings, Fairleigh-Dickinson U., April, 1977.
8. Holland, T., S. Person, W. Snipes & A. Keith. Inhibition of Herpes Simplex Virus Growth by 2-Adamantanone. Am. Soc. Microbiologists, New Orleans, May, 1977.
9. Kousoulas, G., S. Person, T. Holland & R. Knowles. The Influence of Inhibitors of Cell Fusion Produced by a Syncytial Mutant of Herpes Simplex Virus Type I. Third Internatl. Meeting (Symposium) Oncogenesis and Herpesviruses, Boston, July, 1977.
10. Pollard, E. C., S. Person, M. Rader & D. J. Fluke. Relation of Ultra-violet Light Mutagenesis to a Radiation-Damage Inducible System in Escherichia coli. Photobiology Symposium, Radiation Research Society, Puerto Rico, May, 1977.

PERSONNEL ON THE PROJECT:

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 T. Holland (NIH Trainee)
 G. Kousoulas (ERDA 6mos.)
 G. S. Read (NIH Trainee)
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 V. C. Bond (ERDA 12 mos.)

Research Aide:

S. Warner (ERDA 10 mos.)

Undergraduate Student:

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MECHANISM OF VIRUS-INDUCED CELL FUSION

(Supported by ERDA Grant E(11-1)3419 and PHS Grant AI-11513)

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Research Aide, S. Warner

Undergraduate Student, R. Murphy

Membrane fusion occurs in a number of endo- and exocytotic processes such as secretion, phagocytosis, and neural transmitter release. Muscle and bone cell development, fertilization, and lysosomal activity also involve membrane fusion. In addition to these examples of membrane fusion, virus-induced cell fusion is caused by a wide variety of enveloped animal viruses. In order to study the molecular mechanism of membrane fusion, our laboratory is investigating the process of cell fusion which occurs during a productive viral infection with mutants of Herpes Simplex Virus, type 1 (HSV-1), that cause cells to fuse. We have isolated eight syncytia-producing (syn) mutants from a strain of HSV-1 (KOS). Unlike the wild type strain, the mutants cause extensive cell fusion in an otherwise normal infection. Although fusion caused by syn mutants is extensive, we have found that a small but detectable and reproducible amount of fusion occurs during wild type infection. Fusion starts in the wild type infection and stops shortly thereafter. Using SDS gel electrophoresis we have noted an inverse correlation between the amount of cell fusion produced by syn mutants, and the amount of two HSV-1 glycoproteins detected on the cell surface by ^{125}I -lactoperoxidase labeling. One model to explain these data and others is that a fusion factor exists in wild type infected cells but very little fusion occurs because of the early appearance of glycoprotein inhibitors. The syn mutation, which operationally results in extensive cell fusion, may be an alteration in fusion factor, an inhibitor glycoprotein, or in a molecule that causes a change in either or both of the activities.

GENETIC ANALYSIS OF CELL FUSION INDUCED BY SYNCYTIA-PRODUCING MUTANTS OF HERPES SIMPLEX VIRUS TYPE 1: G. S. Read.

Although wild type virus does not produce syncytial plaques when plated on human embryonic lung cells, it does induce a limited amount of cell fusion which begins 5-6 hrs after infection and stops abruptly after 20% of the cells have fused. Fusion induced by the syn mutants begins at the same time as for wild type and is characterized by an exponential decrease in the number of unfused cells. For four of the mutants this decrease continues until at least 85% of the cells have fused; the other mutants are characterized by a sudden decrease in the rate of fusion when 40-60% of the cells still remain unfused. Studies of the kinetics of fusion of cells mixedly infected with a syn mutant and wild type indicate that for seven of the mutants the fusion phenotype is recessive. Results from similar experiments using cells mixedly infected with two syn mutants indicate that none of the mutants complement each other. Related studies show that syn mutant-infected cells fuse well with each other and with uninfected cells, but not with wild type-infected cells. Recombination experiments with six of the syn mutants show that the mutations are clustered (recombination frequencies range from 0.2%-1.6%).

A CORRELATION BETWEEN THE INHIBITION OF CELL FUSION AND THE APPEARANCE OF SURFACE GLYCOPROTEINS IN HSV-1 INFECTED CELLS: R. W. Knowles, S. Person.

Two cell surface proteins, labeled with ^{125}I during viral infection, co-migrate on SDS-polyacrylamide gels with two viral glycoproteins that are synthesized after virus infection. The kinetics of appearance of these glycoproteins on the cell surface was compared with the kinetics of cell fusion produced by several cell fusion-producing mutants of HSV-1. For all of the mutants, as well as the wild type strain from which the mutants were derived, fusion starts 5-6 hrs after infection and proceeds at similar rates. The mutants are differentiated from each other and from the wild type by the time at which the rate of fusion abruptly decreases. The appearance of the two glycoproteins was correlated with the sudden decrease in the rate of fusion. One explanation is that these glycoproteins are inhibitors of cell fusion.

CHARACTERIZATION OF THE KINETICS OF CELL FUSION WITH A SYNCYTIA-PRODUCING MUTANT OF HSV-1 FOR A VARIETY OF EXPERIMENTAL CONDITIONS: G. Kousoulas, S. Person.

The dependence of virus-induced cell fusion on protein, RNA and DNA synthesis and on temperature was determined. We have found that cell fusion is completely inhibited if protein synthesis is blocked by the addition of cycloheximide up to 4 hrs after virus infection. Some inhibition of fusion is observed when the drug is added $4\frac{1}{2}$ hrs after infection. Cell fusion is inhibited by the addition of actinomycin D at 0 or 3 hrs post-infection, but is completely normal for addition at 4 hrs post-infection. Cell fusion is not inhibited by the DNA synthesis inhibitor, phosphonoacetic acid, added 0 hrs post-infection. Fusion can also be inhibited by shifting the incubation temperature from 34°C to 41°C 2 hrs after infection, but normal fusion was observed when the shift was at 4 hrs post-infection.

FLUORESCENCE ENHANCEMENT ASSAY OF CELL FUSION: P. Keller.

Cell fusion induced by a syncytia-producing mutant of Herpes Simplex Virus type I was monitored by measuring the resonance energy transfer between two probes which consisted of fluorescent molecules conjugated to saturated hydrocarbon chains to insure their localization into cellular membranes. Resonance energy transfer requires that the two probes be close together and that there be an overlap of the emission spectrum of the donor probe and the absorption spectrum of the acceptor probe. At appropriate wavelengths resonance energy transfer can be measured by preferentially exciting the donor probe and observing the fluorescence of the acceptor probe. Two cell populations, each labeled with one of the probes, were infected with virus, mixed in equal proportions, and the fluorescence of the acceptor probe measured as a function of time after infection. An increase in fluorescence was observed beginning at the time of onset of cell fusion. An investigation of the distance dependence indicated that the increase in fluorescence was mainly due to resonance energy transfer and not to photon emission and reabsorption. The possible application of this assay to other types of membrane fusion was noted.

GLYCOLIPIDS IN CELLS INFECTED WITH SYNCYTIA-PRODUCING MUTANTS OR WILD TYPE HSV-1: M. Ruhlig, S. Person.

Neutral sphingoglycolipids (glycolipids) were studied in human embryonic lung cells that were either mock infected, infected with a wild type strain of HSV-1 or with one of a number of syncytia-producing (syn) mutants derived

from the wild type strain. Infected cells were labeled with radioactive galactose after infection, the extracted glycolipids were separated using thin layer chromatography, and the resulting autoradiograms compared. Glycolipids from wild type infected cells contained shorter oligosaccharide chains than did uninfected cells, and a small but definite shift to longer oligosaccharide chains was observed for most syn mutant infected cells relative to wild type infected cells.

HERPES SIMPLEX VIRUS AND MEMBRANE PERTURBERS: T. Holland.

During the infectious cycle of HSV-1 a number of membrane-related events take place: virus penetration, synthesis and intracellular transport of membrane-bound proteins, and the envelopment and release of the progeny virions at the inner nuclear membrane. In addition, syn mutants of HSV-1 have been derived which cause fusion of the plasma membranes of adjacent cells, leading to multinucleated cells. A number of membrane-perturbing drugs have been shown to disrupt membrane-related processes in various systems. In this laboratory, it has been shown that several derivatives of adamantane, a small, spherical, hydrophobic molecule, are able to inhibit the fusion of cells produced by the fusion-causing mutants of HSV-1. Adamantanone, one of the derivatives which blocks fusion, has also been found to inhibit virus growth. This inhibition is reversible and appears to occur at a late stage of the infectious process. A working hypothesis is that adamantanone blocks envelopment of the capsid at the inner nuclear membrane.

IS VIRUS-INDUCED CELL FUSION CAUSED BY VIRUS ACTIVATION OF CELL GENES?

R. Murphy, S. Person.

Since human muscle cells fuse during embryogenesis all human cells possess the genes for cell fusion. Therefore, virus-induced cell fusion may result from virus-induced activation of host cell fusion genes. We are testing this possibility by the selective inactivation of the host genome so that host DNA can no longer be used for transcription. This is being done by incorporation of 5-bromodeoxyuridine (BUdR) into cellular DNA and exposing the BUdR-containing cells to 313 nm light. Light of this wavelength causes strand breaks in the DNA at the site of the incorporated BUdR. Treated cells will be infected with cell fusion-causing mutants of HSV-1 and the kinetics of cell fusion determined by measuring the decrease in the number of single cells as a function of time after infection. Studies on the amounts of BUdR and 313 nm light required have been completed and studies on cell fusion will begin soon.

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APPENDIX 7

The Effect of Metabolic Inhibitors on Cell Fusion

In order to understand the molecular mechanisms of membrane fusion we are investigating the process of cell fusion that occurs during the infection of cells with syncytia-producing mutants of HSV-1. In the present study we have characterized macromolecular synthesis and other requirements for fusion with the use of metabolic inhibitors. Human embryonic lung (HEL) cells were infected with a mutant of HSV-1, syn 20, that causes extensive syncytia formation during an otherwise normal infection. To measure fusion, cells were harvested (trypsin-EDTA solution) from a monolayer and counted using a Coulter counter. A size (threshold) interval was used so that only small, single cells were counted. Cells are considered to be fused if they are not separated into small, single cells by the harvesting procedure. After the onset of fusion, the number of unfused cells decreases exponentially with time. The slope of the curve of cells remaining unfused vs. time after infection is a measure of the rate of cell fusion. When metabolic inhibitors were present they were added to the infected cultures at the times indicated and left in the cultures thereafter.

Cycloheximide (0.36 mM), an inhibitor of protein synthesis, was added to parallel cultures at 2, 4 and 5.5 hours after infection. Cultures were harvested and assayed for fusion as indicated. Untreated control cultures were also harvested and the extent of fusion assayed. Data are shown in Fig. 1. The time dependence of the inhibition of fusion is shown in Fig. 2. Percent inhibition is defined as $100\% - \% \text{ fusion}$. Percent fusion is defined as the ratio of the slopes of the treated to untreated fusion kinetics curves multiplied by 100.

The time dependence for the various inhibitors is in the order expected, but some points are worth noting.

- (1) The temperature-sensitive step occurs either late in protein synthesis or early in glycosylation.
- (2) NH_4^+ and the membrane perturber 2-adamantanone act after macromolecular synthesis and glycosylation. Other studies show that the NH_4^+ block probably precedes the 2-adamantanone block. NH_4Cl does not affect protein or glycoprotein synthesis. Perhaps NH_4Cl influences the transport and/or incorporation of glycoproteins into plasma membranes. Presumably 2-adamantanone acts by directly influencing fluidity or structure.
- (3) The metabolic inhibitors - cycloheximide, NH_4Cl , 2-adamantanone and probably high temperature - act reversibly. Actinomycin D and 2dG act irreversibly. PAA was not tested.
- (4) The glycosylation-sensitive step occurs $1\frac{1}{2}$ hr after protein synthesis, a longer time interval than expected. This and other data indicate that the syn mutation may cause a delay in the processing of glycosylated molecules.

App 7, Fig 1.

App. 7., Fig 2

APPENDIX 8

Effects of 2-Adamantanone on Virus Growth

2-adamantanone is a compound that partitions efficiently into cellular membranes and increases membrane fluidity (data not shown). Figure 1 shows the effect of 2-adamantanone at 5 mM on virus growth when added 1, 8, 12 and 16 hr after infection. HEL cells were infected with wild type virus at a multiplicity of infection of 10 plaque-forming units per cell. Virus was released from infected cells by freeze-thawing at the indicated times. The virus titers were determined by a plaque assay on monolayers of HEL cells. If 2-adamantanone is added even at 12 hr after infection the virus titers decreased by 80%.

The reversibility of the lethal effect of 2-adamantanone is indicated in Fig. 2. In these experiments the membrane perturber originally present at 5 mM was removed from cultures at the times indicated. Cells were infected as for Fig. 1 and 2-adamantanone was added to all cultures, including the ones marked control, at 1 hr after infection. At 23 hr after infection cycloheximide (0.36 mM) was added to one set of cultures and 2-deoxyglucose (10 mM) to another set. One hour later the medium was changed so that cycloheximide or 2-deoxyglucose, but not 2-adamantanone, was present. At the indicated times thereafter viruses were harvested and titered as indicated above. The virus titer increases rapidly and dramatically upon removal of 2-adamantanone in the presence or absence of cycloheximide or 2-deoxyglucose. The inhibitory effect of 2-adamantanone on viral growth is to some extent reversible late in virus infection. The reversibility does not require subsequent protein synthesis or glycosylation of glycoproteins or glycolipids. This is one of the major evidences that leads us to conclude that 2-adamantanone may affect virus assembly.

Fig 1

App. B, Fig 1