JOURNAL OF BACTERIOLOGY
Vol. 87, No. 2, p. 363-369 February, 1964

ENZYMATIC VARIANTS OF INFLUENZA VIRUS

III. FUNCTION OF NEURAMINIDASE IN THE VIRAL GROWTH CYCLE

BILLIE L. PADGETT AND DUARD L. WALKER

Department of Medical Microbiology, University of Wisconsin Medical School, Madison, Wisconsin

Received for publication 23 September 1963

ABSTRACT

PADGETT, BILLIE L. (University of Wisconsin, Madison), AND DUARD L. WALKER. Enzymatic variants of influenza virus. III. Function of neuraminidase in the viral growth cycle. J. Bacteriol. 87:363-369. 1964.—Multiplication of a slowly reacting enzymatic variant of influenza B virus, strain LEE, was compared with that of parent virus in ovo. At 35 C, although their final yields were equal, variant virus reached its peak concentration in the allantoic fluids later than parent virus. At 39 C, multiplication of both viruses was slower, parent virus requiring 4 hr and variant virus 8 hr longer to reach infectivity levels comparable with those at 24 hr at 35 C. Variant enzyme activity in vitro can be controlled by altering the temperature and calcium concentration. Growth curves of these viruses in pieces of chorioallantoic membrane (CAM) in culture under conditions in which the variant should be as active as the parent revealed only minor differences between them. Under conditions in which variant enzyme activity would be much slower than the parent, the release of variant virus from the CAM was delayed and the rate of release was slower. Under the most adverse conditions, 39 C and no calcium, formation of infectious variant virus ceased after 5 hr, and the hemagglutination inhibitor in the cells was not degraded although hemagglutinins were produced. These findings are discussed in relation to the function of neuraminidase during viral multiplication.

All myxoviruses exhibit neuraminidase activity. This enzyme is believed to act at some stage in the multiplication cycle of these viruses, but its exact function has not been established. We have isolated a variant of influenza B strain LEE virus which differs from the parent viral population only in characteristics related to this enzyme (Padgett and Walker, 1957, 1958). The enzymatic activity of the variant virus in vitro can be manipulated by changes in temperature and calcium ion concentration so that its rate in one case is equal to that of the parent virus and in another case is markedly slower than that of the parent virus. The multiplication cycles of the parent and variant viruses were compared under various conditions to determine whether there were differences between them and to see whether changes in the environment previously shown to alter the rate of variant enzyme activity would affect a particular step in its multiplication.

MATERIALS AND METHODS

Viruses. The LEE strain of influenza B virus, referred to as stock LEE virus, and the variant 1 virus isolated in this laboratory, referred to as variant virus, were used. Stock LEE seed virus was prepared by inoculating 10-day embryonated eggs intraallantoically with 0.2 ml of virus diluted 10^{-4} and incubating them at 35 C for 26 hr. The allantoic fluids were harvested individually, and those with a hemagglutination (HA) titer of 512 or greater were pooled. Samples of the pool were stored in glass ampoules at −70 C. The 50% egg infectious dose (EID_{50}) of this pool was 10^9/0.2 ml. A pool of variant virus was prepared similarly from eggs inoculated with a 10^{-6} dilution of virus and incubated for 40 hr. The EID_{50} of the variant seed virus was $10^{8.63}$/0.2 ml.

Culture medium. The modified glucosol of Fulton and Armitage (1951), consisting of glucose and sodium, magnesium, and calcium chloride buffered at pH 7.28 with a phosphate buffer, was used. Glucosol without calcium chloride was used where indicated. Penicillin, 10 units/ml, and streptomycin, 40 μg/ml, were added to the medium just prior to use.

Chorioallantoic membrane (CAM) cultures. The procedure used to obtain pieces of CAM from 10-day embryonated eggs was similar to that reported by Tamm, Folkers, and Horsfall (1953). Pieces of CAM with a surface area of 2.9 cm^2 were cut out with a circular steel punch. Each piece of CAM was washed in the appropriate medium, stripped from the shell, and placed in a large (25 × 150 mm) rubber-stoppered test tube con-

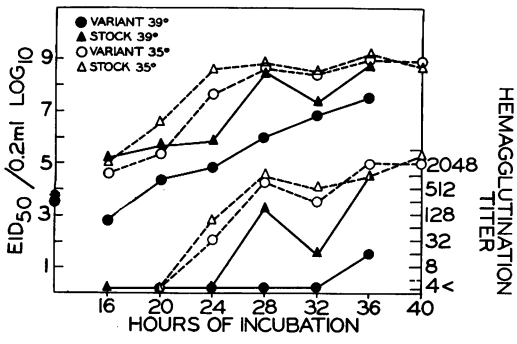

FIG. 1. *Multiplication of stock LEE and variant viruses in ovo at 35 and 39 C. The titers of infectious virus (upper lines) are based on 0.2-ml volumes, and the hemagglutination titers (lower lines) on 0.4-ml volumes. Each point represents a sample from the pooled allantoic fluids of four eggs.*

taining 0.9 ml of culture medium. The pieces of CAM were randomized as follows. Six eggs were selected at random, and from each egg six pieces of CAM were obtained. Each piece of CAM from a single egg was placed in a different group; thus six groups of cultures were formed, each group having one piece of CAM from each of the same six eggs. Three of these groups were infected with stock LEE virus and three with variant virus by the addition to each tube of 0.1 ml of seed virus diluted to the desired concentration in cold medium. The culture tubes were incubated upright in a water bath with continuous horizontal shaking for 1 hr. The membranes, in groups, were removed from the tubes and washed by serial transfer through three petri dishes containing the appropriate medium. Each piece of CAM was placed in another test tube containing 1.0 ml of medium and reincubated. When harvested, the fluids of each group were pooled. Samples were prepared by adding 0.1 ml of heat-inactivated (56 C for 30 min) horse serum to 0.9 ml of the pool and were stored at −70 C until used for infectivity titrations. The rest of the pool was stored at 4 C until used for HA titrations. The membranes were also pooled in groups and stored at −70 C.

Virus titrations. Infectivity titrations were done in ovo at 35 C, with a postinoculation incubation time of 72 hr. The EID_{50} was calculated by the method of Reed and Muench (1938).

HA titrations were performed in tubes, and the HA titer was based on the pattern of sedimented red blood cells (RBC). For titrations of allantoic fluids, 0.4-ml volumes and 1% chick RBC were used. Titrations of CAM suspensions and CAM culture fluids were based on 0.5-ml volumes and 0.125% RBC. The HA inhibitor present in CAM suspensions was destroyed with periodate, as described by Granoff (1955).

CAM suspensions. The pooled membranes were thawed, suspended in 2 ml of glucosol without calcium chloride, and ground for 10 min in a motor-driven Teflon grinder. The grinder was rinsed twice with 2 ml of the medium, making a total volume of 6 ml. The suspension was centrifuged lightly, and the supernatant fluid was used for virus titrations.

Hemagglutination-inhibition (HI) titrations. Portions (1 ml) of CAM suspensions were heated in a boiling-water bath for 10 min, then cooled in an ice bath. Serial twofold dilutions, with 0.2 ml of the heated suspensions, were made in phosphate-buffered saline (pH 7.2), and 0.2 ml containing 4 HA units of heated (56 C for 30 min) LEE virus was added to each tube. After 30 min at room temperature, 0.4 ml of a 0.125% suspension of chick RBC was added, and the pattern of sedimented RBC was read after 45 min. The HI titer is expressed as the reciprocal of the last dilution showing complete inhibition of HA.

RESULTS

Growth curves of stock LEE and variant viruses in ovo. Comparisons were made of the multiplication of these viruses in ovo at 35 and 39 C by following the accumulation of infectious virus and hemagglutinins in the allantoic fluids. A relatively small inoculum was used so that multiple growth cycles would occur. Small differences in their growth would therefore be cumulative and would be detected more readily. Dilutions of seed stock LEE and variant viruses calculated to contain 10^4 EID_{50} were each inoculated intraallantoically into a large group of 10-day chick embryos. The eggs were incubated at 35 C in one experiment and at 39 C in another. At 4-hr intervals, beginning at 16 hr after inoculation, four eggs from each group were refrigerated; 2 ml of allantoic fluid from each of the four eggs were pooled, and samples were immediately stored at −40 C. The results of infectivity and HA titrations with these samples are shown in Fig. 1.

The concentration of infectious variant virus in the allantoic fluids at 35 C was below that of stock LEE virus at 16 hr; at 24 hr, when hemagglutinins were first detected, the HA titer of the

variant was below that of stock LEE virus. After 16 hr, both viruses accumulated in the fluids at similar rates, and their final yields were equal; but, the variant reached this maximal concentration later than did stock LEE virus. At 28 hr, both viruses were present in comparable concentrations, as measured by infectivity and HA. At 39 C, the concentration of infectious variant virus in the allantoic fluids increased steadily over the 36-hr period, but its rate of accumulation was markedly slower than at 35 C. At 36 hr, the level of infectious variant virus at 39 C was comparable only to that attained in 24 hr at 35 C. Stock LEE virus at 39 C was affected similarly but not to the same extent as the variant. At 39 C, the concentration of stock LEE virus at 28 hr was comparable to that reached in 24 hr at 35 C. It is difficult to compare the rates of increase of infectious stock LEE and variant viruses at 39 C, and the reason for the decrease in infectivity and HA titers of stock LEE virus at 32 hr is not known. Hemagglutinin production at 39 C, however, shows marked differences between stock LEE and variant viruses. Stock LEE virus was detected by HA at 28 hr, but the variant was not detected until 36 hr. At 35 C, both viruses had produced measurable hemagglutinins at 24 hr.

These results demonstrate that, at 35 C, the final yields of the two viruses were equal, but the variant took longer to complete its growth than did stock LEE virus. At 39 C, although both viruses appeared in the allantoic fluids at slower rates than at 35 C, the variant was affected to an appreciably greater degree. The EID_{50}/HA ratios indicated that the variant was not producing a greater proportion of noninfectious virus particles than was stock LEE virus, and that the slower rate of accumulation of infectious virus particles of both viruses at 39 C was not the result of increased heat inactivation nor of increased production of noninfectious virus particles.

Single-step growth curves of stock LEE and variant viruses in CAM cultures. Since the experiments in ovo demonstrated that the stock LEE and the variant virus differed in their multiplication, a more detailed study of their growth cycles was undertaken by using pieces of CAM in shake culture. A more exact control of the environment was possible in vitro, and therefore a clearer definition could be obtained of the effects on the various phases of viral replication of altering environmental factors. Previous work had shown that when sufficient calcium was present in the medium the rate of neuraminidase activity of the variant in vitro was comparable to that of stock LEE virus, and its activity, like that of stock LEE virus, was enhanced by increasing temperature. If calcium was absent, the activity of the variant was markedly slower than that of stock LEE virus; the activity was depressed by increasing temperatures, while that of stock LEE virus was unaffected.

Single-step growth curve experiments with stock LEE and variant viruses were performed at 35 and 39 C in the presence and absence of calcium to determine whether alterations in the calcium content of the medium would affect the growth of these viruses. Pieces of CAM, 2.9 cm^2 in size, were arranged in groups. Half of the groups were inoculated with 10^8 EID_{50} of stock LEE virus and half with 10^8 EID_{50} of variant seed virus. The CAM cultures were incubated at 35 or 39 C, and at intervals one group of membranes infected with each virus was harvested. The fluids and membranes were collected separately and stored for infectivity and HA titrations. In one experiment, the pieces of CAM were incubated in glucosol containing 0.009 M calcium chloride, and in another they were incubated in glucosol without calcium chloride.

In glucosol with calcium chloride, an environment in which the enzymatic activity of the variant should be comparable with that of stock LEE virus, the growth curves showed that stock LEE virus was produced in the membranes at a faster rate than the variant virus, but both viruses were released into the fluid at about the same rate. In glucosol without calcium chloride, an environment in which the enzymatic activity of the variant would be much slower than that of stock LEE virus, the growth curves revealed marked differences between the two viruses. These latter results are shown in Fig. 2, together with the curve for variant virus at 39 C in glucosol with calcium chloride for comparison. The results will be described in terms of the various phases of viral replication.

In some cases, a high level of residual virus tended to obscure the time of the first increase in infectious virus, but the variant virus appeared to have the same latent period as stock LEE virus in all the environments tested. Their latent periods in the membranes were 3 hr at 35 C, 2 hr at 39 C when calcium was present, and 3 hr at 39 C when calcium was absent.

The rates of production of the two viruses were

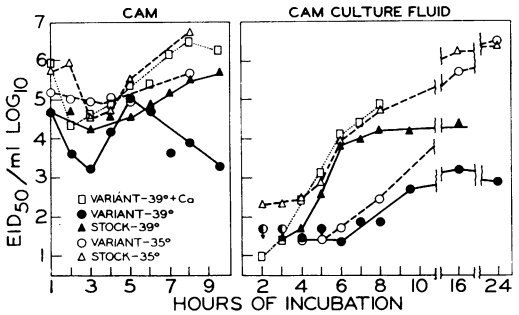

FIG. 2. *Multiplication of stock LEE and variant viruses in chorioallantoic membrane (CAM) shake cultures at 35 and 39 C in glucosol without calcium chloride. Also included (□) is the growth curve of variant virus at 39 C in glucosol with calcium chloride. Each point represents a pool of six CAM cultures. The volumes of the CAM suspensions and pooled culture fluids were each 6 ml.*

different, and they were affected differently by changes in the environment; but, in all cases, stock LEE virus accumulated in the membranes at a faster rate than the variant virus under the same conditions. At 35 C, the absence of calcium had no effect on the rate of production of either virus in the membranes. The curves shown in Fig. 2 for virus in the membranes at this temperature were the same in all respects whether calcium was present or not. Increasing the temperature of incubation to 39 C with calcium present did not affect greatly the rate at which stock LEE virus accumulated in the membranes, but the rate of production of variant virus was increased. In the absence of calcium, however, increasing the temperature of incubation from 35 to 39 C adversely affected the multiplication of both viruses. The quantity of stock LEE virus present at 8 hr at 39 C in the absence of calcium was about 10% of that produced at 35 C, but the membranes did support a steady production of infectious stock LEE virus over the 9.5-hr period. This was not true for the variant virus in the same environment. An increase in the level of infectious variant virus was detected at the same time as for stock LEE virus; but, after 5 hr, there was an apparent halt in the formation of infectious virus, and the level of variant virus in the membranes dropped precipitously. At 8 hr, the amount of variant virus in the membranes at 39 C in the absence of calcium was about 3% of the stock LEE virus produced under similar conditions, and about 1% of the variant virus produced at 35 C.

The release of variant virus into the culture fluid was profoundly influenced by the environmental conditions, but that of stock LEE virus was affected very slightly. In the presence of calcium, both viruses were liberated at about the same rates irrespective of temperature, in spite of the fact that variant virus accumulated in the membranes at a slower rate than did stock LEE virus. The curve shown for variant virus at 39 C in glucosol with calcium is representative of the results obtained with both viruses. The time of appearance of virus in the fluid paralleled events in the membranes and indicated that there was little delay in the initiation of release of the viruses.

In the absence of calcium, although stock LEE virus accumulated at very different rates in membranes at 35 and 39 C, the initial rates of release of the virus were the same at both temperatures, and these rates were about the same as when calcium was present. At 39 C, however, the virus reached a peak in the fluid at 8 hr, but at 35 C it continued to accumulate at a decreasing rate and did not reach a peak until about 16 hr. This reflected the more extensive multiplication of stock LEE virus in the membranes at 35 C. When calcium was absent, the initiation of release of variant virus at 35 C was delayed at least 2 hr, and its rate of release was strikingly slower than when calcium was present in spite of the fact that production of virus in the membranes was the same as when calcium was present. The amount of variant virus released during 8 hr at 35 C in the absence of calcium was only about 1% of that released when calcium was present, but there was a steady production and release so that at 24 hr the level of variant virus in the fluid equalled that of stock LEE virus. Increasing the temperature to 39 C in the absence of calcium resulted in a further delay in the initiation of release of variant virus, but the initial rate of release was the same as at 35 C. Very little variant virus appeared in the fluid under these conditions, and it should be noted that, although the amount of infectious variant virus in membranes at 39 C decreased after the 5th hr, an equivalent amount of infectious virus did not appear in the fluid.

The results of the HA titrations (Tables 1 and 2) show general trends which corroborate the findings with infectious virus. In the absence of calcium at 39 C, stock LEE hemagglutinins were detected in the membranes at 6 hr and in the fluid at 8 hr. Variant hemagglutinins were not detected

TABLE 1. *Hemagglutination (HA) titers of stock LEE and variant viruses in chorioallantoic membranes (CAM) and culture fluids incubated at 35 and 39 C in the presence of calcium*

Time after inoculation	HA titer* of CAM				HA titer of fluid			
	35 C		39 C		35 C		39 C	
	LEE	Variant	LEE	Variant	LEE	Variant	LEE	Variant
hr								
4	<2	<2	<2	<2	<2	<2	<2	<2
5	<2	<2	4	<4	<2	<2	2	<2
6	—	—	16	8	—	—	8	2
7	—	—	64	32	—	—	32	8
8	32	64	64	32	64	64	64	8
9.5	—	—	128	32	—	—	128	16
24	—	—	<2	4	—	—	256	32

* HA titers are expressed as the reciprocal of the last dilution showing 2+ agglutination.

TABLE 2. *Hemagglutination (HA) titers of stock LEE and variant viruses in chorioallantoic membranes (CAM) and culture fluids incubated at 35 and 39 C in the absence of calcium*

Time after inoculation	HA titer* of CAM				HA titer of fluid			
	35 C		39 C		35 C		39 C	
	LEE	Variant	LEE	Variant	LEE	Variant	LEE	Variant
hr								
5	<2	<2	<4	<4	<2	<2	<2	<2
6	—	—	8	<2	—	—	<2	<2
7	—	—	8	<2	—	—	±	<2
8	16	8	16	2	32	<4	2	<2
9.5	—	—	16	4	—	4	4	<2
16	32	32	—	—	512	256	32	<2
24	—	—	4	<2	1,024	512	64	2

* HA titers are expressed as the reciprocal of the last dilution showing 2+ agglutination.

in the membranes until 8 hr, and they did not appear in the fluid until 24 hr. These results are in striking contrast to those obtained at 39 C in the presence of calcium, where stock LEE hemagglutinins were detected at 5 hr in both the membranes and the fluid, and variant hemagglutinins appeared in the membranes at 6 hr and in the fluid 1 hr later. At 35 C in the presence of calcium, the hemagglutinins of the two viruses were present in both the membranes and fluids in comparable amounts at 8 hr. In the absence of calcium at 35 C, stock LEE hemagglutinins were present in the membranes and fluid in comparable amounts at 8 hr, and at this time variant hemagglutinins were detected in the membranes but not in the fluid. At 16 hr, however, the hemagglutinins of both viruses were present in the fluids in comparable amounts. In general, the multiplication of stock LEE virus at 39 C resulted in the production of more hemagglutinins than did that of variant virus, and in both experiments there was evidence that noninfectious hemagglutinins were present.

Disappearance of intracellular HA inhibitor from CAM infected with stock LEE and variant viruses. The allantoic cells of the CAM contain a mucoprotein substrate for the influenza neuraminidase. During the multiplication of the virus in these cells, as the enzyme is being produced, this substrate is degraded (Edney and Isaacs, 1950). Since the previous experiments demonstrated that these viruses differed in their multiplication cycles, and since they were known to differ in their enzymatic activities in vitro, their enzymatic activity during the course of multiplication was followed by determining the time of disappearance of the intracellular substrate. The results of HI titrations with the CAM suspensions are shown in Table 3.

In the presence of calcium, the inhibitor disappeared at the time or just before the time that viral hemagglutinins could first be detected, that is, at 5 hr at 39 C. At 35 C, no samples were taken

TABLE 3. *Hemagglutination inhibition (HI) titers of membranes infected with stock LEE and variant viruses and incubated at 35 or 39 C*

Calcium in medium	Time after inoculation	HI titers* of membranes			
		35 C		39 C	
		LEE	Variant	LEE	Variant
	hr				
Present	1	32	32	16	16
	4	16	16	16	16
	5	16	16	<8	<8
	8	<8	<8	<8	<8
Absent	1	32	16	16	16
	4	16	32	8	16
	5	16	16	<8	16
	7	—	—	<8	16
	8	<8	<8	<8	8
	9.5	<8	<8	<8	8

* HI titers are expressed as the reciprocal of the last dilution showing complete inhibition.

between 5 and 8 hr. At 5 hr, the inhibitor had not decreased and there were no hemagglutinins detectable, but at 8 hr the inhibitor had been degraded and both viruses had produced measurable amounts of hemagglutinins. In the absence of calcium, the results at 35 C were the same as those with calcium, but at 39 C the results were quite different. With stock LEE virus, the inhibitor disappeared at about the time that hemagglutinins were first detected. With the variant virus, however, although the amount of substrate decreased at 8 hr when hemagglutinins were detected, it did not disappear completely and was still detectable at 9.5 hr. Except for this one instance, the substrate was completely degraded by the time hemagglutinins could be detected. These results suggest that the enzyme of the variant virus was not as active in vivo under these conditions, 39 C and absence of calcium, as it was under other conditions, or as was the enzyme of stock LEE virus.

DISCUSSION

Neuraminidase has been considered to act at some stage or stages in the multiplication of influenza viruses. Various suggestions have been made concerning the exact function of this enzyme, and two of the proposals are supported by some experimental evidence. Hoyle (1950) suggested that the enzyme attacked the cell membrane, resulting in the release of newly formed virus particles. Experimental support for this proposal has been provided by Cairns and Mason (1953) and by Ackermann and Maassab (1954). Schlesinger and Karr (1956), however, found that degradation of intracellular inhibitor in the CAM of infected eggs was stepwise under conditions permitting cycles of viral multiplication and that it occurred during the periods of synthesis of infectious virus. Release of virus was not cyclic, and, at the time when large quantities of virus were being released, the level of intracellular inhibitor was constant or actually increased. They suggested that the viral enzyme was involved in the synthesis of new viral material.

The two virus strains used in this work were known to differ only in properties associated with the viral neuraminidase. The results presented show that they also differ in their multiplication in ovo and in pieces of CAM. The multiplication of the variant was affected to a greater extent by certain changes in the environment, and the differences between the two viruses were greatest when there was no calcium in the medium, a situation in which the rate of variant enzyme activity in vitro is markedly slower than that of stock LEE virus. Although one cannot be certain that the environmental factors employed in this work affected multiplication of the variant virus mainly through their effects on enzymatic activity, the parallelism between the influence of temperature and calcium concentration on the enzymatic activity of the variant in vitro and their influence on its multiplication supports this premise.

The experiments in ovo were limited to following the appearance of virus in the allantoic fluids, the end result of viral multiplication. Although these experiments demonstrated that the overall growth cycle of the variant was slower than that of stock LEE virus, the differences could not be attributed to a particular step in the multiplication cycle. The experiments concerned with multiplication of the viruses in pieces of CAM, however, suggest that the viral enzyme is active during liberation of the virus from infected cells. When calcium is present, the rate of variant enzyme activity in vitro is equivalent to that of stock LEE virus, and the variant is released from the CAM at the same rate as stock LEE virus

even though it is produced at a slower rate. If calcium is absent, the rate of variant enzyme activity in vitro is much slower, and the virus is released from the CAM much more slowly even though its rate of production is unchanged. In addition, the results suggest that the enzyme may function at some step prior to the release process. Stock LEE virus multiplied steadily in membranes incubated at 39 C in the medium without calcium, but variant virus did not. The inability of the variant virus to multiply at 39 C under these conditions therefore was not due to some insufficiency on the part of the cells, but rather is referable to the virus itself. That the activity of the variant enzyme was impaired in vivo under these conditions is shown by the fact that intracellular substrate was still detectable after variant hemagglutinins were formed. In contrast, at 35 C in the absence of calcium, the variant enzyme was active and the intracellular substrate was degraded. Under these conditions, variant virus multiplied at a slower rate than stock LEE virus, but it produced a comparable final yield of infectious virus. The fact that the duration of the latent period was the same for both viruses in the same environments, even under conditions in which their rates of enzyme activity in vitro were quite different, suggests that the enzyme does not participate in the processes which take place during adsorption, penetration, and the eclipse phase. The effect of a deficiency of calcium on production of virus in membranes at 39 C may explain the results in ovo at this temperature, as the amount of calcium available may be below the level needed to support the maximal rate of virus production.

ACKNOWLEDGMENTS

This work was supported by a research grant from the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service. The work was performed during the tenure by one of us (B. L. P.) of a Wisconsin Alumni Research Foundation Postdoctoral Fellowship.

LITERATURE CITED

ACKERMANN, W. W., AND H. F. MAASSAB. 1954. Growth characteristics of influenza virus. Biochemical differentiation of stages of development. J. Exptl. Med. **100**:329–339.

CAIRNS, H. J. F., AND P. J. MASON. 1953. Production of influenza A virus in the cells of the allantois. J. Immunol. **71**:38–40.

EDNEY, M., AND A. ISAACS. 1950. Interference between inactive and active influenza viruses in the chick embryo. III. Inhibitor of virus haemagglutination in the chorioallantoic membrane. Australian J. Exptl. Biol. Med. Sci. **28**:603–612.

FULTON, F., AND P. ARMITAGE. 1951. Surviving tissue suspensions for influenza virus titration. J. Hyg. **49**:247–262.

GRANOFF, A. 1955. Noninfectious forms of Newcastle disease and influenza viruses. Studies on noninfectious virus occurring within cells that are producing fully infectious virus. Virology **1**:516–532.

HOYLE, L. 1950. The multiplication of influenza viruses in the fertile egg. J. Hyg. **48**:277–297.

PADGETT, B. L., AND D. L. WALKER. 1957. Enzymatic variants of influenza virus. I. Isolation and characterization of slowly reacting enzymatic variants of influenza B virus. J. Exptl. Med. **106**:53–68.

PADGETT, B. L., AND D. L. WALKER. 1958. Enzymatic variants of influenza virus. II. Effect of environmental factors on enzymatic characteristics of a variant of influenza B virus. J. Exptl. Med. **108**:651–664.

REED, L. J., AND H. MUENCH. 1938. A simple method of estimating fifty per cent endpoints. Am. J. Hyg. **27**:493–497.

SCHLESINGER, R. W., AND H. V. KARR. 1956. Influenza virus and its mucoprotein substrate in the chorioallantoic membrane of the chick embryo. II. Stepwise inactivation of substrate and its relation to the mode of viral multiplication. J. Exptl. Med. **103**:333–349.

TAMM, I., K. FOLKERS, AND F. L. HORSFALL, JR. 1953. Inhibition of influenza virus multiplication by alkyl derivatives of benzimidazole. II. Measurement of inhibitory activity by hemagglutination titrations. J. Exptl. Med. **98**:229–243.