GROWTH CHARACTERISTICS OF INFLUENZA VIRUS: THE INFLUENCE OF A SULFONIC ACID*

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By a combination of enzymatic and viral techniques, it has been possible to follow the effects of certain inhibitors on host-cells as well as on the synthesis of virus and to correlate quantitatively the degree of metabolic alteration produced with the rate of viral production (1, 2). These studies have served to emphasize the role of the metabolism of the host-cell in the biosynthesis of virus; some biochemical reactions essential to the process have been uncovered, and something has been learned of the types of chemical structures which can influence viral propagation (3, 4). Now it is fitting that some effort be expended to correlate these specific activities of the host-cell with particular phases of the development of the virus.

It is the purpose of this paper to present an analysis of the effect of a viral inhibitor upon the growth cycle of influenza virus. An early stage in the latent period is described which is sensitive to the action of α-amino-*p*-methoxyphenylmethanesulfonic acid. Some evidence is presented which indicates that the processes of viral liberation and penetration share some common character.

Materials and Methods

Virus and Tissue.—The PR8 strain of Type A influenza virus selected for these studies has undergone 7 passages in ferrets, 593 passages in mice, and 141 passages in eggs. The host tissue was obtained from the chorioallantoic membrane of 14 to 16 day embryonate eggs.

Immune Serum.—The immune serum used to remove excess virus from infected tissues was prepared by intravenous inoculation of ferrets with allantoic fluid containing the PR8 strain of influenza virus. It had a hemagglutination-inhibition titer of 4096 and was diluted 10-fold prior to use.

α-Amino-p-Methoxyphenylmethanesulfonic Acid.—This inhibitor was prepared in this laboratory by the method previously described (4).

Warburg Flask Culture.—The host-virus system was maintained in a Warburg apparatus using a modified Simms solution. Each reaction vessel contained 400 mg. of chorioallantoic membrane which was used without mincing. The cultures were incubated as routine with shaking at 37°C. A description of the method has been reported elsewhere (1, 3), and details of the individual experiments are found in the corresponding figures.

Virus Titrations.—The amount of virus was estimated by determining the infectious titer for eggs. For this purpose 10-fold serial dilutions were prepared in broth, and 4 to 6 eggs were

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inoculated with 0.1 ml. of each dilution. After 3 days of incubation at 37°C., samples of allantoic fluid were removed from each egg and tested for virus by the addition of red blood cells. The 50 per cent infectious titer was calculated using the method of Reed and Muench (5).

EXPERIMENTAL

I. Some Normal Characteristics of Viral Development

Influenza virus will propagate rapidly in isolated sections of chorioallantoic membrane maintained in the reaction vessels of a respirometer (1, 3). Not only are excellent yields obtained, but the method permits one to follow with facility in a single culture the rate of viral development as a function of time (6). The amount of virus in the medium is easily estimated at various times by repeated sampling of the extracellular fluid. If a series of cultures is used, samples of tissue can be removed at various times, and the appearance of cellular virus can also be followed. Experiments of this general type and the resulting data are described in the following section. They were designed to supply reference data for the study of the action of specific substances which influence viral development.

Viral Development Consequent upon a Small Inoculum.—

A relatively small inoculum of virus was added to a culture containing tissue and medium, and at hourly intervals thereafter samples of extracellular fluid (0.1 ml.) were removed and titered in eggs. The aliquot was replaced with an equal volume of fresh medium. In this way the development of the virus was followed over a 15 hour period. The details of the experiment and the data obtained are found in Fig. 1.

It will be noted there is a constant or latent period of 3 to 4 hours followed by a liberation period of at least 3 hours. For some time after, the titer of the fluid is again constant before a second release period is seen. This second latent period is often shorter and less distinct than the first (Fig. 1).

The observations are most intelligible if one assumes that a limited number of cells are infected initially and several cycles of multiplication result. Distinct cycles are observed only because under these conditions virus is not completely adsorbed by the cells. Since the unadsorbed virus can be estimated by injecting limiting dilutions into embryonate eggs, it is obviously viable. These data are similar to those reported by other workers who used *intact* embryonate eggs (7, 8). However, they differ in that here the repeated cycles are more clearly discernible, the latent period is shorter, and there is no evidence of a general "burst" phenomenon (7). The virus is liberated or released from the tissue over a considerable period of time at what appears to be an exponential rate rather than a linear one. It should be emphasized that these findings will not permit conclusions to be drawn regarding the release of virus from individual cells.

As will be shown later, in this type of experiment it is important to be able

to recognize these features of the growth cycle, but particular significance must not be attributed to the numerical values assigned from it. The rate of release of virus increases with time, and the first virus to appear is small in quantity. Only when it becomes considerably greater in amount than the residuum of inoculated virus in the medium can it be detected. Thus, the earliest release of virus is obscured by the residual inoculum, and the length of the latent period may be only an illusion. By the same token, the release of virus after the maximal rate has been achieved is difficult to detect with this type of experiment. While a clearer picture obtains from the use of re-

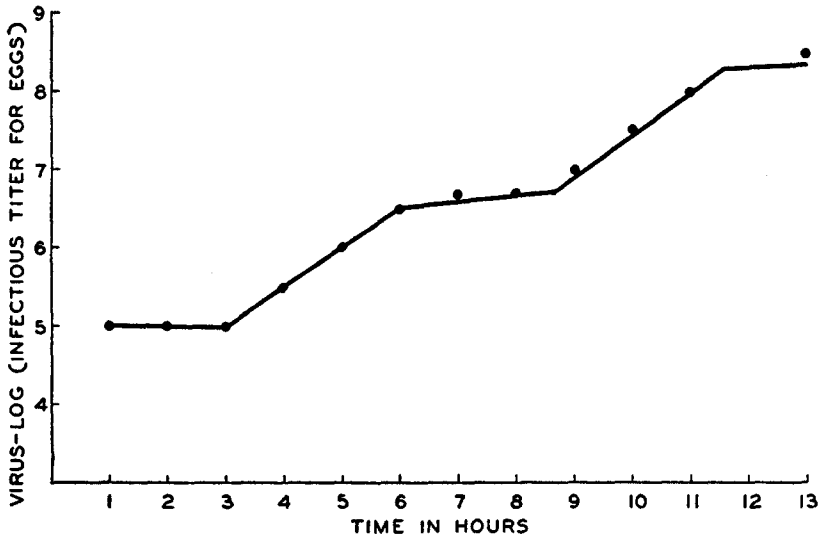

FIG. 1. Growth curve for influenza virus determined for single *in vitro* culture. The tota volume of the culture was 3.0 ml. It contained 400 mg. of tissue and 0.3 ml. of inoculum with a titer of $10^{-6.0}$ which was added at zero time.

ceptor-destroying enzyme (9) or irradiated homologous virus (8), these procedures are not without their own complicating effects. One can study single cycles of infection by such methods, as well as by others described below, but the value of the present system lies in its comparability with the natural infectious process in which all susceptible cells of an animal are rarely infected simultaneously. This is borne out by the rather interesting effects already noted by the application of metabolic inhibitors to cultures potentially capable of undergoing several cycles of infection (6).

Viral Development Following a Massive Inoculum.—

The data in Fig. 2 were obtained from a single culture which had been heavily inoculated in an effort to infect all susceptible cells. To approximately 5×10^7 cells were added 5×10^9

egg infectious doses of influenza virus, *i.e.* about 100 EID_{50} per cell. After 1 hour at 37°C. the tissue was removed and washed 3 times in 20 ml. volumes of cold saline followed by one washing in a 1 to 2 dilution of immune serum and 3 more washings with saline. This treatment was found necessary to remove large amounts of virus held superficially to the tissue. The membrane was then placed into fresh culture fluid which contained no virus.

In a short time the minute amount of residual virus had been distributed between the tissue and the medium. The titer of the fluid was then $10^{-2.5}$. At hourly intervals the extracellular fluid was titered for virus. The results are plotted in Fig. 2.

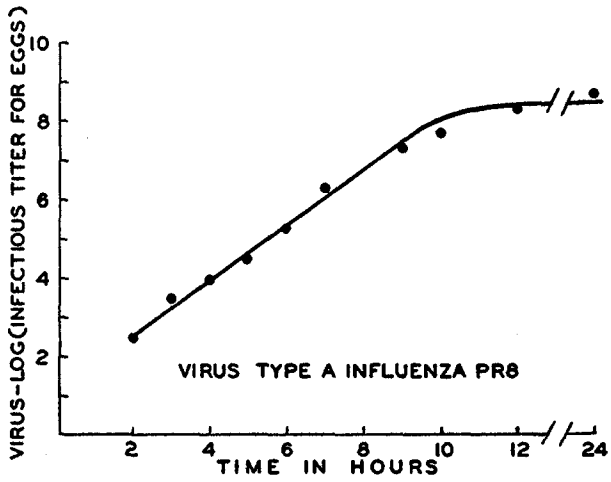

FIG. 2. Premaximal rate of virus production. The total volume of the culture was 3.0 ml It contained 400 mg. of tissue and 0.3 ml. of undiluted allantoic fluid which had a titer of $10^{-9.3}$. The inoculum was added at zero time and washed from the tissue after 1 hr. of incubation at 37°C. The tissue was placed in fresh medium and sampled at hourly intervals.

The first release of virus occurred between 2 and 3 hours. It continued at a rapid rate for the next 7 hours. By the 12th hour, the substantial part of the viral yield had been obtained since no further significant increase in titer was measured at 24 hours. When contrasted to the curve which resulted from a small inoculum (Fig. 1), one notes that the latent period is shorter (2 hours or less) and the release period is longer. The curve has all appearances of a single growth cycle. While the obscuring effect of the residual virus inoculum on the latent period has been reduced by the technique of the experiment, the large yields of virus obtained by the 12th hour still prevent the measurement of the postmaximal rate of virus release.

Measurement of the Postmaximal Rate of Viral Production.—

To test if the culture continued to produce virus after the maximal rate had been attained, a culture was prepared and infected as described in the experiment of Fig. 2. When the maxi-

mal rate of viral production had been achieved, the tissue was again washed in the manner employed to remove the residual inoculum. The tissue was placed in a new flask with fresh medium containing no virus. The extracellular fluid was sampled at 2 hour intervals and titered in eggs. The data are shown in Fig. 3.

The original titer of the fluid was $10^{-2.5}$. By the 13th hour the titer had increased to $10^{7.7}$ and remained essentially constant until the 15th hour. This indicated that the maximal rate of virus release had been achieved. After washing the tissue the titer of the extracellular fluid was $10^{-3.3}$. The titer of the fluid again increased between the 16th and 23rd hour until it reached $10^{-5.5}$.

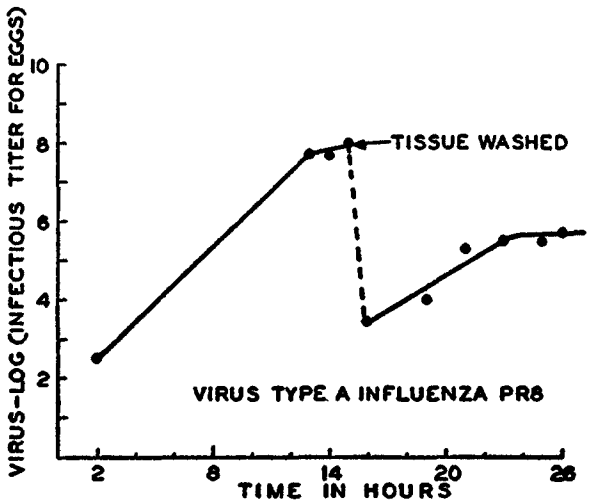

FIG. 3. Postmaximal rate of virus production. The culture contained 400 mg. of tissue and initially 0.3 ml. of undiluted allantoic fluid which had a titer of $10^{-9.3}$. The residual inoculin was washed from the tissue after 1 hr. of incubation at 37°C., and the tissue was placed in fresh medium. After 15 hours of incubation the tissue was again washed and placed in fresh medium.

If there was further viral production after that point, the rate was less than $10^{-5.5}$. Obviously it is the limitation of the method used to estimate influenza virus which requires the second washing procedure in order to demonstrate this postmaximal development of virus.

II. Action of an Inhibitor of Viral Development

The growth curve of Fig. 2 is produced by a population of cells; hence the latent period observed may represent a small segment of the culture, and the virus produced at 10 or 12 hours may derive from cells with a much longer latent period which is obscured by virus of the earlier yielding cells. Equally possible it is that the majority of cells may have a latent period of about 2 hours in which no increase in mature virus appears, and then all cells produce

and release virus over an extended subsequent period. In the latter case all reactions required for the synthesis of virus are taking place in the tissue during the entire liberation period, while the reactions of the latent period may represent those involved in the process of adapting the cell to its new role of viral host. This adaptation process may take a number of biochemical forms—the inhibition of some cell processes, the initiation of others, or the accumulation of certain metabolites. A comparison of the sensitivity of the different phases to the influence of specific metabolic inhibitors may offer a method of analyzing the biochemical significance of the latent and release periods.

TABLE I

Sensitivity of Phases of the Latent Period to the Action of AMPS

Experiment No.	Control titers* at various times			Experimental titers* at 12 hrs. following the addition of AMPS at various times‡							
	0 hrs.	6 hrs.	12 hrs.	0.00	0.25	0.50	0.75	1.00	2.00	3.00	4.00
I§	4.7	—	9.0	4.7	—	—	—	5.3	5.3	5.0	6.2
II§	4.7	5.5	6.7	4.5	4.7	4.5	5.3	5.6	—	—	—
III‖	5.0	6.3	7.5	4.5	4.3	5.5	6.3	6.5	—	—	—

* Titers are expressed as the negative log of the limiting dilution infectious for eggs.

‡ Time of addition of AMPS expressed in hours and measured from the addition of virus.

§ Titers of the extracellular fluid.

‖ Titers of the fluid plus tissue.

Effect of Time of Addition of AMPS on Viral Development.—

A series of flasks was prepared using a small inoculum as described in the experiment of Fig. 1. At hourly intervals from 0 to 5 hours, after inoculation with virus, 1 mg. of α-amino-p-methoxyphenylmethanesulfonic acid (AMPS) was added to individual flasks. The titers of the supernatant fluids were determined after 12 hours of incubation.

While the culture to which AMPS was added at zero time showed no increase in titer, all other cultures to which the sulfonic acid was added at 1 hour or later gave evidences of growth. However, in each case the increase of titer was comparable but less than that of the control which contained no inhibitor. These data are summarized in Table I.

In a second experiment of this type the inhibitor was added at quarter hour intervals up to 1 hour. Titrations of the fluids at 12 hours showed a complete suppression produced by the 0 and 15 minute additions, while cultures receiving AMPS at 30 minutes or later produced a yield of virus somewhat smaller than that of the controls (Table I).

The second experiment was repeated, but after the incubation period, the tissue was ground with alundum and added to the medium of the appropriate

flask. In this way any virus not released from the tissue by 12 hours could also be measured. The early addition of AMPS again produced a complete inhibition of the production of virus while later additions gave only partial inhibition (Table I). The reduced viral yield thus observed was the result of inhibited production rather than impaired release.

These data show that stages exist in the latent period which vary in sensitivity to the action of AMPS. Some process which is inhibited by the action of the sulfonic acid takes place quite early in this period. If that process is allowed to go to completion, then a yield of virus will result even in the presence of the inhibitor. Since a small inoculum was used in the experiments, the cultures were capable of several cycles of multiplication. Hence, if the cycles are truly the same process repeated successively, then when AMPS is added after the completion of the sensitive phase of the first cycle, it will be present at the beginning of the second cycle. Thus the addition of AMPS to the culture 1 hour after the virus would permit the first cycle to be completed but would prevent the development of successive cycles. The earliest additions would give complete inhibition while later additions would result in partial suppression. By a comparison of the growth curves of a control culture and one in which there is partial inhibition, it should be possible to determine whether the viral yield derives from a single cycle of growth.

Growth Curves of Partially Inhibited Cultures.—

The data of Fig. 4 represent four separate experiments. A small inoculum was used and the growth curves were followed by repeatedly sampling the medium from single cultures as described under Fig. 1. For each experiment two flasks were employed. To one flask AMPS was added 1 hour or more after the addition of virus, while the second served as a control. Because of the similarity of the normal growth curves from these four experiments and for brevity, the data for each point were averaged and a composite curve is presented for the control cultures (Fig. 4). However, the growth curves observed in the presence of AMPS are presented separately.

As shown in the previous type of experiment (Table I) the addition of AMPS at zero time prevented an increase in the viral titer of the fluid even when the culture was observed as long as 24 hours. The addition of the sulfonic acid to the culture 1, 2, or 4 hours after the virus allowed a yield of virus which could be detected in the fluid. It is apparent from the data (Fig. 4) that a single step growth curve is obtained by these later additions of AMPS. However, when the growth curves are followed, it is seen that the viral yield occurs in the medium at 9 or 11 hours instead of 3 or 4 hours after inoculation. The apparent latent period is thus extended almost 3-fold.

Since the results of Fig. 4 are obtained by titration of the extracellular fluid of the cultures, these growth curves may not necessarily parallel the synthesis of cellular virus. To determine whether AMPS really extends the true intra-

cellular latent period or merely prevents the release of mature virus, the development of virus in the tissue was also followed.

Measurement of the Development of Cellular Virus.—

Nine flasks were prepared as in the experiment of Fig. 1. They were inoculated at zero time with a small amount of virus, and 2 hours later 1 mg. of AMPS was added to each flask. At hourly intervals from the 2nd to 10th hour, cultures were removed from the water bath, and

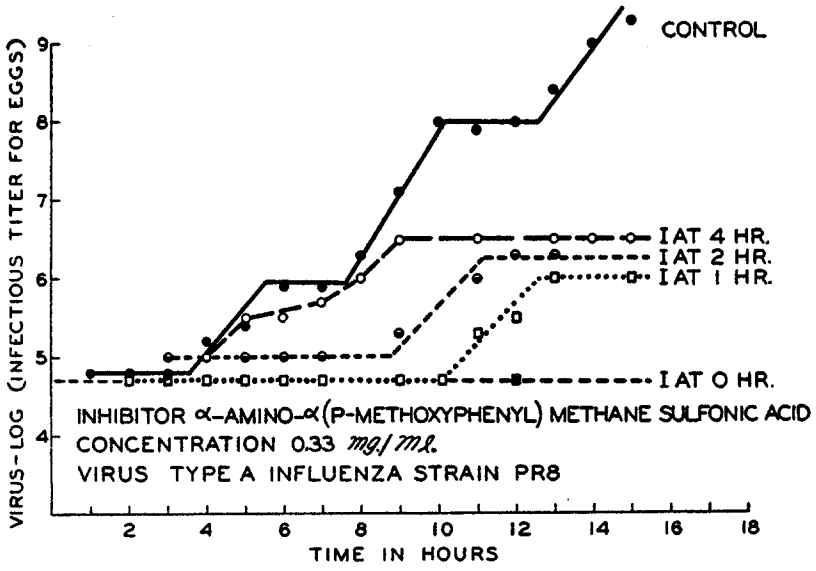

FIG. 4. Effect of an inhibitor on the lag phase of virus development. Each culture contained 400 mg. of tissue. The inoculum was added at zero time in each case and consisted of 0.3 ml. of medium with a titer of $10^{-6.0}$. The titrations represent values obtained for the extracellular fluid. The control curve is a composite made from 4 similar experiments. One flask was used for each curve.

the tissues and fluids were titered separately. In this way the development of the virus in the tissue and the appearance of virus in the fluid could be followed.

From the data plotted in Fig. 5, it can be seen that the titer of the fluid was constant until the 7th hour. Between the 7th and 11th hours, the latter titer increased from $10^{-4.5}$ to $10^{-5.7}$. In contrast, the rise in the titer of the tissue occurred at 4 hours, and by the 7th hour it had increased from $10^{-4.5}$ to $10^{-6.3}$. As the titer of the fluid began to rise, there was a corresponding fall in the titer of the tissue.

It is apparent that the increased latent period as measured by observing the fluid is not paralleled by a corresponding delay in the intracellular production of virus. The action of the delayed addition of AMPS is directed primarily

toward preventing the release of mature virus from the host cell rather than increasing the true latent period. Since virus is not released until the 7th or 8th hour, this technique permits the study of the rate of intracellular viral synthesis. It will be noted from these data (Fig. 5) that the cellular virus appears also over a considerable period of time and at what seems to be an exponential rate.

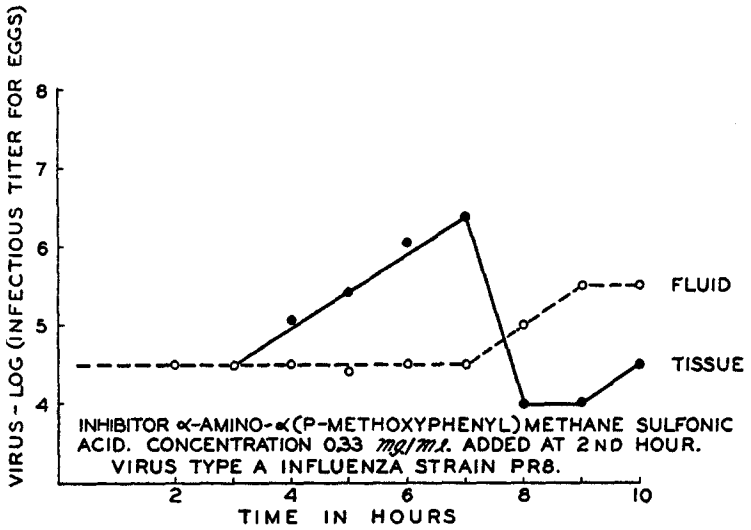

FIG. 5. Effect of inhibitor on intracellular viral development. Each culture contained 400 mg. of tissue. The inoculin was added at zero time and consisted of 0.3 ml. of medium with a titer of $10^{-6.7}$. One culture was used to obtain data for the points at each time interval. The tissues were ground separately with alundum, and the titers obtained represent the infectious titer expressed per gram of tissue. The titers of the fluids are recorded on a per milliliter basis.

Early Rate of Viral Synthesis with AMPS.—If the synthesis of virus proceeds at the normal rate in the presence of a delayed addition of AMPS, the total viral content of control and treated cultures should be similar.

To make this comparison, 14 flasks were prepared with virus and tissue as in the experiment of Fig. 1. AMPS was added to 7 of these at the 2nd hour. At hourly intervals from 2 to 9 hours tissues were removed from one control and one experimental flask and ground separately with alundum. The corresponding fluids were added and the mixture titered to estimate the total virus content of each flask. The resulting data are found in Fig. 6.

Throughout the early stages the rate of viral production was, within the accuracy of the experiment, the same in the control and treated cultures. The similarity of the curves when the total virus is measured is in contrast to the differences seen in the fluids. It is clear from the results of Figs. 4 to 6

that the early addition, up to 30 minutes, of AMPS prevents the multiplication of virus in the tissue, while the later addition permits multiplication but interferes with the liberation process.

Effect of AMPS in the Presence of a Massive Inoculum.—In view of the effects of the delayed addition of this sulfonic acid, it was of interest to test it under conditions in which only one cycle of growth could take place, namely under conditions of a saturating level of inoculum.

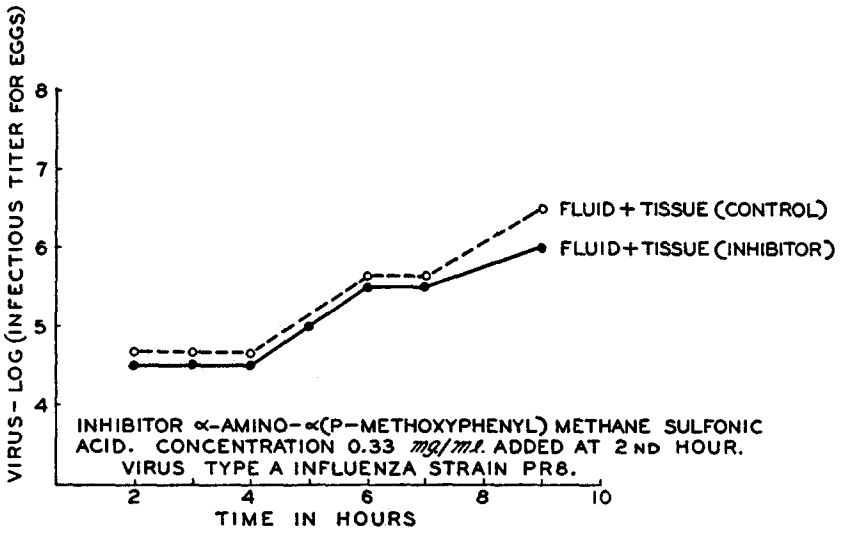

FIG. 6. Effect of inhibitor on total yield of virus production. One culture was used for each point obtained. Each culture contained 400 mg. of tissue which was removed, ground with alundum, and combined with appropriate fluid. The original inoculum was 0.3 ml. and had a titer of $10^{-5.7}$.

Two flasks were prepared with tissue and large inoculum of virus as in the experiment of Fig. 2. After 1 hour and 35 minutes, the tissues were removed and washed in saline and immune serum as described before (Fig. 2). One tissue was placed into fresh medium containing 1 mg. of AMPS while the second served as a control. The fluids were sampled periodically, and the resulting data and details of the experiment are recorded in Fig. 7.

The titer of the fluid in the control began to rise after a latent period of 2 hours and showed a 10^6-fold increase at 12 hours when the maximum was attained. The culture containing AMPS had a latent period of 5 to 6 hours which was followed by an abrupt rise in the titer of the fluid. Between 6 and 12 hours a further gradual rise in titer was observed until the maximum was reached. It appears that while a considerable rate of viral synthesis can take place in the presence of the sulfonic acid, the maximum titer ($10^{-7.5}$) is something less than the control ($10^{-8.3}$). This may result from a true effect upon

virus production or from incomplete release from the tissue. Since the early synthetic rate appears identical in the control and treated cultures (Fig. 5), these results may show some inhomogeneity in what appears to be a one step growth curve. When a large inoculum is used, not only is the latent period of the control shortened but also that of the inhibited culture. However, with both large and small inocula the percentage effect is about the same.

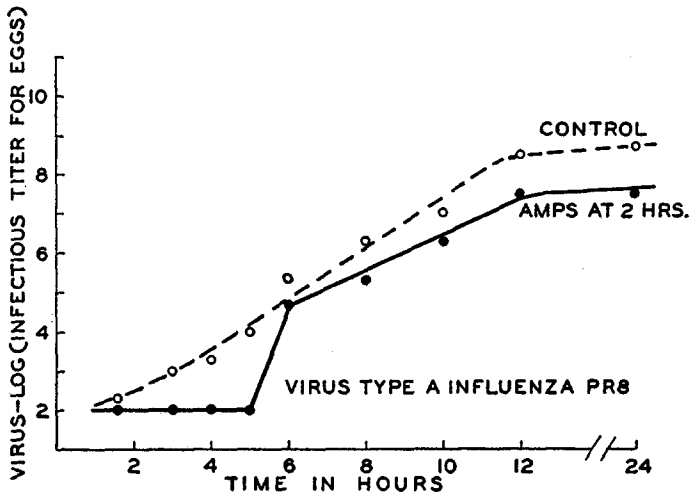

FIG. 7. Effect of AMPS on a culture with a massive inoculum. Each curve represents data obtained with a single flask. Each culture contained 400 mg. of tissue and 0.3 ml. of undiluted allantoic fluid with a titer of $10^{-9.3}$. The residual inoculum was washed away after 1 hour and 35 minutes of incubation at 37°C. The sulfonic acid at a concentration of 0.33 mg./ml. was added at the end of the 2nd hour.

DISCUSSION

That the liberation of influenza virus does not parallel the burst phenomenon seen with bacterial viruses was recognized several years ago on the basis of respiration data obtained with infected tissues (1). It was concluded that the virus was released without concurrent destruction of the membrane of the infected cell. The results presented here showing the liberation of virus over a period of many hours (Figs. 2 and 3) also support this concept and are not inconsistent with recent reports by other workers (10). The finding raises many interesting questions as to the biochemical mechanisms involved in the transfer of large particles through the semipermeable membrane of such animal cells.

It is apparent that AMPS inhibits some process occurring early in the latent period which may represent one step by which the normal cell is adapted for the production of virus. Unfortunately the site of action of this sulfonic

acid at the molecular level is not known (4). That information would allow the identification of an important reaction. Since the process occurs so soon after inoculation, some influence upon adsorption or penetration is suggested. At first sight the data obtained would imply a dual action of AMPS, one to prevent the synthesis of virus and a second to affect the release or liberation. However, it is quite likely that the initiation of infection, *i.e.* adsorption and penetration of the parent virus, may involve some of the same reactions important later in the release of the progeny. In considerations of this phenomenon of liberation-inhibition, it may not be of particular value to assume at this time that the barrier to the release is necessarily the cellular membrane. If other intracellular structures represent the site of viral multiplication, the penetration and release of the virus may involve the limiting parts of those structures (11).

It is clear in each experiment (Figs. 4, 5, and 7) that the effect of the delayed addition of AMPS is to impair rather than prevent the liberation of virus. After a time, the influence of AMPS appears to be overcome and the release of virus proceeds rapidly. This waning of the effect is not readily explained on the basis of the inactivation of the sulfonic acid. The question arises as to whether the virus produced in the presence of AMPS is identical with the normal virus. The delay in liberation could result from an impaired ability of the virus to alter some cellular structure. This implies an active role of the virus in the liberation process.

While the mechanism of action of AMPS is unknown, it is clear that a wide variety of important cellular activities are not affected. This is shown by the excellent rate of synthesis of viral protein, nucleic acid, and lipid which can take place in the presence of the inhibitor when it is added 1 hour after the virus. This fact may serve to explain the low toxicity of the compound *in vivo*. Its site of action in the growth cycle of the virus seems ideal for a minimum of interference with normal cellular activity, and though the molecule does not have the proper chemical and physical properties of a useful drug, it may be worthy of consideration as a model for further chemotherapeutic studies.

SUMMARY

The growth characteristics of influenza virus in an isolated tissue maintained *in vitro* have been described. When compared with previously reported results using the embryonate egg, a considerably shorter latent period was observed. The release or liberation of the virus occurred throughout a period of many hours. There was no evidence of a general "burst" phenomenon, and the destruction of a cellular membrane did not seem to be essential to or concomitant with the release of virus.

An early phase in the development of virus was described which is sensitive to the action of α-amino-*p*-methoxyphenylmethanesulfonic acid and it is by virtue of this that virus multiplication is prevented. If this phase was allowed to go on to completion, replication of virus occurred even in the presence of the sulfonic acid, but the release of virus from the tissue was impaired.

It is suggested that the sulfonic acid may interfere with the adsorption or penetration of the virus and that the initiation of infection and the liberation of new virus may be processes which share some common character.

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