STUDIES ON HOST-VIRUS INTERACTIONS IN THE CHICK EMBRYO-INFLUENZA VIRUS SYSTEM*

IX. THE PERIOD OF LIBERATION OF VIRUS FROM INFECTED CELLS

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In the preceding paper of this series (1) certain technical problems have been outlined which are encountered in the study of the growth cycle of influenza virus in the intact chick embryo. It has been shown there that some of the difficulties these present can be overcome in part by the use of deembryonation technics. Of particular interest was the observation that liberation of progeny from infected cells appeared to be a lengthy process in contrast to previous estimates (2, 3). This phenomenon has now been analyzed further by various technics and the results indicate that once a cell has been infected it may produce and liberate virus at nearly constant rates for periods of 30 hours or longer. These experiments, which have been summarized briefly elsewhere (4), will be described in detail below.

Methods and Materials

The PR8 strain of influenza A virus was used throughout. The deembryonation (DE) technics have been recorded in detail, and the methods used for preparation of the seeds, titration of infectivity, and hemagglutinin assay have been referred to in the preceding paper (1). Hemagglutinin titrations were carried out within 24 hours after harvest. For infectivity titrations the fluids were diluted in 10 per cent normal horse serum and stored in glass sealed ampuls at —65°C. until the tests could be performed. Further experimental details are presented in the text.

EXPERIMENTAL

In order to study the liberation of virus from 1 set of infected entodermal cells of the allantois, two approaches can be used. When a small inoculum of

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virus is injected allantoically, which is insufficient to infect all the available cells, the remaining susceptible cells must be rendered resistant to infection in order to prevent initiation of further infectious cycles (adsorption, multiplication, and liberation) by the progeny liberated from the cells originally infected. This can be achieved by induction of the interference phenomenon in the remaining susceptible cells (2), or more conveniently by treatment of these cells with the receptor-destroying enzyme (RDE) of Vibrio cholerae (5, 1). With a sufficiently large inoculum of virus (>10^6 ID_50) presumably all cells become infected directly and thus only one infectious cycle will result. The period of liberation of virus progeny has been studied under the two sets of conditions employing the “differential growth curve technic” in eggs deembryonated after infection (1). With this technic the period of adsorption of seed virus can be limited to a few minutes and by regular exchanges of the medium the hourly yields of virus can be measured. In Experiment 1 a relatively small dose of seed was used and the infectious process was held to 1 cycle by the addition of RDE to the medium. In Experiment 2 the infecting dose was large enough to yield only 1 infectious cycle.

**Experiment 1.**—A number of chick embryos were each injected with approximately 10^6 ID_50 of virus. After allowing 5 minutes for adsorption, 8 eggs were deembryonated by the usual technic. To 1 set of 4 modified glucosol solution was added and to another set of 4 the same medium containing 1 per cent RDE or a total of approximately 100 units of the enzyme. Thereafter the eggs were incubated at 37°C. on the rotating device and at each hour for 19 hours the fluids were exchanged by fresh medium of the respective kinds. The membranes were not washed at these periods. From the 19th to the 36th hour the media were replaced every 2nd hour. The fluids thus obtained were titrated for hemagglutinating activity within 24 hours and for infectivity after storage at −65°C. for 4 days to 2 weeks.

The results of these titrations are presented in Fig. 1. The solid dots represent the data obtained in the RDE-free series. As can be seen, virus liberation became demonstrable only at the 6th hour. It is possible, however, that liberation of small amounts of virus might have been detected by the 4th or 5th hour if the titrations had been started with lower dilutions. The amount of virus released per hour increased up to the 8th hour when a temporary plateau was reached which was maintained for about 4 hours. Thereafter the hourly yields increased again, presumably on account of liberation from a second group of cells infected by virus released from those originally infected by the seed, and a second plateau was reached. There was suggestive evidence of a third step after the 15th hour, but since most of the cells apparently became infected in the 2nd cycle the increase in hourly yields in the 3rd step was relatively small. From the 16th to 17th hour on, when presumably all cells had been infected, liberation proceeded at only a slightly decreasing rate up to the 36th hour, the longest interval tested. Since from the 19th hour on the medium was exchanged only every 2nd hour, the titration values per hour were obtained by dividing
Fig. 1. Experiment 1. The hourly yields of ID₅₀ and HA units (differential growth curves) from eggs deembryonated 5 minutes after infection with approximately 10⁶ ID₅₀ and the effect of addition of RDE to the medium.
The liberation of influenza virus was studied through infectivity titrations. Hemagglutinins became detectable with the second step in the infectivity titrations, and their liberation proceeded at a nearly constant rate from the 17th to 36th hour.

The results obtained in the RDE-series are represented by the circles on the chart. As can be seen, a relatively high infectivity titer was obtained in the 3-hour medium. This amount of virus represented presumably superficially adsorbed seed virus released from the membrane by the action of RDE (6; 7). In the 4-hour sample only 1/4 of the amount of virus was found. Definite evidence of liberation of new generations of virus was obtained by the 5th hour. Thus, in the RDE series, liberation could be detected 1 hour earlier and the amounts released in the next 2 hours were larger than in the corresponding samples of the RDE-free series. This presumably indicated that the RDE was sufficient to remove the receptors from the remaining susceptible cells so that most of the virus liberated remained free in the medium. A plateau was reached by the 6th hour which was comparable in height to the first plateau seen in the control series. No further step in the hourly yields became apparent in the subsequent samples and the rate of liberation remained nearly constant up to the 36th hour. The slight undulations of the curve may indicate possibly some periodicity in liberation. On the other hand, the variations in titer seem to fall well within the accuracy of the infectivity titrations as performed (8; 9). Thus, it would seem that the RDE has prevented further spread of the infection to cells not infected originally by the inoculum. In line with the relatively low levels of infectious virus released is the fact that hemagglutinins were not detectable in any of the preparations.

The experiment was accompanied by cumulative growth curves in deembryonated eggs as well as in the intact chick embryo, i.e., some of the eggs after infection were deembryonated, but instead of replacing the medium at hourly intervals, small aliquots were removed after 12, 24, and 36 hours. From other groups of eggs, which were not deembryonated, allantoic fluid was collected after 12, 24, and 36 hours respectively. In order to compare the results obtained with these specimens with those of the differential growth curves, the individual titers of the latter were added (calculated cumulative results). These are presented in Fig. 2. As can be seen, the values obtained in the deembryonated eggs by the cumulative technic fell fairly close to the curve of the calculated cumulative values derived from the differential series. In agreement with previous observations (1), the infectivity titers of the cumulative series were somewhat lower than those of the differential set indicating possibly some inactivation of virus at 37°C. In the former. In contrast, the hemagglutinin titers of the cumulative series exceeded somewhat those of the differential curve suggesting that the total amount of virus material liberated may have been somewhat larger when the medium was not exchanged. The allantoic fluids of the intact chick embryos showed higher titers of both infectivity and...
Fig. 2. Experiment 1. Comparison of the calculated cumulative yields of ID<sub>50</sub> and HA units derived from the differential growth curves (Fig. 1) with the yields of cumulative growth curves in deembryonated eggs and in intact chick embryos.
hemagglutinins and they reached high levels at an earlier date as compared to the DE series. Thus, as has been suggested in the preceding paper (1), the infectious process in the intact chick embryo appears to proceed at a faster rate, a greater total amount of virus is produced and the differences observed cannot be ascribed entirely to the greater total area of allantois in the intact chick embryo as compared to that of the deembryonated egg.

Experiment 2.—The same type of experiment as described above was carried out again, but instead of using only about $10^6$ as inoculum more than $10^6$ ID$_{50}$ were injected 5 minutes prior to deembryonation. Also, in addition to the 2 groups (medium with or without RDE) a 3rd set of eggs was included in which at each time interval when the RDE-free medium was exchanged, the membranes were washed twice with warm glucosol solution by filling and decanting. The wash fluids were saved and also assayed for virus activity.

The results are presented in Fig. 3. As can be seen, not all of the residual non-adsorbed seed virus had been removed by the DE procedure, leaving a threshold level between $10^5$ and $10^6$ ID$_{50}$/ml. in the early harvests of the RDE-free series (solid dots). After the 4th hour liberation became apparent by a sharp increase in titer and thereafter the yields per hour remained at a high level for the 36 hours of the experiment. However, a gradual slight decline in liberation over this period was apparent. The liberation of virus in the RDE series was of the same order throughout the experiment except that high titers were found already in the 3 hour sample. This virus represented in part presumably seed virus removed from the membranes by RDE; in part possibly newly formed and liberated virus. In the 3rd series in which the membranes were washed at each interval the infectivity titers were similar to those of the other 2 series and the wash waters retained only fractions of the virus present in the medium at the corresponding periods.

The hemagglutinin data likewise revealed no marked differences among the 3 groups except that in the RDE series some activity was measurable already at the 3rd hour which either represents seed hemagglutinin dislodged from the membranes by RDE or newly formed virus which remained free since the remaining receptors presumably had been destroyed by RDE thus preventing its adsorption. Furthermore, in the washed series the hemagglutinin titers were consistently higher in the early period as compared to the corresponding preparations of the unwashed series; and these were somewhat higher than those of the RDE series. These differences were slight and may represent chance observations. The wash waters contained some hemagglutinins at various times but in each instance the titers were considerably lower than those found in the medium prior to the washing procedure.

As in the preceding experiment sets of eggs were deembryonated for cumulative data and others, for in ovo growth curves, were not deembryonated. The results with the 12, 24 and 36 hour harvests of these groups are compared in Fig. 4 with the calculated cumulative data of the differential harvests described.
Fig. 3. Experiment 2. The hourly yields of ID₅₀ and HA units (differential growth curves) from eggs deembryonated 5 minutes after infection with approximately 10⁸ ID₅₀ and the effects of addition of RDE to the medium and of repeated washing at the time of exchange of the medium.
Fig. 4. **Experiment 2.** Comparison of the calculated cumulative yields of ID$_{50}$ and HA units derived from the differential growth curves (Fig. 3) with the yields of cumulative growth curves in deembryonated eggs and in intact chick embryos.
above. Again it is seen that the allantoic fluids of the in ovo series gave higher infectivity titers but the hemagglutinin assays in this case fell close to the curve of the calculated cumulative titers of the differential series. The infectivity titers obtained in the cumulative DE series again were somewhat lower than the calculated cumulative values of the differential set, indicating some inactivation of infectivity upon prolonged incubation of the deembryonated eggs, whereas the reverse was seen with respect to the HA titers.

In the above 2 experiments groups of deembryonated eggs were employed in each series and the media collected at each time interval were pooled before analysis. It was of interest to determine the variations encountered in individual preparations. Also, the previous experiments obviously had not reached the end of liberation and longer study was indicated. The answer to these 2 questions was sought in the following experiment.

Experiment 3.—In order to dovetail the test, 3 eggs were infected in the evening with about 10⁶ ID₅₀ of virus, deembryonated 1 hour later, and rotated for 12 hours without exchange of medium. Another set of 3 eggs was infected the next morning with the same seed and deembryonated 1 hour later. The medium was then exchanged in both sets at each hour for 12 hours and thereafter the liberated virus was allowed to accumulate for 12 hours overnight. The next day hourly exchanges of medium were again performed. Thus, hourly collections were obtained over a 46 hour period. This change in procedure did not significantly affect the results. Only representative samples (6 hour intervals) were assayed for infectivity and hemagglutinins up to the 36th hour, but all the samples collected thereafter.

It can be seen in Fig. 5 that the titers obtained with both sets varied to some extent from egg to egg, yet they all seemed to fall within the limits of accuracy of the titration technics. Thus, there was no striking difference in the response of individual eggs. Up to the 30th hour liberation proceeded apparently at a nearly constant rate. After the 36th hour the liberation decreased markedly and rapidly and by the 46th hour it was less than 1 per cent of the maximum.

The preceding experiments indicated that liberation of virus from 1 set of infected allantoic cells may proceed at a nearly constant rate for prolonged periods of time. It was conceivable that after deembryonation the viability and nutrition of the tissue decreased rapidly thereby slowing down the infectious process and prolonging the period of virus production and liberation. It was of interest therefore to determine whether the infectious process in the intact chick embryo would take a similar course. It was thought that an answer to this question might be obtained by permitting the infectious process initiated by a large dose of virus to proceed in the intact chick embryo for increasingly longer periods of time before deembryonation. In Experiment 4, the deembryonated eggs were incubated only for 1 hour corresponding to the “combined in ovo–DE technic” (1). In Experiment 5, differential harvests were made over prolonged periods after deembryonation and the various differential curves obtained were compared both with respect to the time after infection and the time after deembryonation.
Experiment 4.—Two series of chick embryos were inoculated with 10^4 ID₅₀ per egg at an interval of 12 hours. The 2 series were then studied in parallel, series 1 supplying the data for the 12 to 25 hour period, series 2 those for the first 12 hours. One group of 6 eggs was de-embryonated 2 hours after infection and the yield of virus from this group was permitted to accumulate up to the 7th hour, but several samples of the medium were withdrawn during this period. Other groups of 6 eggs each were deembryonated at each hour from the 7th to the 16th hour following infection and these were incubated on the rotating machine for only 1 hour thereafter (combined in ovo-DE technic (1)). Finally, several groups of eggs were not deembryonated and the allantoic fluids were collected from these at intervals during the 24 hour period.

The results of this experiment are shown in Fig. 6. The 1 hour yields from the combined in ovo-DE series (solid dots) were closely similar over the 7 to 16 hour period studied both with respect to infectivity and hemagglutinins, again indicating a nearly constant rate of liberation. The first 7 hours following infection were covered by cumulative data, represented by the circles. The 7 hour values found were taken as the starting point for calculating the cumulative curves of the 1 hour yields obtained in the combined in ovo-DE series
(crosses). As can be seen the calculated cumulative hemagglutinin curve approached that of the values found in the allantoic fluid of intact chick embryos (solid squares), indicating similar yields of virus material. On the other hand, the calculated cumulative infectivity curve fell considerably below the values found in the allantoic fluid titrations. The latter values remained almost constant during the experimental period and the fact that the 2 hour sample gave titers of the same order as the others indicates that it represented largely non-adsorbed seed virus. Yet, there was a marked increase in hemagglutinins. This result is in agreement with data presented by von Magnus (10) who demonstrated that after infection of intact chick embryos with massive doses of virus the yields will show low ID₅₀/HA ratios as a result of diminished production of infectious virus, whereas the formation of hemagglutinins is not, or only little, affected. Indeed, the ID₅₀/HA ratios obtained in the deembryo-
nated eggs were of the order of $10^8$. If this ratio is applied to the hemagglutinin titers found in the allantoic fluids it is readily seen that the amount of infectious virus liberated was of the order of $10^9$ and that this quantity could not have been detected as an addition to the high threshold level of non-adsorbed seed virus of slightly less than $10^6$. This interpretation is supported by Bernkopf (11) who likewise showed that under similar conditions liberation of infectious virus could be demonstrated in deembryonated eggs when its detection was prevented in the intact chick embryo.

These data indicate that liberation in ovo from 1 set of infected cells also proceeds at an almost constant rate for at least 17 hours. It could be argued, however, as far as the yields of infectious virus are concerned, that the quantities found in the media 1 hour after deembryonation may reflect insufficient washing at the time of setting up of the preparations. In general the washing procedure decreased the titers by at least 3 log$_{10}$ units and often by 4, when the
Fig. 8. Experiment 5. Comparison of the 2-hourly yields of ID_{50} and HA units from eggs deembryonated at increasingly longer intervals after infection with approximately 10^7 ID_{50} in relation to time after infection.
allantoic fluid titers prior to deembryonation were compared with the final medium of the DE preparation before incubation (1). This is seen also in the 2 hour DE preparation in Fig. 6. The ID₉₀ values found in the 1 hour yields of the combined in ovo-DE series were only about 2 log₁₀ units lower than the allantoic fluid titers and therefore some liberation of infectious virus presumably has occurred during the 1 hour period of incubation following deembryonation. This point is more adequately proven in Experiment 5.

Experiment 5.—A number of 12 days old chick embryos was set aside for this experiment. Groups of these were infected with approximately 10⁶ ID₉₀, 48, 40, 32, 24, 16, 8, and 1 hour prior to deembryonation. From these 7 sets of eggs differential growth curves were obtained by exchanging the media at 2 hourly intervals. Hemagglutinin titrations were performed on all media obtained but only representative samples were selected for ID₉₀ titrations.

In Fig. 7, the results were plotted in relation to the time after deembryonation. It is apparent that with an increase in the interval between infection and deembryonation from 1 to 24 hours the period of subsequent nearly constant liberation of hemagglutinins decreased, although the maximal rates of release were of a similar order. With further prolongation of the in ovo incubation period from 32 to 48 hours, the rates of liberation were found already to be reduced immediately after deembryonation. Furthermore, the yields thereafter decreased the more rapidly the longer the preceding incubation in the intact chick embryo. The ID₉₀ values followed roughly the same pattern, although greater fluctuations in titers were noted than in the hemagglutinin assays, which in part may be ascribed to several unsatisfactory batches of chick embryos received during this period and used for the titrations.

The data were replotted in relation to the time after infection. As seen in Fig. 8, most of the hemagglutinin values fell closely on 1 curve except those derived from the groups of eggs deembryonated late after infection. In these the titers appeared to decrease more rapidly than in the comparable preparations deembryonated earlier after infection. This might indicate that production and liberation in ovo proceeds at a somewhat faster rate and that the host cells become exhausted correspondingly somewhat earlier. The infectivity data followed a similar trend, but, as pointed out above, a wider fluctuation in titers became apparent.

DISCUSSION

The experiments presented in this paper concern the liberation of new generations of virus from the entodermal cells of the allantois infected with influenza virus. Previous studies in the intact chick embryo suggested that the period of liberation was rather short; i.e., of the order of 4 to 6 hours (2, 3). It was reasonable to assume that some cells would begin to release virus earlier than the majority of them and a few cells would start liberating late and consequently continue to do so for a somewhat longer period of time. If that
were the case, a bell-shaped curve should be obtained upon plotting of the hourly yields against time. The data presented here did not confirm this view but rather revealed a period of liberation far in excess of that expected according to the previous experiments cited. Under the experimental conditions used in this study when either presumably all the available cells were infected by exposure to an overwhelming dose of seed virus for 5 minutes, or when after infection of relatively few cells within a similarly short period of time the receptors of the remaining susceptible cells were continuously exposed to RDE, liberation from the 1 set of infected cells extended over periods of 30 to 36 hours at almost constant rates and only thereafter did the hourly yields decrease significantly.

It was conceivable that the extent of the liberation period was exaggerated in the deembryonated egg because of a possible decrease in the viability of the tissue. Indeed data presented by Cairns (3) obtained by exchange of the fluid content of the allantoic cavity of the intact chick embryo at half-hourly intervals seemed to indicate a much shorter liberation period. Hemagglutinin assays of the half-hour samples obtained during a 15 hour incubation period revealed 2 peaks of liberation which on extrapolation suggested 2 partially superimposed bell-shaped curves. This result of 2 “peaks” appears to be incompatible with the assumption that upon overwhelming infection all available cells are infected immediately, in which case one would expect only 1 peak. However, the depression between the “peaks” was small and would seem to fall well within the limits of accuracy of the hemagglutination technic used. On the other hand, liberation in the experiments cited was still in progress at the time they were terminated. This problem of apparent discrepancies between the results of in ovo and DE experiments was approached, therefore, by other technics. If eggs were deembryonated at an increasingly longer interval after infection with sufficient quantities of virus to saturate all cells, production and liberation should have been exhausted after a certain time (certainly by the 16th hour) and only small amounts of virus should have been detectable in the media after deembryonation and the yields should have rapidly decreased on successive exchanges of the fluids. Actually, however, considerable amounts of virus were still released into the media of eggs deembryonated 24 hours after infection or later, and liberation continued for some time thereafter. These results failed to support the view that the virus found in the media represented residual amounts not removed by washing of the tissue during deembryonation, but indicated rather that they were actively liberated. Furthermore, as will be shown later (12), comparison of the amounts of virus present in the membranes at the start of a given 1 or 2 hour period with the sum of the total amounts liberated and remaining in the tissue at the end of that period indicated that multiplication still was in progress at a time previously assumed far beyond the end of the infectious cycle. However, the data obtained in Experiment 5 also
indicated that in the intact chick embryo the period of liberation is somewhat shorter than in the deembrionated egg, presumably on account of a slow-down in virus production in the latter.

The question why the extended period of liberation had not been recognized before in experiments with intact chick embryos can be answered by simple calculations. In the face of virus already present in the allantoic fluid the addition of new particles has to be large before it can be detected. Assuming a constant rate of liberation and that this rate has been established at a given time, a doubling of the ID₅₀ titer (+0.3 log₁₀ units) will occur in the next hour, the subsequent 2-fold increases will occur successively later; i.e., after further 2, 4, 8, 16 hour intervals and so forth. In the meantime, the liberated virus is maintained at 37°C, and some inactivation of infectivity almost certainly will occur. Incubation of infected allantoic fluid at 37°C in vitro leads to a considerable loss in titer during such periods (13), but it is not certain to what extent these data apply to allantoic fluid virus in ovo. However, on prolonged incubation of intact infected chick embryos it can be seen that increases in hemagglutinins are still obtained after the infectivity has passed its peak (14, 9). Thus, it is likely that liberation and inactivation may be in balance until inactivation ultimately gains the upper hand.

The available data indicate then that once a cell has been infected production and liberation of virus continue at a nearly constant rate for a considerable period of time. This concept does not require a change in interpretation of one-step growth curves except that the infectious cycle can no longer be said to terminate when the plateau has been reached in about 8 to 10 hours as was previously assumed (2), and the "50 per cent liberation time" offered as a more accurate figure for the average length of a cycle (3) has also lost its significance. Cells originally infected by the seed virus continue to liberate virus for periods much longer than 8 to 10 hours. If not all of the cells are infected by the seed virus, they will subsequently become infected by virus liberated from the first set of cells and when they start to liberate virus a second step will result in the growth curve. These cells continue to produce and liberate virus again for periods in excess of the cycle time previously assumed. This second step can be prevented by the destruction of the cell receptors of the remaining susceptible cells by the action of RDE and thus liberation from the 1st set of cells can be followed for extended periods of time. It is clear that an infectious cycle extends over more than 36 hours.

The question arises as to how the virus may be liberated. Obviously, since the cells remain in production for extended periods of time they cannot have been destroyed with the onset of liberation. In this regard it is of interest that histologic sections prepared in this laboratory of the entodermal layer of the allantois obtained less than 48 hours after infection with large doses of virus failed to reveal recognizable lesions. Only after 48 hours some evidence of necro-
sis was found (15). Similarly, electron micrographs of thin sections of infected allantoic membranes failed to show cellular destruction when obtained early after infection (16) but necrosis was apparent in later specimens (17). The virus must leave then by a process which does not immediately destroy the cells. It had been suggested by Hoyle (18), based on darkfield observations of teased fragments of infected allantoic membranes, that the virus material is protruded through the cell walls in filaments and that these are then fractioned into spherical particles which may gradually break off, or that even the whole filament may be cast off. More detailed information has been obtained from electron micrographs of thin sections of infected membranes (16, 17, 19, 20). The cell surface showed many filamentous protrusions of a diameter compatible with that of the virus particle, and these filaments revealed frequently terminal nodules or internal beading, resembling the spherical virus particle in shape and density. As suggested by Hoyle (18) and by Murphy and Bang (16) it would not be difficult to assume that these terminal nodules or filaments break off gradually from the cell and that this might represent the mode of liberation of the virus. Indeed, structures resembling those found on the membrane have been seen free in infected allantoic fluid (19–23). If this represents the course of events, the casting off of the terminal nodules or filaments does not disrupt the cell which might be compared to a self-sealing tank. This mode of liberation would not be expected to be explosive and thus may explain the prolonged liberation period. Only when the cell has become gradually exhausted after prolonged production of virus it may finally die and thus histologic lesions may become apparent only late after infection.

This mode of liberation of influenza virus is in striking contrast to that of bacterial viruses which are released by lysis of the host. On the other hand, data obtained with other animal viruses resemble to some extent those reported here.

It has been shown recently by Dulbecco (24) that the virus of Western equine encephalitis is liberated from infected cells in tissue culture well in advance of the appearance of cytopathogenic effects. Furthermore, the prolonged production of certain viruses in suspended tissue cultures such as poliomyelitis virus may denote a similar mechanism of liberation although in these instances the possibility of formation of new host cells in the culture cannot be excluded.

SUMMARY

The period and rate of liberation of influenza virus from entodermal cells of the allantois have been studied by deembryonating eggs within a few minutes after infection, exchanging the medium thereafter at hourly intervals and assaying the virus concentration in the harvests thus obtained (differential growth curves). If the inoculum was sufficiently large, presumably all available cells immediately became infected and only 1 infectious cycle was expected to
occur. If the inoculum was small, so that only a fraction of the cells adsorbed virus, the infectious process was held to 1 cycle by continuous exposure of the remaining susceptible cells to RDE. In either case, the results obtained indicate that once cells have been infected they produce and liberate virus at nearly constant rates for periods of 30 hours or longer before the yields decrease rapidly. Evidence has been presented which strongly suggests that such prolonged periods of liberation are observed not only in deembryonated eggs but also in the intact chick embryo.

Attempts have been made in the discussion to reconcile these findings with previous estimates of the liberation period and to integrate them with histologic observations and electron micrographs of thin sections of infected allantoic membranes having a bearing on the mode of liberation.

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