GROWTH CHARACTERISTICS OF INFLUENZA VIRUS
BIOCHEMICAL DIFFERENTIATION OF STAGES OF DEVELOPMENT. II*

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(Received for publication, June 30, 1955)

In a multicellular culture and probably in a single cell, the production of virus which follows infection represents a continuous process with many of the stages of development proceeding at the same time (1-3). Even when dealing with a single sequence of infection (3, 4), it is difficult to learn of the interdependence and order in which reactions occur. The possibility of synchronizing the viral synthetic process by the use of phase-specific inhibitors has been explored in the past (5). In the present experiments, the technique is employed to investigate the interrelationship of the function of methionine and phenylalanine in the synthesis of influenza virus.

p-Fluorophenylalanine antagonizes the utilization of phenylalanine and by virtue of this it shows potent antiviral activity in cultures producing either poliomyelitis virus (4), or the agent from Sendai, mentioned in reference 6. The evidence from other biological systems suggests that the fluoroderivative prevents the participation of phenylalanine in the synthesis of protein (7).

Several structural analogues of methionine have been found which will inhibit the multiplication of influenza virus. However, methionine is known to be involved in many biological processes and it is unlikely that the various metabolic antagonists of methionine interfere with the same process (8). The inhibitory action of methoxinine and ethionine can be reversed by methionine (9), but that of methionine sulfoximine can not. Apparently, this latter structural analogue is not a metabolic analogue of methionine. The inhibition of influenza virus induced by this substance is reversed by glutamine, but not by glutamic acid or methionine (10).

The site of action of methoxinine in the infectious sequence has been most studied and has been defined relative to the effects produced by the receptor-destroying enzyme (from cholera vibrio) and α -amino-*p*-methoxyphenylmethane sulfonic acid (5). In the present study the phase specificity of methoxinine is used as a metabolic marker to describe the relative site of action of *p*-fluorophenylalanine. It will be shown that like methoxinine, *p*-fluorophenylalanine is phase-specific, does not interfere with the initiation of infection, but rather acts during the productive period of

* Aided by a grant from The National Foundation for Infantile Paralysis, Inc.

the infectious sequence. The two inhibitors act upon different reactions which for the most part occur simultaneously but are closely interdependent. It has been possible to define two developmental stages which could not be recognized from the usual growth curve.

Materials and Methods

Virus and Tissue.—In all experiments of this study, the PR8 strain of Type A influenza virus was used. It 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-old embryonate eggs.

dl-Methoxinine.—(*dl*- α -amino- γ -methoxybutyric acid) was generously supplied by the American Cyanamid Company.

dl-Fluorophenylalanine.—(FPA)¹ was kindly supplied by Dr. Marvin D. Armstrong at the University of Utah.

Immune Serum.—The immune serum was obtained from ferrets which had received by intranasal instillation allantoic fluid containing the PR8 strain of influenza virus. The hemagglutination-inhibition titer was 4096.

*Receptor-Destroying Enzyme (RDE)*² was a partially purified preparation of cholera vibrio extract obtained from the allantoic inoculation of embryonate eggs. It contains approximately 32,000 units of receptor-destroying enzyme per mg. of nitrogen.

Warburg Flask Culture.—A detailed description of the method has been given elsewhere and details of individual experiments will be found in the experimental section (9, 11). As routine the host-virus system was maintained in a Warburg apparatus using as medium a modified Simms solution. The center-well contained Pardee's mixture. The reaction chamber of the vessel contained 400 mg. of chorioallantoic membrane that was used without mincing. The cultures were incubated with shaking at 37°C.

Virus Titrations.—The amount of virus was estimated by determining the infectious titer for eggs. For this purpose, tenfold serial dilutions were prepared in broth, and four to six eggs were inoculated with 0.1 ml. of each dilution. After 3 days of incubation at 37°C, a sample of allantoic fluid was removed from each egg and tested for virus by the addition of red blood cells. The 50 per cent infectious titer was calculated by the method of Reed and Muench (12). The titers expressed in the paper represent the number of 50 per cent egg infectious doses per 0.1 ml. of sample.

EXPERIMENTAL AND RESULTS

Relation of the Age of Infection to the Inhibitory Effectiveness of FPA and Methoxinine.—A number of inhibitors of viral production have been found to be not only metabolically specific but also phase-specific. When such compounds are added to a culture in which a single sequence of infection is occurring, the ability of the compound to inhibit virus increase depends upon what portion of the sequence has occurred; *i.e.*, upon the age of the infection. This implies the presence of discrete phases in the synthesis of virus. Methoxinine is such a phase-specific inhibitor. While being inhibitory, it does not affect the initiation of infection, those functions for which the RDE-sensitive

¹ FPA, *p*-fluorophenylalanine.

² RDE, receptor-destroying enzyme from a partially purified preparation of cholera vibrio extract.

receptors are required, the process inhibited by α -amino-*p*-methoxyphenyl-methanesulfonic acid, the last stages of viral maturation nor the release of newly formed virus (4). To determine the site of action of *p*-fluorophenyl-alanine (FPA) and to test its phase-specificity, it was studied in conjunction with methoxinine whose influence at a distinct phase of virus development was well identified.

To do this, methoxinine and FPA were added separately to parallel cultures in which a single sequence of infection was induced with a massive viral inoculum. By adding the inhibitors to cultures at various times after infection and determining the per cent inhibition which could be obtained, it was possible to determine whether the reactions inhibited by FPA and methoxinine were occurring at nearly the same stage of the growth sequence.

Replicate cultures were prepared in Warburg vessels with a modified Simms medium in the reaction chamber and Pardee's mixture in the center-well. Each flask contained 400 mg. of chorioallantoic membrane and 0.3 ml. of undiluted allantoic fluid infected with approximately $10^{8.0}$ EID₅₀ per 0.1 ml. After 1 hour of incubation with shaking at 37°C., the tissues were removed, washed six times in cold saline, one time in immune serum, and transferred to a second culture flask containing only Simms's medium. Several of the above cultures, which comprised one series, received a single addition of 0.3 mg. of FPA at some interval between 0 and 7 hours. When the additions were made at 0 hour or 1 hour after virus, they were added necessarily to the first flask in which the infection was initiated. Then the membranes were transferred to a second flask which already contained FPA and in which the production of virus was followed.

The fluid portions of the cultures were all sampled at 2 hours, at the time FPA was added, at 12 hours and at 24 hours. From these observations it was possible to determine the viral increase which occurred before the addition of FPA and also afterward. The per cent inhibition which ensued upon each addition of FPA was calculated from the actual increase observed by the 12th hour and the maximal increase of virus possible as determined by the titer of virus at the time of addition of the inhibitor and the titer of the control culture at the 12th hour.

In a second series *dl*-methoxinine was added to the cultures in the same manner as described for FPA. The additions of methoxinine were 4.0 mg. per flask.

The resulting data from both series are tabulated in Table I. It will be noted that the amount of each inhibitor used was sufficient to produce complete inhibition when added at the same time as the virus and retained throughout the incubation period. Both compounds became less effective when added later in the infectious sequence until finally by the 7th hour no inhibitory effect was evident. When added progressively later in the infectious sequence, there was some indication that methoxinine became less effective at a greater rate than FPA. By the 4th hour, methoxinine would produce only partial inhibition while FPA was almost completely effective. This suggests that the two analogues act upon different phases of development which occur close upon each other. The one sensitive to methoxinine may be completed prior to that influenced by FPA. The absence of clearly separated

periods of sensitivity and resistance is probably due to the lack of synchronization of the synthetic centers and has been discussed previously (5). From these data one cannot tell the order in which the sensitive phases begin and it is there that the two should be most clearly distinguished. This point will be considered next by a different type of analyses.

TABLE I
Relation of Inhibiting Capacity of FPA and Methoxinine to Age of Infection

Time of adding inhibitor	Viral titers* of cultures				Per cent† inhibition at 12th hr.
	at 2 hrs.	At time of adding inhibitor	At 12 hrs.	At 24 hrs.	
<i>p</i> -Fluorophenylalanine‡ 0.1 mg./ml.					
None	3.4	—	8.9	9.2	—
0	3.0	—	3.0	3.3	100
1	3.9	—	3.9	4.4	100
2	3.8	3.8	3.6	4.5	104
3	3.3	4.3	4.5	4.5	98
4	3.5	4.7	5.0	5.5	93
5	3.5	5.0	6.4	6.8	64
6	3.1	6.4	7.6	8.4	52
7	3.1	7.3	8.8	9.4	0.6
<i>d</i> L-Methoxinine 1.3 mg./ml.					
None	3.3	—	8.8	9.4	—
0	3.0	3.0	2.7	3.0	100
4	3.5	4.3	5.5	6.7	73
5	3.3	5.3	7.0	7.9	51
6	3.3	6.4	8.1	8.8	29
7	3.3	7.3	8.9	9.4	0

* Viral titers expressed as $-\log$ of the infectious titer for eggs.

† Per cent inhibition calculated: control titer at 12th hour—titer of treated culture at 12th hour/control titer at 12th hour—titer of treated culture at time of addition of inhibitor.

§ These data are a composite of four closely comparable experiments.

|| These data are taken from two closely comparable experiments.

Increase of Extracellular Virus Following Virostasis Induced with FPA.—When an infected culture of cells is inhibited by a reversible inhibitor and the inhibition is maintained for several hours, much of the individual differences in the response of cells to virus can be reduced and the sequence of infection synchronized. Though some cells may have become infected earlier than others and the development of virus begun sooner or is proceeding more rapidly in some, during the period of virostasis an opportunity is had for the infection in all cells to proceed to the same stage of development at which it is arrested and there to await the reversal of inhibition for further maturation.

tion. When the inhibition is relieved, development proceeds as if all centers of synthetic activity were at the same metabolic point. If virostatics is induced by different inhibitors which act upon phases which begin at different times in the infectious sequence, development in one case may proceed further during virostatics than in another. Upon release of the inhibition, the difference may be manifest in the growth curve as is shown in the following experiments using methoxinine and FPA.

Two cultures were prepared in the usual manner with 400 mg. of chorioallantoic membrane and Simms's solution. Each received 0.3 mg. of FPA and 0.3 ml. of allantoic fluid which

FIG. 1. The tissues were exposed to virus ($3 \times 10^{6.0}$ EID₅₀) in the presence of 0.1 mg. of FPA (*p*-fluorophenylalanine) for 1 hour at 37°C. after which 0.2 ml. of RDE (receptor-destroying-enzyme) was added. The tissues were washed at 2 hours in saline and added to a second flask containing FPA and RDE. At the 7th hour, 0.2 mg. of PA (*d*-phenylalanine) was added to one of the cultures.

supplied $10^{6.0}$ EID₅₀ of influenza virus. After 1 hour of incubation 0.2 ml. of a preparation of RDE containing 32,000 units of enzyme was added. Incubation was continued for a 2nd hour and then the tissues were removed, washed six times with saline, once with immune serum, then replaced into culture flasks containing medium, RDE and FPA. To one of these no further additions were made and the extracellular fluid was titrated for virus at 2.3, 7, and 12, and 24 hours. The second flask received 0.4 mg. of phenylalanine at the 7th hour. The fluid portion of the culture was tested for virus at 2.3, and 7 hours and also at each 0.5 hour interval following the 7th hour.

In the normal growth curve an increase in virus is not detectable until 3 to 4 hours after the addition of the viral inoculum. It will be noted from the data plotted in Fig. 1 that within 30 minutes or less after the addition of phenylalanine, the virus of the fluid had increased 20-fold. Upon release of the inhibition, the production of virus proceeds with little or no lag and further the rate of production is extremely rapid so that the maximum titer is reached

in 3 hours. Apparently, the rate-determining reaction of the viral synthetic process is different from that which operates normally. This indicates that in the usual production of virus the rate-limiting reaction is in the early rather than advanced stages of development.

It is also apparent that in the presence of FPA, infection can be initiated and the function of the receptors sensitive to RDE can be completed. The process which is inhibited by FPA is not irreversibly damaged. The inhibition can be reversed by phenylalanine after a virostatic period of 3, 7, 10, or 24 hours with excellent yields of virus. This is in contrast to the results obtained with poliomyelitis virus propagating in HeLa cells (4). However, it should be

FIG. 2. Each point represents the titer of the tissue and fluid portions of a separate culture. All tissues were exposed to virus ($3 \times 10^{5.0}$ EID₅₀) in the presence of 0.1 mg. of FPA (fluorophenylalanine) and subsequently treated with RDE (receptor-destroying enzyme) PA (phenylalanine) and washed as described under Fig. 1.

noted that if the virostatic period is 3 hours, production of influenza virus is resumed after a clearly detectable lag. When the period of virostatic was 7, 10, or 12 hours, no lag was discernible.

Increase in Total Virus Following Virostasis Induced with FPA.—The sudden increase of virus in the fluid after release of the inhibition suggests virus had accumulated in the tissue during the virostatic period. However, this is apparently not the case as is indicated by the following experiment.

Seven replicate cultures were prepared as described in the previous section with medium, tissue, virus, and FPA. The addition of RDE was made to each culture at the end of the 1st hour of incubation. Each tissue was incubated for 1 hour more and then washed with saline and immune serum. The washed tissues were replaced into vessels containing RDE, FPA, and Simms's solution. At 2.3 hours one tissue was removed, ground with alundum and titrated for virus. The virus of the fluid portion of the curve was also estimated. The remaining six cultures received at the 7th hour 0.4 mg. of phenylalanine. Tissues were removed at

the 7th hour and at each 0.5 hour interval until the 9th hour. The total virus of individual replicate flasks could be determined from the titration of the corresponding fluid and the membrane. The resulting data showing the increase in the total virus as a function of time before and after the addition of phenylalanine are plotted in Fig. 2.

No increase in virus was observed in the virostatic period from 2.3 to 7 hours. Further, within the first half hour after the addition of phenylalanine, an increase in the total virus of the culture could be detected. The results closely parallel the observations made of the extracellular fluid. Apparently, these reactions which must occur in the constant period before there is any production of new virus, occur also when fluorophenylalanine is present.

Viral Increase Following Virostasis Induced by Methoxinine.—Next the

FIG. 3. The data were all obtained by sampling the fluid portion of one culture at various times. The experiment was patterned after that described in Fig. 1. MX (methoxinine) was used at a concentration of 1.3 mg. per ml. and removed by transferring the tissue to a fresh culture free of methoxinine.

effect upon the growth curve of virostatics induced by methoxinine was studied in a series of experiments designed after the pattern described for FPA in the second section. In the place of FPA, 4.0 mg. per culture of methoxinine was used. At the 7th hour the tissue was washed in saline and transferred to a second flask to free the tissue of methoxinine. The appearance of influenza virus in the fluid portion of the culture was followed and the results are plotted in Fig. 3. The experiment and results are similar to that experiment described in a previous publication (3). In the present series of experiments varying periods of virostatics (3, 7, 12 hours) were investigated.

The findings are in contrast to those obtained with FPA. A constant period of at least 2 hours elapsed between the release of inhibition and the first detectable increase in virus titer. It is apparent that a considerable amount of biochemical activity, which must occur during the constant period before the production of any new virus, did not occur in the presence of methoxinine.

Again there is the increased slope of the production portion of the growth curve. The two phases in the viral development which were suggested by the variation in sensitivity of different portions of the infectious sequence to the action of methoxinine and FPA are more clearly distinguished. The data imply that the methoxinine-sensitive phase begins before the phase which is inhibited by FPA.

Sequential Action of Methoxinine and FPA.—The interrelation of these phases of viral development was further illustrated by determining the ca-

FIG. 4. The concentrations employed were: 0.1 mg. of FPA (*p*-fluorophenylalanine) per ml., 1.3 mg. of MX (methoxinine) per ml., 0.2 mg. of PA* (*dl*-phenylalanine) per ml. The orders of addition are presented with the corresponding curves. The data presented for each curve were obtained by repeated sampling of the fluid portion of one culture.

capacity of each analogue to inhibit the viral increase which follows the virostatic periods induced by FPA and methoxinine.

Five culture flasks were prepared containing the usual culture medium, 400 mg. of chick membrane, a massive inoculum (3×10^8 EID₅₀) or influenza virus, and 0.3 mg. of FPA. One of the cultures also received 0.4 mg. of phenylalanine while a second was given 0.4 mg. of phenylalanine plus 4.0 mg. of methoxinine. At the end of 1 hour the tissues were washed to remove residual inoculum and placed into new vessels with the same contents as those in which infection was initiated; however, no virus addition was made. To two of the flasks receiving only FPA at 0 time, further additions were made at the 7th hour. One flask received 0.4 mg. of phenylalanine and to the second was added both 0.4 mg. of phenylalanine and 4.0 mg. of methoxinine. At various intervals from 2 to 12 hours the fluid portions of all cultures were sampled and titrated for virus. The resulting data will be found in Fig. 4.

* PA, *dl*-phenylalanine.

The flask containing FPA throughout and which received no other addition showed no viral increase by 24 hours. Hence, the concentration of FPA used was sufficient to prevent viral multiplication when added at 0 time.

The culture receiving FPA and phenylalanine at 0 time showed a viral growth rate quite similar to the usual untreated infected membrane. The concentration of phenylalanine employed antagonized completely the effect of FPA.

When a culture received FPA, phenylalanine, and methoxinine at the time of addition of virus, no viral increase was detected by the 24th hour. Thus the addition of 4.0 mg. of methoxinine at the time of initiation of infection and in the presence of FPA and phenylalanine, *i.e.* to a culture capable of supporting viral multiplication, is completely inhibitory. The result then can be compared with that obtained when methoxinine is added to a similar culture at the 7th hour.

The fourth culture to which phenylalanine was added at the 7th hour produced a substantial viral increase comparable to the culture receiving PA and FPA at zero time. However, the rate of viral increase, as also shown in Fig. 1, is so rapid that the maximum yield is obtained, after a virostatic period, at nearly the same time as the control.

The fifth culture received both phenylalanine and methoxinine at the 7th hour and it showed a limited amount of growth. Clearly the methoxinine was not a very effective inhibitor when added after the virostatic period induced by FPA. The effectiveness of methoxinine was also tested after 10 and 12 hour FPA virostatic periods. Under such conditions the yields of virus were larger than after the 7th hour period but did not approach the viral yield of the control.

In a similar series of experiments, the inhibitory effect of FPA was tested upon a culture producing virus after a virostatic condition was imposed for 7 hours by methoxinine. The viral multiplication which follows virostatics induced by methoxinine could be completely inhibited by FPA.

DISCUSSION

The observations presented here are intelligible if one assumes that two stages of viral development, A and B, are occurring in the infected multicellular culture. For the most part these occur simultaneously. Reaction A begins early in the latent period; it is inhibited by methoxinine but not by FPA. Further, it can proceed independent of the second reaction. Reaction B is sensitive to the action of FPA but not to that of methoxinine. This reaction cannot proceed unless the first reaction is occurring or has operated for some prior interval. Because of this interdependence, the second reaction can be indirectly prevented by the addition of methoxinine. The inability of the system to bring reaction A entirely to completion when B is inoperative (as in-

dicated by the partial inhibitory effect of methoxinine after virostatics induced by FPA) may be similar to the product-inhibition phenomenon seen in many blocked enzymatic reactions in which there is product accumulation.

It is of interest to note that by this technique it was possible to isolate stages of development which usually occur in multicellular cultures in widely overlapping intervals of the infectious sequence. As the list of carefully studied inhibitors grows, it will be possible to map the viral synthetic pathway in terms of these metabolic markers. The technique will be of particular value in the future study of the problem by classical biochemical methods. For example, it would be of great interest to know whether the interdependent reactions A and B represent the synthesis of nucleic acid and protein respectively.

SUMMARY

A further analysis of the growth curve obtained *in vitro* for influenza virus in chorioallantoic membrane has been made using the viral inhibitor *p*-fluorophenylalanine. It has been found that *p*-fluorophenylalanine is phase-specific, does not interfere with the initiation of infection but rather acts during the productive period of the infectious sequence. The site of action of this fluoroderivative has been described relative to the site of action of methoxinine. By the use of this inhibitor in combination with methoxinine, it has been possible to recognize two stages of development which were not discernible from the usual growth curve. In a multicellular culture these two reactions, A and B, occur for the most part simultaneously, during the latent and productive periods. Reaction A is inhibited by methoxinine, but not by fluorophenylalanine. It begins early in the latent period and can proceed independent of reaction B. Reaction B is inhibited by fluorophenylalanine, but not by methoxinine, and it cannot proceed unless reaction A is proceeding or has operated for some prior interval.

BIBLIOGRAPHY

1. Cairns, H. J. F., *J. Immunology*, 1952, **69**, 168.
2. Dulbecco, R., and Vogt, N., *J. Exp. Med.*, 1954, **99**, 183.
3. Ackermann, W. W., and Maassab, H. F., *J. Exp. Med.*, 1954, **99**, 105.
4. Ackermann, W. W., Rabson, A., and Kurtz, H., *J. Exp. Med.*, 1954, **100**, 437.
5. Ackermann, W. W., and Maassab, H. F., *J. Exp. Med.*, 1954, **100**, 329.
6. Ackermann, W. W., and Maassab, H. F., *Minerva Med.*, 1954, **XLV**, 1, N42, 1.
7. Halvorson, H. O., and Spiegelman, S., *J. Bact.*, 1952, **64**, 207.
8. Pearson, H. E., Lagerborg, D. L., Winzler, R. J., and Visser, D. W., *J. Bact.*, 1955, **69**, 225.
9. Ackermann, W. W., *J. Exp. Med.*, 1951, **93**, 337.
10. Ishida, N., and Ackermann, W. W., unpublished results.
11. Ackermann, W. W., *J. Biol. Chem.*, 1951, **189**, 421.
12. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.