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A Progress Report

to the

U. S. Department of Energy

STUDIES ON VIRUS-INDUCED CELL FUSION

Period covered: August 1, 1977-
June 30, 1978

MASTER

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July, 1978

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ABSTRACT

We have previously postulated that wild-type Herpes Simplex Virus type I (HSV-1) infections are characterized by the presence of a fusion factor and a fusion inhibitor activity. The fusion inhibitor presumably is dominant so that a small fraction of cells fuse in a typical wild-type infection. Furthermore, the *syn* mutants isolated in our laboratory are thought to cause extensive cell fusion because the production of active fusion inhibitor in cell membranes is delayed. If mutations existed that altered both the fusion factor and fusion inhibitor activity then separate viruses containing these two mutations might be able to complement each other, each supplying the defective gene product missing in the other virus. This would produce a wild type and not a syncytial mutant response. Complementation tests between two viruses, tsB5 and *syn* 20, which are thought to contain defects in the production of active fusion factor and fusion inhibitor activity, respectively, were done. A wild-type response was observed indicating that the mutations affecting fusion were in two separate genes. The fusion capacity of *syn* 20-infected cells was found to decrease late in infection, a finding consistent with the defect being due to a delay in the appearance of active fusion inhibitor in cell membranes. An assay to measure and characterize the fusion inhibitor has been developed. The block in virus growth by the membrane perturber adamantanone has been extensively studied. The effect may be an action on the fusion factor or fusion inhibitor glycoprotein. Our current working hypothesis is that adamantanone causes the virion to incorporate incorrect glycoproteins when it buds through the inner nuclear membrane, or that one or more of these glycoproteins cannot be processed in the presence of adamantanone.

PROGRESS REPORT

We are studying the mechanism of cell fusion induced by fusion-causing mutants of Herpes Simplex Virus type I (HSV-1). We have isolated a number of these mutants which, unlike the wild-type strain from which they are derived (KOS), cause extensive cell fusion during an otherwise normal infection. These mutants are called syn mutants because of their ability to form syncytia, cells containing many nuclei, due to cell fusion. Because the syncytia result in plaques that are distinctly different from those produced by wild-type virus, the mutants were first identified as plaque morphology mutants. Using such mutants, our goal has been to genetically and biochemically dissect the fusion process in HSV-1 infected cells.

A year ago the progress report included a summary of work on the timing of some of the molecular events required for cell fusion. Viral DNA, RNA, protein synthesis and the glycosylation of glycoproteins were inhibited at various times after infection. The resulting effect on cell fusion was determined. It was found, for example, that protein synthesis was not required for cell fusion after $5\frac{1}{2}$ hr after virus infection. This is the time of onset of fusion. This research has been accepted for publication and is included as Appendix 1. Reprints of work in press or manuscripts submitted for publication a year ago are included as appendices:

Appendix 2 - Ammonium chloride inhibits cell fusion
Appendix 3 - A study of neutral glycolipids of mock-infected, syn mutant infected and wild type infected cells
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This year we proposed to (1) determine the fusion capacity of syn mutant-infected cells, (2) characterize fusion inhibition in wild-type infected cells, (3) examine the fusion process using electron miscroscopy, and (4) identify the step in virus growth that is blocked by adamantanone. Substantial progress has been made on the determination of the fusion capacity of syn mutant cells and experiments have been begun on the characterization of fusion inhibition. The blockage of virus growth by 2-adamantanone has been extensively studied. All of these studies are expected to be completed by the end of the current granting period. Examination of the fusion process by electron microscopy, although an important project, has not been initiated as of this time. Because of recent developments in Dr. Patricia Spear's laboratory (U. Chicago), we initiated and completed a study on genetic complementation of the syn mutants isolated in our laboratory with a mutant isolated by Dr. A. Buchan (U. Birmingham). A discussion of this research follows.

We have noted previously that wild-type infections appear to be characterized by both a fusion factor and a fusion inhibitor activity. The action of the inhibitor is evidently dominant so that only a small fraction of cells fuse. Furthermore, we hypothesized that the syn mutants isolated in our laboratory contained a defect in the expression of fusion inhibitor activity (see Grant Proposal). Manservigi, Spear and Buchan (PNAS 74:3913-3917, 1977) reported that a mutant, tsB5, derived from another strain of HSV-1, was temperature-sensitive for the production of fusion factor. It seemed worthwhile to determine if these mutants, one presumed to contain a defect in fusion inhibitor production, and the other presumed to contain a defect in the fusion factor production, would complement each other. A positive result of a complementation test was considered a good possibility because each virus may be able to supply the gene function missing in the other virus. If this occurred a wild-type fusion

response would be observed.

Cell fusion was assayed using the Coulter counter assay which measures the number of unfused single cells. As already known, one of the mutants isolated in our laboratory, syn 20, produced extensive fusion at 34° and 38°C. tsB5 produced extensive fusion of human embryonic lung (HEL) cells at 34° but not at 38°C. Since tsB5 causes extensive fusion of HEL cells at 34°C, in contrast to the parent strain from which it was derived, the temperature-sensitive mutation in tsB5 presumably produces an altered fusion factor that can cause fusion even in the presence of an unaltered fusion inhibitor. Therefore, we postulated that tsB5 would cause fusion in mixed infections with wild-type virus at 34°C, unlike syn 20. Fusion was not expected at 38°C because the temperature-sensitive mutation results in defective fusion factor at that temperature. When HEL cells were simultaneously infected with wild-type and tsB5 virus these predictions were confirmed.

In order to test for complementation between two,mutants, both must be recessive in mixed infections with wild-type virus. Although tsB5 does not meet this condition at 34 it does at 38°C. Therefore a satisfactory test for complementation between tsB5 and syn 20 would be to examine the kinetics of fusion at 38°C following a mixed infection of HEL cells. These experiments were done and fusion curves typical of wild-type virus, and not of mutant viruses, were observed. Other syn mutants isolated in our laboratory, syn 8, 30, 31, 32 and 33, were also tested for complementation with tsB5. Although fusion was only scored qualitatively in these latter experiments, in all cases the mixed infections with tsB5 produced a wild-type response. Apparently the defects in the syn mutants and in tsB5 are in different genes and are able to complement each other. This provides genetic evidence for the existence of two viral gene products that affect cell fusion, and is in agreement with the

biochemical evidence provided by Manservigi, Spear and Buchan. It is hoped taht future experiments will confirm that the two genes are ones affecting the production of active fusion inhibitor and active fusiȯn factor.

The purpose of experiments on the fusion capacity of syn mutant-infected cells is to determine if fusion capacity decreases late in infection. This may be the case because we suspect that fusion inhibitor production is delayed, but not permanently turned off, in syn mutant infections. Furthermore, we suspect that the delay is different for each of the syn mutants. The fusion capacity experiments would provide an independent test of these ideas.

In order to determine the fusion capacity of syn mutant infected cells, cells at a low cell density are infected with a particular syn mutant. The cells do not fuse during incubation under this condition because the cell-cell separation distances are too great. At various times after infection an excess of uninfected indicator cells is added so that fusion can be scored subsequently between infected and uninfected cells. Cycloheximide is added along with the indicator cells to minimize changes in the plasma membranes after the addition of the indicator cells. We have found that cycloheximide does not interfere with the attachment of uninfected cells, nor does it prevent the fusion of syn mutant cells with the indicator cells. The extent of fusion is determined after a prolonged incubation of sparse cells with the indicator cells in order to allow all possible fusion events to occur. Infected cells are specifically marked by prior labeling of these cells with ^{3}H-thymidine and preparing radioautographs prior to scoring for fusion. The fusion of cells containing labeled nuclei in the cells containing unlabeled nuclei was determined as a function of time after infection (time of addition of cycloheximide and indicator cells). Typical data are shown here for cells infected with syn 20 virus (Fig. 1). It is clear that the capacity of the infected cells to cause fusion (decrease

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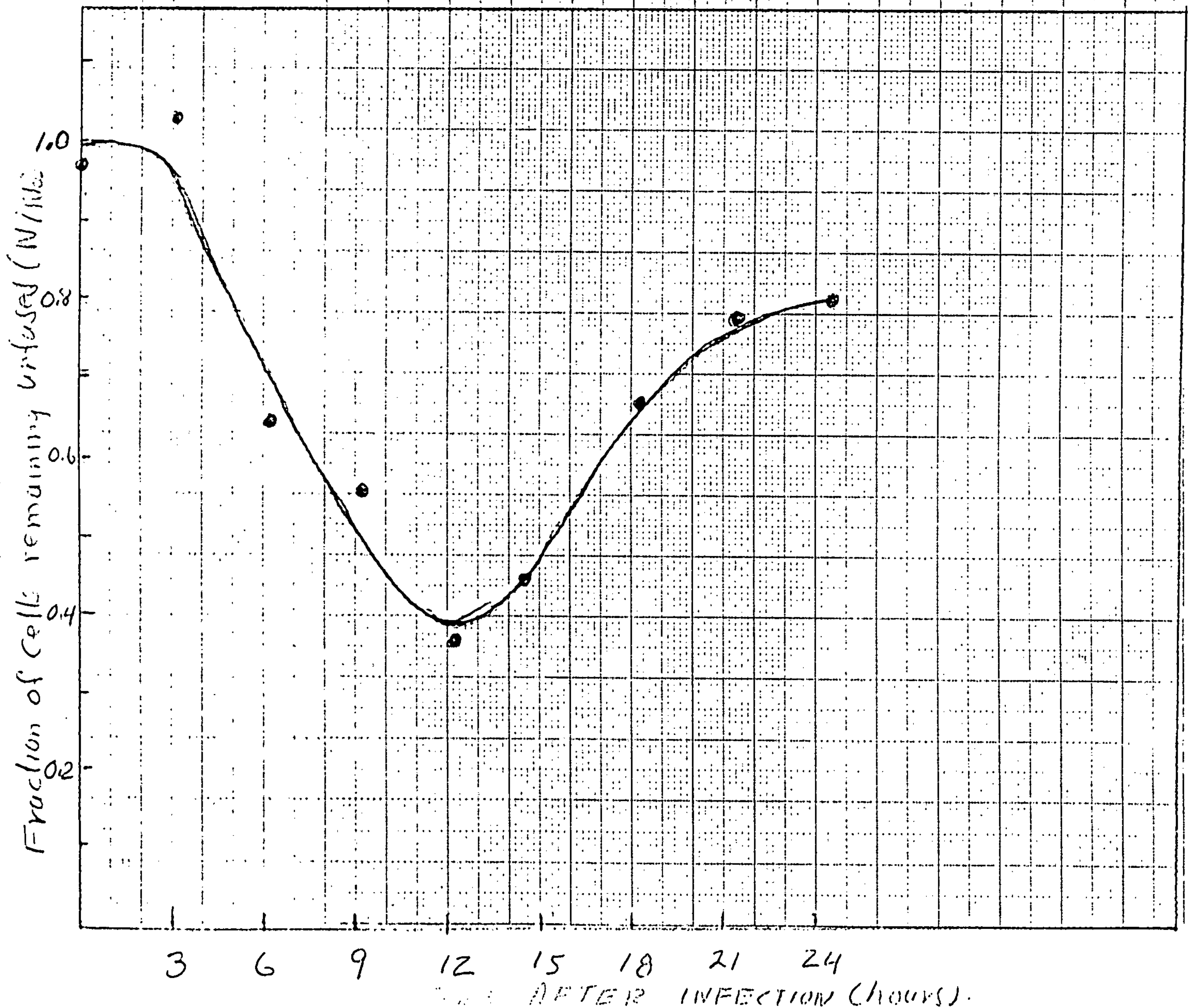
Fusion capacity of *syn* 20 infected cells at various times after infection. At the indicated times an excess of uninfected cells were added to sparse *syn* 20 infected cells. Cycloheximide was added at the same time as the uninfected cells. Cultures were incubated overnight in the presence of cycloheximide and then scored for fusion (see text).



in the number of infected cells that are not fused, N/N_o) increases after the onset of fusion, reaches a maximum at about 12 hrs after infection, and then decreases rapidly later in infection. These data are consistent with the data from fusion kinetics curves which show an onset of fusion at 5½ hrs after infection followed by a marked decrease in the rate of fusion beginning at 12 hrs after infection. Since the Coulter counter assay of fusion kinetics depends on harvesting cells without rupturing syncytia, the current experiments are a much more valid indicator of fusion capacity since the scoring is done using a microscope so that the cells are not harvested. We are currently characterizing various syn mutants for their fusion capacity throughout infection using this technique.

In a similar way the fusion of sparse syn 20-infected cells incubated to produce maximum fusion capacity (12 hrs) can be assayed when the excess indicator cells have been infected with wild-type virus. The variable in these experiments will be the time of incubation of the wild-type virus-infected cells prior to their addition to the syn mutant-infected cells. In these experiments similar results are obtained if sparse syn mutant cells are added to the wild-type infected cells or vice versa, but the addition of the syn mutant cells is preferred. In this protocol the harvesting procedure is not applied to the wild-type infected cells, which are the cells of interest. The purpose of these experiments is to determine when active fusion inhibitor is present at the cell surface of the wild-type infected cells. When it appears it will decrease the extent of fusion of syn mutant-infected cells with wild-type infected. Earlier experiments have already established that syn mutant cells do not fuse extensively with wild-type infected cells.

In addition to measuring the time of appearance of fusion inhibitor on the cell surface of wild-type infected cells, the nature of the fusion inhibitor can also be studied. For example, cycloheximide can be added to wild-type infected cells at various times to determine the time course of synthesis of fusion inhibitor. Finally, the indicator cells can be other syn mutant-infected cells so that one can measure the time of appearance of fusion inhibitor on the surface of these cells. Preliminary experiments using wild-type infected cells show that full inhibition is expressed by 8 hrs after infection. Experiments on the nature of the inhibitor molecule are just beginning.

The growth of the KOS strain of HSV-1 in inhibited by adamantanone and by adamantaneacetic acid. Although the latter is the more potent inhibitor, the former has been more extensively studied. Both compounds are thought to act by perturbing cellular membranes.

It has been determined that adamantanone acts at a late point in the virus life cycle. It inhibits further virus growth even when added quite late in infection, its effect beginning immediately after its addition to the infected cells. Its effect is partially reversible when it is removed from the culture at 24 hrs after infection, which is the peak of virus growth in untreated cells. This reversibility is not blocked by cycloheximide or 2-deoxyglucose. Therefore, the adamantanone-sensitive step does not require protein synthesis or glycosylation. Since it is also partially reversible late in infection and since adamantanone is a membrane perturber, we suspected that it acted at some step in the acquisition of the viral envelope.

Adamantanone has no general effect on protein synthesis in infected or uninfected cells as measured by an examination of protein and glycoprotein profiles following their separation by SDS gel electrophoresis. However, it does affect the post-translational processing of at least two HSV-1 glycoproteins.

Interestingly, these two glycoproteins correspond to the fusion inhibitor and the fusion factor. It appears to cause an increase in the mobility of the glycoprotein that is thought to be the fusion inhibitor, and causes a small decrease in the ratio between the glycoprotein thought to be the fusion factor and another glycoprotein that is thought to be a precursor to the final fusion factor. These differences are not thought to be responsible for the decrease in virus production by adamantanone since virus strains completely lacking fusion inhibitor exist, and because the effect on fusion factor and its precursors is small. Nonetheless, it may bear on the fusion inhibition that has also been observed for adamantanone.

Nuclei of infected cells have also been examined by SDS gel electrophoresis. It has been found that adamantanone does not inhibit the translocation of viral polypeptides to the nucleus, which is the site of capsid assembly and envelopement. An interesting result of these experiments is that the nuclei contain only the fusion factor precursor, but not the mature fusion factor which is found in the virion. This may mean that although the virion acquires its membrane by budding through the inner nuclear membrane, it must undergo further maturation, presumably in the cisternae of the endoplasmic reticulum or the Golgi complex.

Adamantanone has been found to only slightly inhibit the assembly of virus capsids in the nucleus, and particles with properties similar to virions have been found in the cytoplasm of adamantanone-treated infected cells, although their number is substantially reduced. The nature of these particles is being investigated. Our working hypothesis is that adamantanone either causes the virion to fail to incorporate the correct glycoprotein when it buds through the inner nuclear membrane, or that these glycoproteins cannot be processed in the presence of adamantanone, as discussed above.

PUBLICATIONS (published, in press or submitted for publication):

1. Holland, T. and S. Person. (1977) Ammonium chloride inhibits cell fusion induced by syn mutants of Herpes Simplex Virus Type I. J. Virol. 23, 213-215.
2. Keller, P. M., S. Person and W. Snipes. (1977) A fluorescence enhancement assay of cell fusion. J. Cell Sci. 28, 167-177.
3. Kousoulas, K. G., S. Person and T. Holland (in press) The timing of some of the molecular events required for cell fusion induced by Herpes Simplex Virus Type 1. J. Virol.
4. Pollard, E. C., S. Person, M. Rader and D. J. Fluke. (1977) Relation of ultraviolet light mutagenesis to a radiation-damage inducible system in Escherichia coli. Radiat. Res. 72, 519-532.
5. Ruhlig, M. A. and S. Person. (1977) Alterations of neutral glycolipids in cells infected with syncytium-producing mutants of Herpes Simplex Virus Type 1. J. Virol. 24, 602-608.

Manuscripts in preparation:

1. Read, G. S., S. Person and P. M. Keller. A genetic analysis of cell fusion induced by Herpes Simplex Virus type 1.
2. Person, S., S. C. Warner and D. Bzik. Genetic evidence for the existence of two viral genes that affect cell fusion in Herpes Simplex Virus type 1 infected cells.
3. Person, S. (Review) Biological Consequences of Tritium Decay: Transmutation effects for mutagenesis, DNA single-strand breaks and DNA crosslinks.

Talks presented at meetings, seminars:

1. Holland, T. C., Person, S., Snipes, W. and Keith, A. Inhibition of herpes simplex virus growth by 2-adamantanone. Am. Soc. Microbiol., New Orleans, May, 1977.
2. Kousoulas, K. G., Person, S., Holland, T. C. and Knowles, R. W. The influence of inhibitors of cell fusion produced by a syncytial mutant of herpes simplex virus type I. Oncogenesis and Herpes Viruses: Third Internatl. Mtg., Boston, July, 1977.
3. Person, S. (invited) Biological consequences of tritium decay: Transmutation effects for mutagenesis, DNA crosslinks and DNA strand breaks. 9th Intern. Hot Atom Chem. Symp., Blacksburg, VA, Sept., 1977.
4. Person, S. (poster) Cell fusion induced by herpes simplex virus: Evidence for the involvement of two viral gene products. Biophysics Reunion, PSU, November, 1977.

5.-8. Person, S. Cell fusion induced by herpes simplex virus: Evidence for the involvement of two viral gene products. (Seminars)
Dept. of Microbiology & Cell Biology, PSU, Dec., 1977
Depts. of Chemistry and of Physics, Lehigh U., Dec., 1977
Dept. of Bacteriol. and Immunology, U. N. C., Chapel Hill, 1978
Dept. of Zoology, Duke U., 1978.

PERSONNEL ON THE PROJECT:

Graduate Students:

A. Bhagwat (other funds)
T. Holland (DOE - 6 mos)
G. Kousoulas (DOE - 6 mos)
V. C. Bond (DOE - 3 mos)
D. Bzik (DOE - 3 mos)

Research Aide:

S. Warner (PHS grant)

Undergraduate Student:

G. Fortier (not supported)

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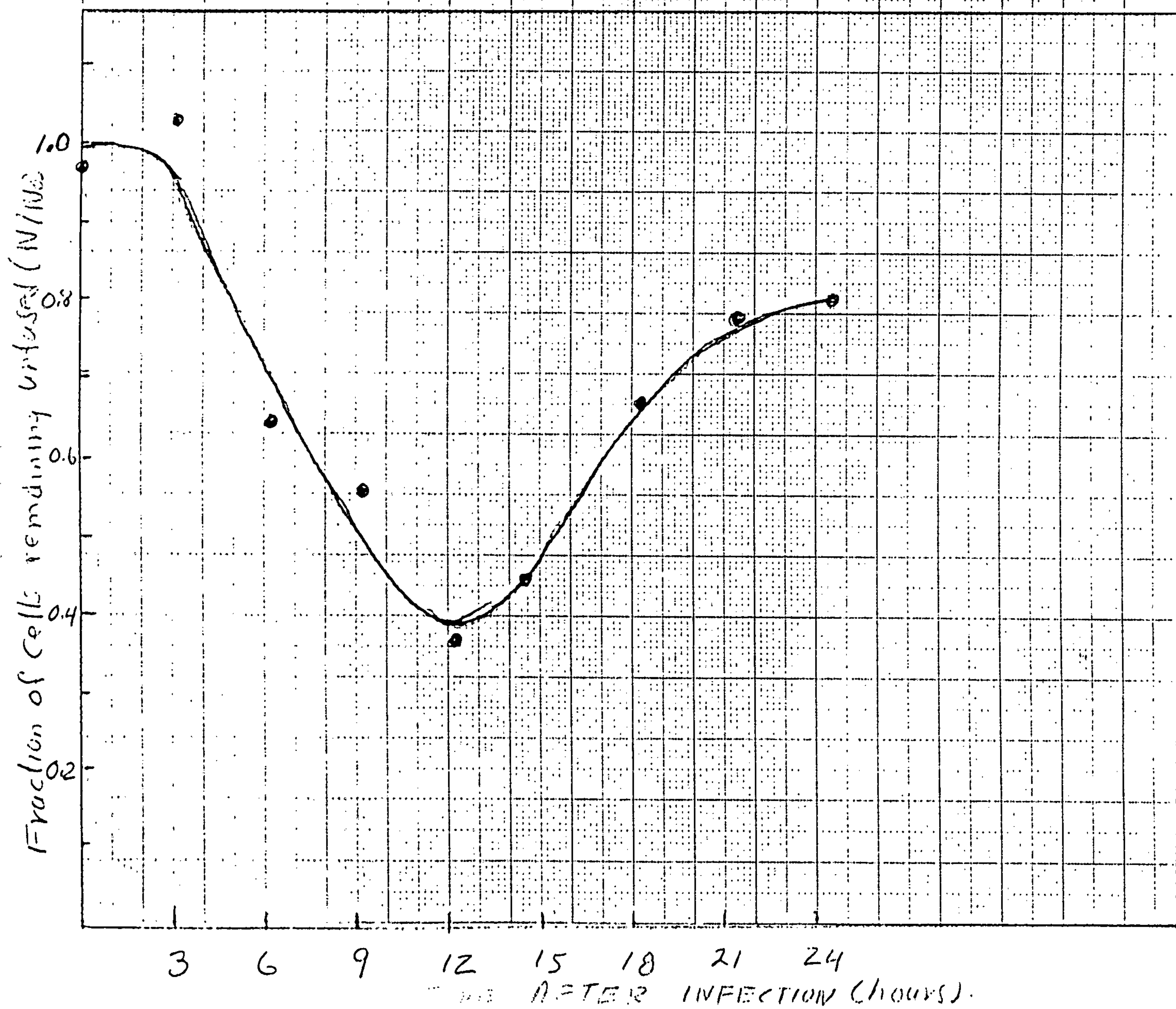

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1. Holland, T. and S. Person. (1977) Ammonium chloride inhibits cell fusion induced by syn mutants of Herpes Simplex Virus Type I. J. Virol. 23, 213-215.
2. Keller, P. M., S. Person and W. Snipes. (1977) A fluorescence enhancement assay of cell fusion. J. Cell Sci. 28, 167-177.
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Manuscripts in preparation:

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2. Person, S., S. C. Warner and D. Bzik. Genetic evidence for the existence of two viral genes that affect cell fusion in Herpes Simplex Virus type 1 infected cells.
3. Person, S. (Review) Biological Consequences of Tritium Decay: Transmutation effects for mutagenesis, DNA single-strand breaks and DNA crosslinks.

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5.-8. Person, S. Cell fusion induced by herpes simplex virus: Evidence for the involvement of two viral gene products. (Seminars)
Dept. of Microbiology & Cell Biology, PSU, Dec., 1977
Depts. of Chemistry and of Physics, Lehigh U., Dec., 1977
Dept. of Bacteriol. and Immunology, U. N. C., Chapel Hill, 1978
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Appendix I.

The Timing of Some of the Molecular Events Required for Cell Fusion Induced by Herpes Simplex Virus Type 1

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Running Head: Cell fusion induced by Herpes Simplex Virus Type 1

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The timing of some of the molecular events that are required for cell fusion was investigated. Cell fusion was produced by a mutant of Herpes Simplex Virus type 1 (HSV-1) that causes extensive cell fusion during infection. The timing of molecular events required for fusion was established by the use of blocking agents. Phosphonoacetic acid (PAA) blocks viral DNA synthesis; actinomycin D blocks RNA synthesis; cycloheximide blocks protein synthesis; 2-deoxyglucose blocks glycosylation of glycoproteins; high temperature, NH_4Cl and adamantanone block unknown steps required for cell fusion. For cells infected at a low multiplicity of infection (MOI) PAA decreased the rate but not the final amount of fusion, but at an MOI of 10 it had no effect on the rate of cell fusion. RNA synthesis is required for fusion until 4 h after infection, protein synthesis until 5½ h after infection, and glycosylation until 7 h after infection. The temperature-dependent step occurs before 6 h after infection, whereas NH_4Cl and adamantanone act at steps that occur until 8 h after infection. Cycloheximide, temperature, NH_4Cl and adamantanone act reversibly; actinomycin D and 2-deoxyglucose act irreversibly. The same order of action of the inhibitors was also determined by using pairs of inhibitors sequentially. These experiments also indicated that fusion factor was not an α-polypeptide. Virus growth and cell fusion were both found to be highly dependent on temperature in the range of 30 to 40°C. Wild type infections

are apparently characterized by the presence of a fusion factor and a fusion inhibitor. The fusion-blocking agents were added to wild type infected cells under a variety of conditions in an attempt to selectively block the production of the fusion inhibitor molecule, and thereby cause extensive cell fusion. However, fusion was not observed in any of these experiments.

It now appears that wild type Herpes Simplex Virus type 1 (HSV-1) infections are characterized by the presence of fusion factor and fusion inhibitor activity (10, 15, 16; Person and Warner, unpublished observations). The action of the inhibitor dominates so that only a small fraction of cells fuse (13). We have isolated mutants that cause extensive fusion and these may be altered in the expression of fusion inhibitor activity (15). Using a qualitative assay for cell fusion and some of the inhibitors employed here, others have examined the requirement and timing of macromolecular synthesis for cell fusion (3, 4, 8). The present study employs a quantitative assay for cell fusion and extends the previous studies.

MATERIALS AND METHODS

Cell cultures and virus stocks. The growth and maintenance of HEL cell cultures and virus stocks were described previously (13). The HSV-1 strain, KOS, was kindly provided by Priscilla Schaffer (Sydney Farber Cancer Institute, Harvard Medical School, Boston, MA). The mutant of KOS used here, syn 20, causes extensive syncytia formation. The procedure for mutant isolation was described previously (13).

The growth medium used in these experiments was a modified F12 containing 10% fetal bovine serum. The saline solution used to wash cells and dilute virus suspensions was a tricine-buffered saline (TBS). Both were described previously (13). At various times after infection cells were harvested with trypsin and EDTA and counted using a Coulter counter. The extent of fusion was determined by measuring the number of cells with pulse heights corresponding to the size of small single cells. Cells disappear from this interval as they fuse with other cells and shift to a larger threshold interval (13).

Metabolic inhibitors. Inhibitors were dissolved in growth medium and the pH adjusted to 7.3 when necessary. Cell monolayers were washed once with TBS prior to the addition of growth medium containing the inhibitor. Metabolic inhibitors were added to cultures at various times and remained in the cultures during subsequent incubation except where otherwise indicated.

Phosphonoacetic acid (PAA) was obtained from Richmond Organics, Richmond, VA; actinomycin D and 2-D-deoxyglucose from Sigma Chemical Co., St. Louis, MO; cycloheximide from Calbiochem, San Diego, CA; 2-adamantanone from Aldrich Chemical Co., Milwaukee, WI; and ammonium chloride (NH_4Cl)

from Fisher Scientific Co., Fairlawn, NJ. In preliminary experiments actinomycin D, cycloheximide and 2-deoxyglucose were found to selectively inhibit RNA synthesis, protein synthesis or glycosylation, respectively, at the concentrations employed. These results are consistent with those reported by others (5, 9, 11, 14).

Equilibrium sedimentation analysis. Duplicate cultures of HEL cells were seeded in 16-ounce prescription bottles (2.5×10^4 cells/cm^2 and grown for 40 h at 37°C (about 90% confluent). Immediately after virus adsorption 4 µCi/ml of [^{3}H]thymidine was added to the cell cultures and PAA was added to one-half of the cultures. After 10 h incubation at 34°C the cells were scraped into growth medium, pelleted and washed twice in the following solution: 10^{-1} M NaCl, 10^{-3} M EDTA and 10^{-2} M Tris-HCl at pH 7.4. Uninfected cells labeled with [^{14}C]thymidine (0.1 µCi/ml) were harvested and washed in the same way and mixed with either PAA-treated or untreated, [^{3}H]thymidine labeled, infected cells. The cells were lysed in 12 ml of lysing solution (0.5% of sodium dodecyl sulfate and sodium lauryl sarcosine in 2×10^{-3} M dithiothreitol and 2.5×10^{-2} M EDTA for 15 min at room temperature) and the DNA deproteinized with autolysed (30 min at room temperature) pronase (100 µg/ml) for 30 min. The lysate was added to solid cesium chloride (CsCl) to give a mean density of 1.707 g/cm^3 and centrifuged for 64 h at 30,000 rpm in a Beckman type 50 titanium rotor. After centrifugation fractions were collected, calf thymus carrier DNA was added to each fraction, and DNA was precipitated with 5% (w/w) trichloroacetic acid (TCA). Precipitates were collected onto Millipore filters (0.22 µM pore size), washed with

distilled water, air dried and the filters suspended in Omnifluor liquid scintillation fluid. The ^{3}H and ^{14}C radioactivity was determined in a liquid scintillation spectrometer.

Radioactive chemicals and Omnifluor liquid scintillation fluid were from New England Nuclear Corp., Boston, MA; sodium lauryl sarcosine, dithiothreitol and Tris from Sigma Chemical Co. NaCl and EDTA from Mallinkrodt, St. Louis, MO; sodium dodecyl sulfate from Pierce Chemical Co., Rockford, IL; pronase from Calbiochem; CsCl from Metallgellschaft Ag., Frankfurt, W. Germany; TCA from Fisher Scientific Co.; and Millipore filters from Millipore Corp., Bedford, MA.

RESULTS

Effect of PAA on DNA replication and cell fusion. PAA blocks HSV-1 DNA replication as measured by equilibrium sedimentation of labeled DNA (Fig. 1). Cells were infected with *syn* 20 and labeled with [^{3}H]thymidine prior to sedimentation in CsCl. Data are shown for virus infections in the presence and absence of PAA. In the presence of PAA, viral DNA synthesis is almost completely eliminated while cellular DNA synthesis is only decreased by about 25% (see Fig. 1). A large amount of cellular DNA was observed because the label was added immediately after virus adsorption.

When cells were infected with *syn* 20 at an MOI of 0.2 or 1.0, and PAA was added immediately after infection, the rate of fusion was decreased (Figs. 2, 3). After the onset of fusion (about 5 h after infection) a semi-logarithmic plot of the fraction of cells in a culture remaining unfused as a function of time after infection yields a straight line whose slope is a measure of the rate of fusion. The percent inhibition of fusion is defined as one minus the ratio of the slopes of the fusion kinetics curves, in the presence and absence of an inhibitor, times 100. At an MOI of 1 the percent inhibition of fusion by PAA was 50% when PAA was added immediately after infection, and decreased to 10% when PAA was added 4 h after infection (Fig. 4).

Data presented in Fig. 4 could reflect a requirement for transcription of some minimum number of DNA genomes, and the translation of the resulting mRNAs, in order to achieve a maximum rate of fusion. This idea was tested by determining the inhibition of fusion as a function of the adsorbed

MOI. The percent inhibition of fusion was found to decrease as the MOI increased, reaching zero at an MOI of 10 (Fig. 5). Furthermore, by extending the fusion kinetics curves to longer times extensive fusion was ultimately observed even for infection at the lower MOI (data not shown).

Effect of actinomycin D, cycloheximide, temperature shift, 2-deoxyglucose, NH_4Cl and adamantanone on cell fusion. If one of these inhibitors was added shortly after infection, fusion was completely blocked but the fusion blocking was limited to a specific time interval during infection. Data which support this conclusion are shown for infected cells incubated in the presence of cycloheximide (Fig. 6), 2-deoxyglucose (Fig. 7), and for a temperature shift from 34° to 41°C during incubation (Fig. 8). Similar results were obtained using actinomycin D, NH_4Cl and adamantanone (data not shown). Results of many fusion kinetics curves using these blocking agents are most simply expressed in diagrammatic form (Fig. 9). A shaded rectangle is used to show the time interval during which fusion inhibition changes from 100% to 0% for a particular blocking agent. The left end of the rectangle represents the latest time after infection that an agent completely inhibits fusion. The right end of the rectangle represents the earliest time after infection that an agent has no effect on fusion. For example, the addition of cycloheximide prior to 3 h after infection gives complete inhibition of fusion and when added after 5½ h gives no inhibition of fusion. Using this criterion the temporal order of agents that block synthetic processes required for fusion is actinomycin D, cycloheximide, temperature shift, and 2-deoxyglucose. Blocking agents,

NH_4Cl and the membrane perturber adamantanone, presumably block post-synthetic events required for fusion. The accuracy of determination of the timing for 100% and 0% inhibition of fusion in Figure 9 is probably $\pm \frac{1}{2}$ h. That is, occasionally a single fusion kinetics curve would be obtained that differed from the average values by $\pm \frac{1}{2}$ h (compare curve for 5.5 h after infection [Fig. 8] with average data [Fig. 9]).

The temperature dependence of cell fusion and virus growth were determined and the percents of the maximum values were plotted as a function of incubation temperatures (Fig. 10). Both curves have a maximum between 34° and 38°C and decrease rapidly at higher and lower temperatures. Although the overall data are somewhat similar there is a more rapid decrease in the rate of cell fusion than for virus growth between 38° and 39°C.

An independent method of establishing the order of action of blocking agents. Consider a two-step pathway consisting of a reactant A, an intermediate B, and a product C in which the production of B and C may be blocked by the agents a and b, respectively ($A \xrightarrow{a} B \xrightarrow{b} C$). If b is present the intermediate B will accumulate. Replacing b with a will then allow the conversion of B to C. If the addition of a precedes its replacement with b, the product C will never be formed. Pairs of blocking agents were used in this way to establish the order of action of the blocking agents. If two agents block the same step in a pathway, or if the agent that blocks the later step acts irreversibly, one cannot determine the order of action of the blocking agents.

Experiments were performed by infecting monolayers of cells in petri dishes and scoring fusion 24 to 36 h later by a qualitative microscope assay. One blocking agent was present from 1 to 9 h after infection and was replaced by a second blocking agent at that time. Results are shown in Table 1. Cycloheximide, NH_4Cl, adamantanone, and the temperature-dependent step were reversible whereas actinomycin D and 2-deoxyglucose were not (Table 1, lines 1-6). The cycloheximide-sensitive step preceded the 2-deoxyglucose, NH_4Cl, adamantanone, and perhaps the temperature-sensitive step. The temperature-dependent step occurred earlier than the NH_4Cl-sensitive step. The overlap between NH_4Cl and adamantanone blocking was not resolved, but a small amount of fusion was observed when adamantanone preceded the use of NH_4Cl. Cycloheximide is known to act at a later step than actinomycin D and cycloheximide was shown to act reversibly. Nonetheless, when cycloheximide was added before actinomycin D, no fusion was observed. The same results were obtained when the reversible protein inhibitor puromycin was used (data not shown).

From studies of mutants of HSV-1 that affect cell fusion (10, 15, 16; Person and Warner, unpublished observations) it is thought that wild type infections are characterized by the presence of a fusion inhibitor. By adding agents that block fusion at several times after infection and at several concentrations, some of which were less than those required to block fusion, it was hoped to selectively block fusion inhibition in wild type infections. Then wild type infections would result in the extensive fusion characteristic of syn mutant infections. These experiments were done, but extensive fusion was not observed in any of the experiments (Table 1 and data not shown).

In order to determine if PAA blocked the synthesis of fusion inhibitor it was added to wild type infected cells (MOI of 10) at 0, 1 and 2 h after infection. Again extensive fusion was not observed (data not shown). As a control, *syn* 20 infected cells were similarly treated and extensive fusion was observed.

DISCUSSION

We have studied the timing of some of the molecular events required for cell fusion using metabolic blocking agents and a quantitative assay for cell fusion. Our data is largely in agreement with previous data which was based on a qualitative assay for cell fusion. The order of action for the blocking agents used is actinomycin D, cycloheximide, temperature shift, 2-deoxyglucose, NH_4Cl and adamantanone. There is considerable uncertainty about the assignment of the order of NH_4Cl relative to adamantanone.

The onset of DNA replication and of cell fusion in HSV-1 infections occurs at roughly the same time. Falke found that actinomycin D and cycloheximide depressed DNA replication and fusion by similar amounts and speculated that DNA replication might be a trigger for cell fusion (3, 4). However, the inhibition of DNA replication by mitomycin C (12) or cytosine arabinoside treatment (8) did not block cell fusion. Using PAA, which specifically blocks viral DNA polymerase (11), we have shown that the rate but not the final amount of fusion is decreased when a low MOI is used. At an MOI of 10 there is no influence of PAA on cell fusion. We conclude that DNA replication *per se* is not required for cell fusion but that the presence of about 10 HSV-1 genomes/cell (PFU/cell) is required to achieve the maximum rate of fusion. Since extensive fusion occurs in the absence of DNA replication virus production is not required for cell fusion.

In contrast to the results for PAA, actinomycin D and cycloheximide added early in infection completely block fusion for an extended period

of time. RNA synthesis is required for cell fusion until 4 h after infection; protein synthesis is required until 5 h after infection. Considering differences in virus and cell strains, media, and temperature of incubation, these results are in good agreement with results reported by Falke and by Keller (3, 4, 8). The action of cycloheximide is readily reversible, while the removal of actinomycin D does not result in the production of fusion scored 24 h later. It has been reported previously that 2-deoxyglucose blocks cell fusion and the glycosylation of HSV-1 glycoproteins (1, 8, 9). Here we show that it acts irreversibly and that glycosylation is required until 7 h after infection.

Adamantanone and NH_4Cl, which block fusion reversibly, appear to act at post-synthetic steps. A small amount of fusion was observed when adamantanone preceded the use of NH_4Cl. This may indicate that the NH_4Cl-sensitive step precedes the adamantanone-sensitive step. Since adamantanone is a known perturber (2) it is possible that both of these agents block fusion by action at the plasma membrane. NH_4Cl has little influence on viral growth (6) but adamantanone reversibly inhibits a late step in virus replication, perhaps at envelopement (T. C. Holland, unpublished observations).

We have observed a temperature-dependent step for cell fusion; it occurs early in infection and is reversible. In order to determine if the temperature dependence was specific for cell fusion, we determined the temperature dependence of fusion and compared it to the temperature dependence of virus growth. Both have maximum values between 34° and 38°C and decline rapidly at lower and higher temperatures. Although the data for both fusion and growth do not differ strikingly, there is a precipitous

decrease in cell fusion between 38° and 39°C. The temperature-dependent step for virus growth may be required for cell fusion. It would be of interest to know more about the effect of temperature for both functions. A temperature-dependent step for the growth of a bovine herpesvirus has been reported (17, 18). It was thought that the critical step was a block in DNA replication, but we note that DNA replication may be somewhat dependent on RNA and protein synthesis (4).

Honess and Roizman have defined three types of peptides following the infection of cells with HSV-1: α, β and γ (7). If cycloheximide and actinomycin D are used sequentially, as in the experiments reported in Table 1, then only α-polypeptides are made; β- and γ-polypeptides require the prior synthesis of α-polypeptides. Since this same protocol blocks fusion we conclude that fusion factor is not an α-polypeptide.

We found that fusion inhibitor production in wild type infected cells did not require viral DNA replication, and that fusion inhibitor activity could not be selectively inhibited by the application of a wide variety of concentrations and times of addition of the blocking agents. Apparently the production of fusion factor and fusion inhibitor activity have similar metabolic and temporal requirements.

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TABLE 1. The order of action of fusion-blocking agents

Blocking agent	Time (h) of addition of blocking agent						
	wt infected cells			*syn* 20 infected cells			
	>1	>9	1→9	>1	>9	1→9	1→9, >9
Actinomycin D	-	-	-	-	++	-	
Cycloheximide	-	-	-	-	++	++	
2dG	-	-	-	-	++	-	
NH_4Cl	-	-	-	-	++	++	
Adamantanone	-	-	-	-	++	++	
Temperature shift	-	-	-	-	++	+	
Actinomycin D, cycloheximide							-
Cycloheximide, actinomycin D							-
Cycloheximide, 2dG							-
2dG, cycloheximide							-
Cycloheximide, NH_4Cl							-
NH_4Cl, cycloheximide							++
Cycloheximide, adamantanone							-
Adamantanone, cycloheximide							++
NH_4Cl, adamantanone							-
Adamantanone, NH_4Cl							+-
Cycloheximide, temperature shift							-
Temperature shift, cycloheximide							+-
Temperature shift, NH_4Cl							-
NH_4Cl, temperature shift							++

Figure legends

FIG. 1. Effect of PAA on DNA replication. Cultures of HEL cells were infected with _syn_ 20 at an MOI of 1 and labeled with [^{3}H]thymidine from 0 to 10 h after infection. Infected cultures were lysed, the DNA was centrifuged to equilibrium in CsCl, and TCA-insoluble radioactivity was determined for each fraction. The radioactivity in each fraction is plotted as a function of the fraction number;the direction of sedimentation is from right to left. The arrow indicates the position of cellular DNA which was determined by mixing [^{14}C]thymidine-labeled uninfected cells with infected cells prior to cell lysis.

FIG. 2. Effect of PAA on the kinetics of cell fusion. Cultures were infected with _syn_ 20 at an MOI of 0.2 and incubated for the times indicated. Cells were harvested with a trypsin-EDTA solution and the number of small single cells was measured using a Coulter counter assay described in Materials and Methods. The fraction of single cells remaining unfused is plotted as a function of time after infection for cultures incubated in the presence and absence of PAA.

FIG. 3. Effect of PAA on the kinetics of cell fusion; PAA was added at 0 and 2 h after infection. Cells were infected with _syn_ 20 at an MOI of 1 and assayed for cell fusion as in Fig. 2. The fraction of cells remaining unfused as a function of time after infection is shown for PAA added at 0 and 2 h after infection.

FIG. 4. The inhibition of cell fusion by PAA as a function of time after infection. Fusion kinetics curves for cells infected with _syn_ 20

at an MOI of 1 were determined as described for Figs. 2 and 3. PAA was added to the cultures at the indicated times after infection and left in the cultures during subsequent incubation. The percent inhibition of fusion is defined as one minus the ratio of the slopes of the fusion kinetics curves, in the presence and absence of the blocking agent, times 100.

FIG. 5. The inhibition of cell fusion by PAA as a function of MOI. PAA was added to cultures immediately after infection with syn 20 at the indicated MOI. The inhibition of cell fusion was measured as described for Fig. 4.

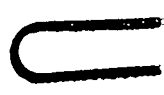
FIG. 6. The effect of cycloheximide on cell fusion. Cells were infected with syn 20 at an MOI of 10 and assayed for cell fusion as indicated for Fig. 2. The fraction of cells remaining unfused is plotted as a function of time after infection.

FIG. 7. The effect of 2-deoxyglucose on cell fusion. Cells were infected with syn 20 at an MOI of 10 and assayed for cell fusion as indicated for Fig. 2. The fraction of cells remaining unfused is plotted as a function of time after infection.

FIG. 8. The effect of temperature shift on cell fusion. Cells were infected with syn 20 at an MOI of 10 and incubated at 34°C. At the times indicated cultures were shifted to 41°C. Cells were assayed for cell fusion as indicated for Fig. 2. The fraction of cells remaining unfused is plotted as a function of time after infection.

FIG. 9. The timing of molecular events required for cell fusion. The inhibitors used are shown at the left of the diagram and the events blocked by the inhibitors are shown at the right of the diagram. A time scale is plotted as the horizontal axis. The times for virus adsorption and the onset of fusion are shown as shaded rectangles at the top of the figure. For any blocking agent the left-hand edge of a shaded rectangle represents the latest time after infection at which an agent completely inhibits fusion. The right-hand edge of the rectangle represents the earliest time after infection at which an agent has no effect on fusion. The concentrations of blocking agents used were: Actinomycin D, 8 μm; cycloheximide, 0.36 mM; 2-deoxyglucose, 10 mM; NH_4Cl, 20 mM; adamantanone, 7 mM.

FIG. 10. The effect of temperature on cell fusion and virus growth. The rate of cell fusion and extent of virus growth are plotted as a percent of the respective maximum values. For fusion the slope of the fusion kinetics curve is taken as the rate of fusion; a maximum rate, 100%, was obtained at 36° and 38°C. For virus growth, optimal incubation times for virus growth were determined and this value was plotted for each temperature. Virus growth was measured by plaque formation on HEL cells; maximum growth (100%) was obtained at 34° and 36°C.



MOI = 1

● No PAA added

○ 1.4 mM PAA added at 0 hours

Counts Per Minute in Each Fraction x 10^{-3}

20

15

10

5

10 15 20 25 30 35

Fraction Number

Fig. 2.

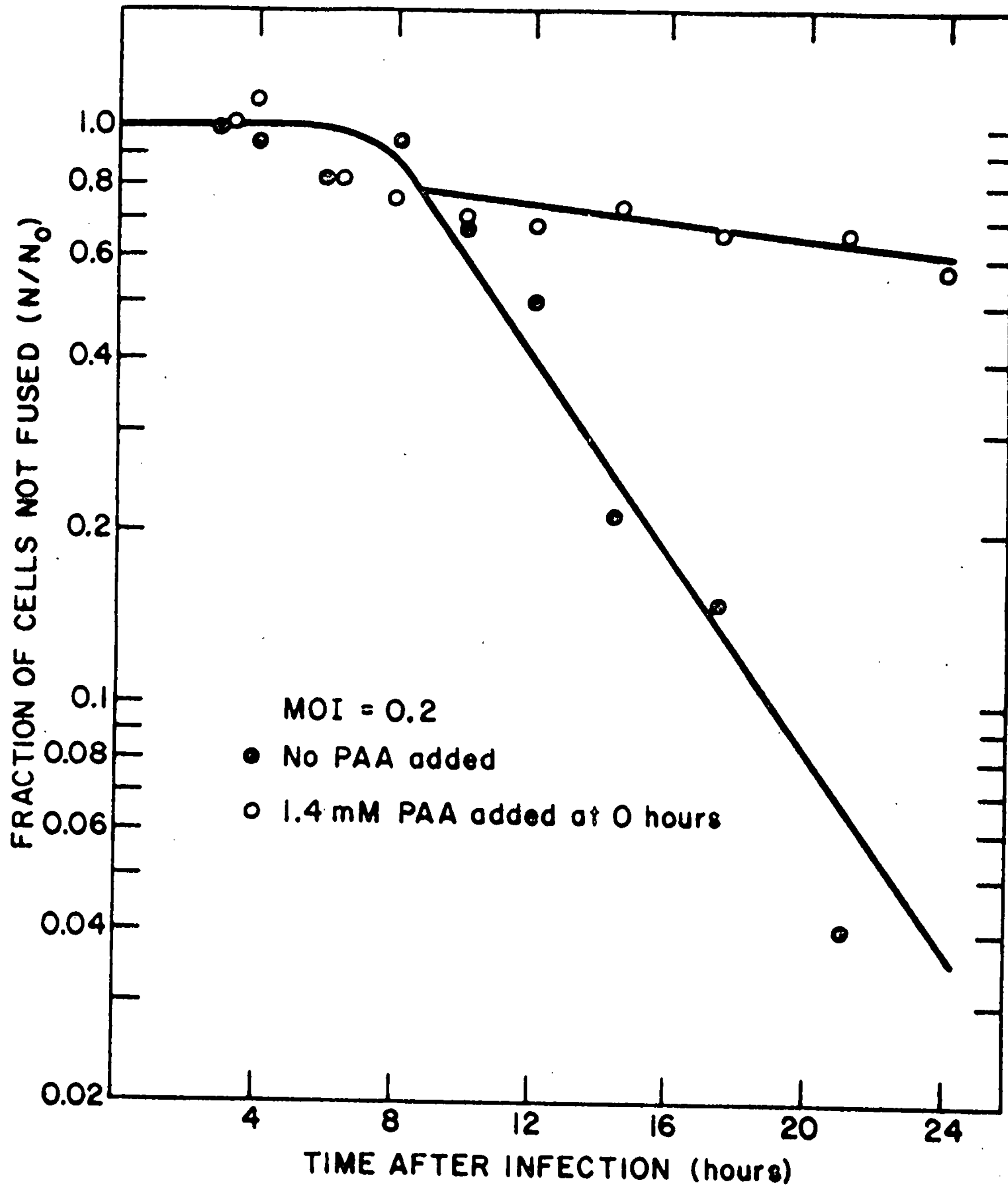


Fig. 3

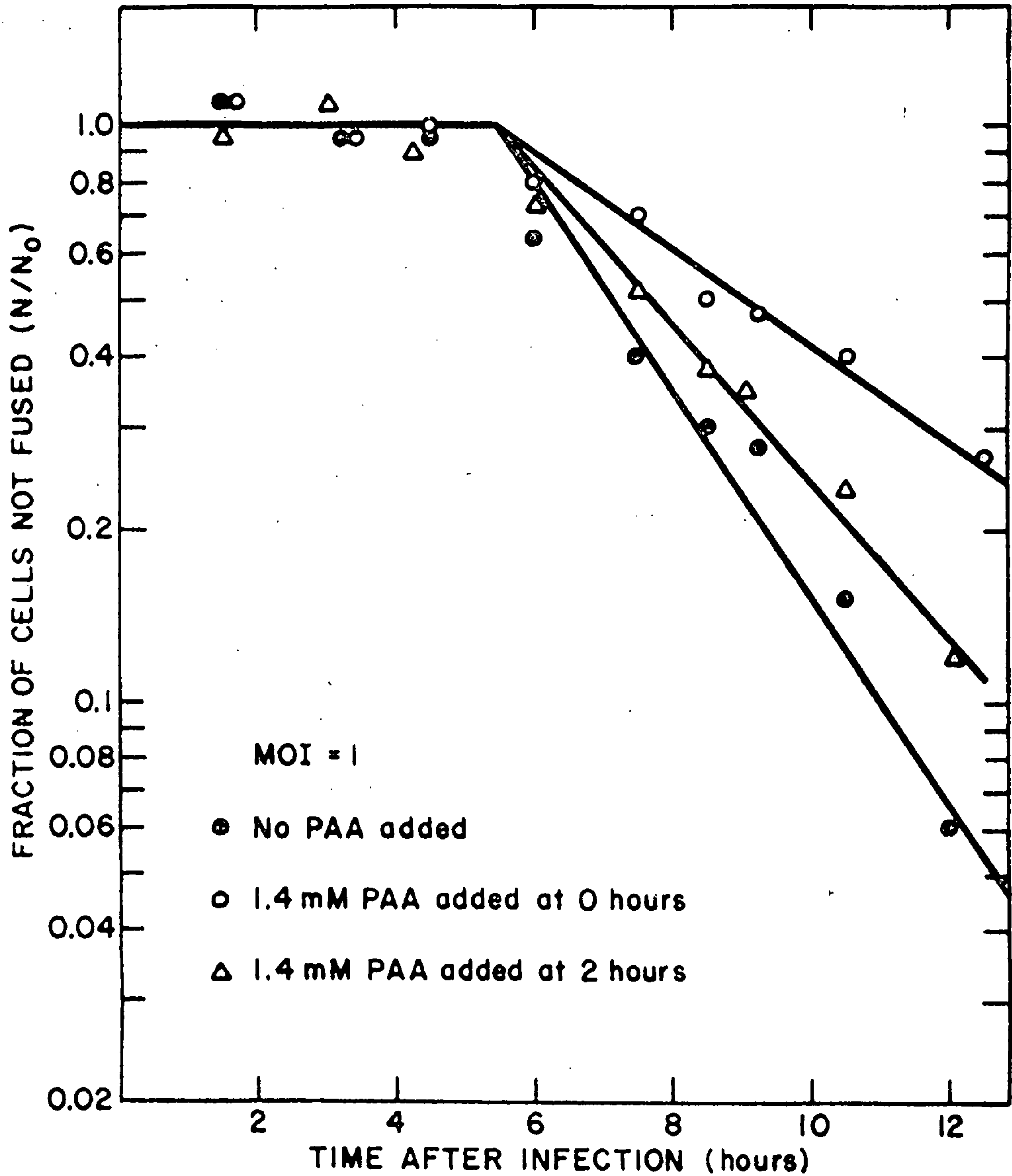


Fig. 4

INHIBITION OF CELL FUSION (PER CENT)

100
80
60
40
20

1
2
3
4

TIME AFTER INFECTION (hours)

Fig. 5

INHIBITION OF CELL FUSION (PER CENT)

100

80

60

40

20

2 4 6 8 10

ADSORBED MOI (PFU/CELL)

Fig. 6

FRACTION OF CELLS NOT FUSED (N/N_0)

1.0
0.8
0.6
0.4
0.2
0.1
0.08
0.06
0.04
0.02

● No cycloheximide added

○ 0.36 mM cycloheximide added at 2 hours

□ 0.36 mM cycloheximide added at 4 hours

■ 0.36 mM cycloheximide added at 5.5 hours

2 4 6 8 10 12

TIME AFTER INFECTION (hours)

Fig. 7

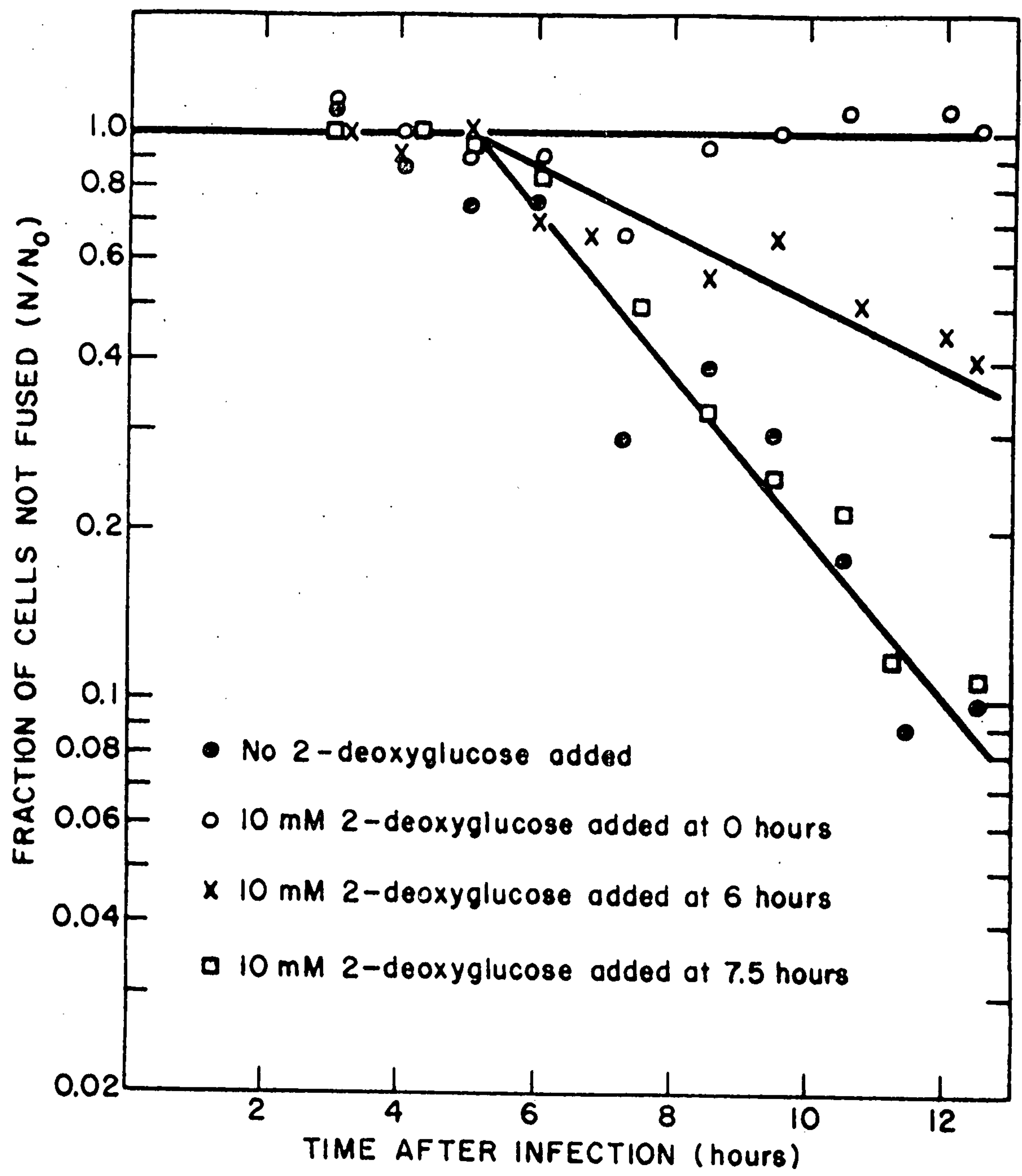


Fig. 8

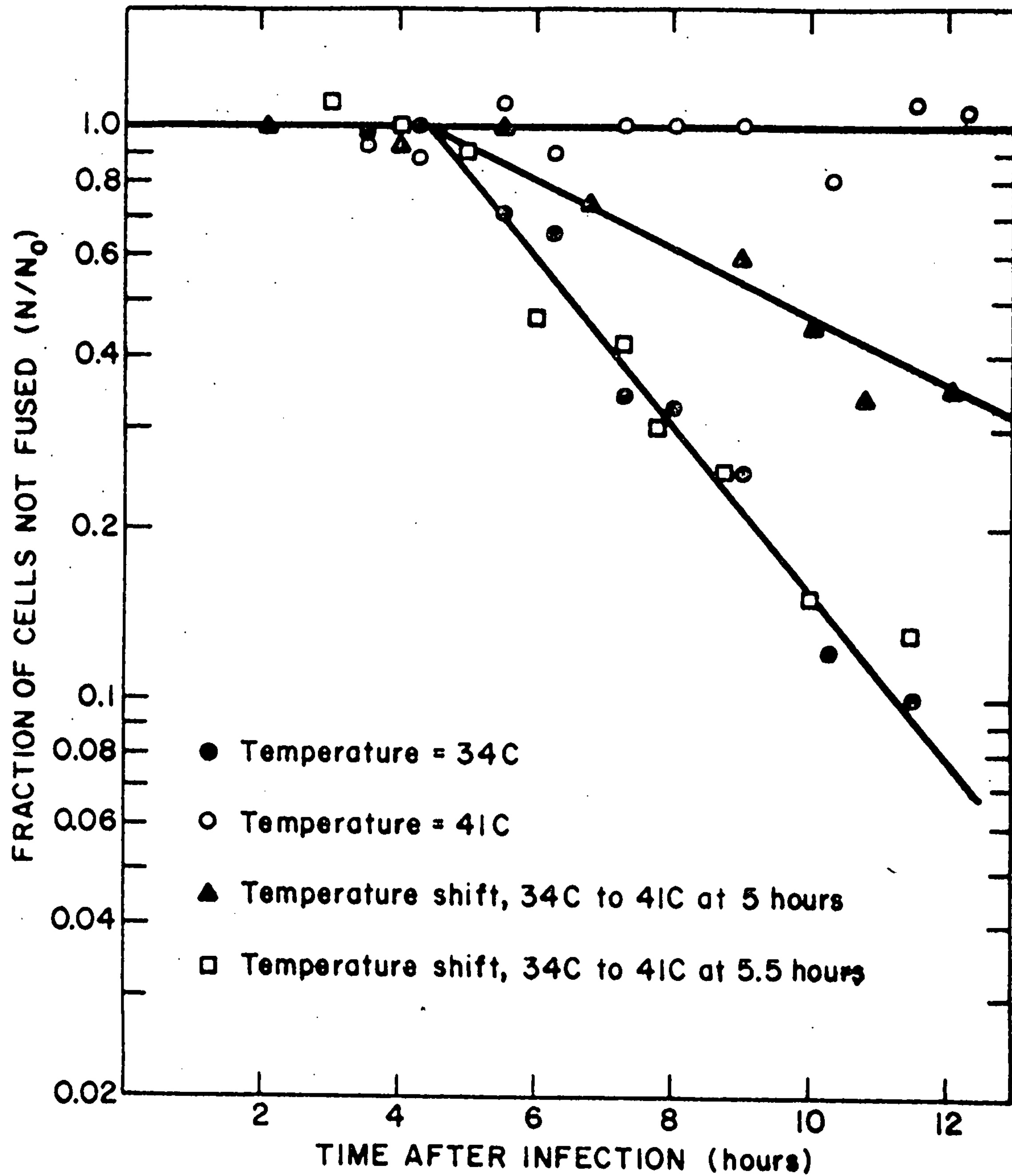


Fig.
9

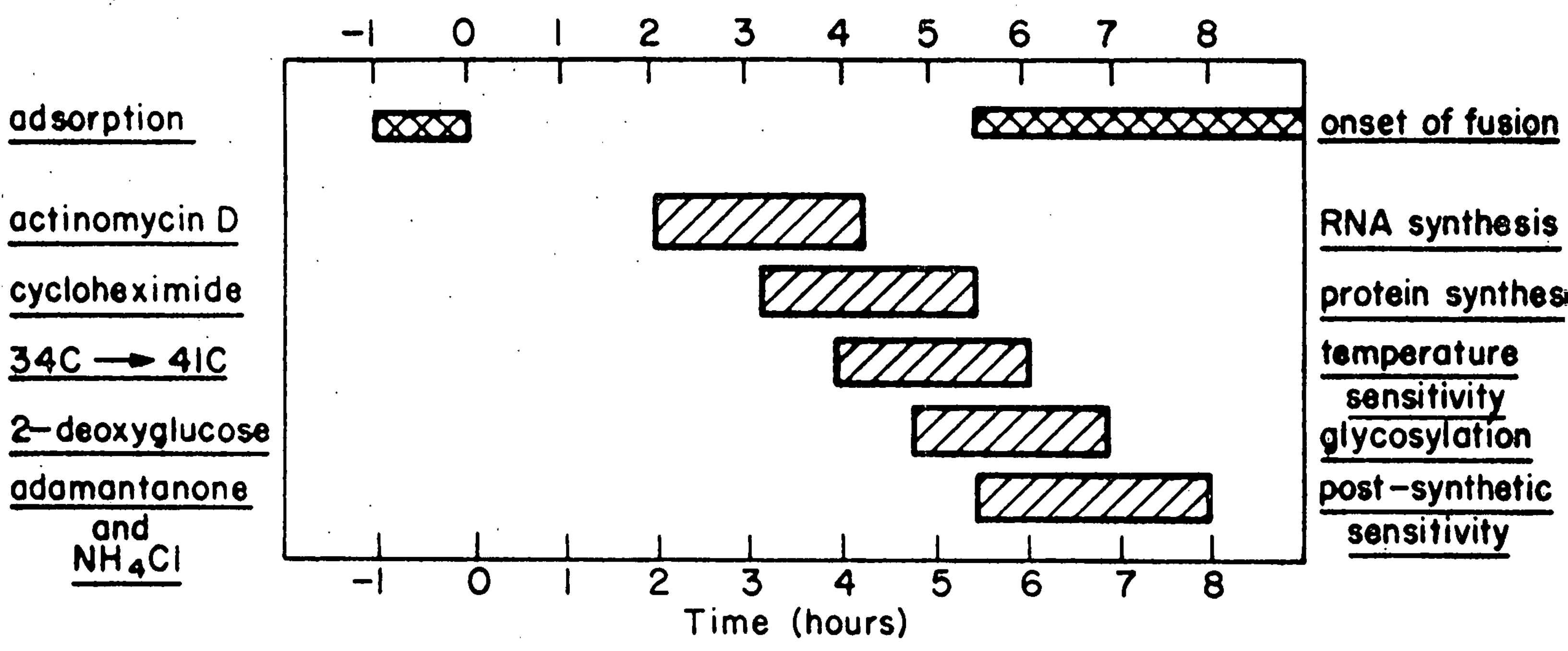

-1
0
1
2
3
4
5
6
7
8
adsorption
onset of fusion
actinomycin D
RNA synthesis
cycloheximide
protein synthes
34C → 41C
temperature sensitivity
2-deoxyglucose
glycosylation
adamantanone and NH_4Cl
post-synthetic sensitivity
Time (hours)

Fig. 10

● cell fusion
○ virus growth

Per Cent of Maximum

100
80
60
40
20
0

25 30 35 40

Temperature (C)