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

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Heat-stable inhibitors (HI) of hemagglutination by influenza viruses have been extracted from the chorioallantoic membrane (CAM) of chick embryos, and in the preceding paper (1) it was shown that their general characteristics resembled those of inhibitors isolated from other biological materials which have been identified as mucoprotein in nature. Inhibitory activity of CAM extracts against heat-inactivated influenza viruses was used as a measure of the allantoic cells' total supply of substrate susceptible to enzymatic breakdown by active virus or by receptor-destroying enzyme of *Vibrio cholerae* (RDE).

In this paper, experiments are described in which fluctuations in the level of soluble substrate (HI) extracted from CAM infected with influenza viruses were related on a time scale to different phases in the infectious process.[1]

Materials and Methods

This section has been fully covered in the preceding paper (1).

EXPERIMENTAL

Early in this work, it was expected that changes in titer of hemagglutination inhibitor (HI) of the infected CAM might be comparable in nature to those previously found in experiments on mouse brain (3, 4). It had been shown that HI in mouse brain underwent a consistent and progressive decrease in the course of infection with either neurotropic or non-neurotropic strains of influenza virus. In mice inoculated intracerebrally with egg-adapted strains, such as PR8, it was found that the HI titer remained abnormally low for as long as 13 days after inoculation (4). Because such drastic changes were

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[1] A preliminary discussion was published earlier (2).

associated with a severely restricted infectious process, the nature of which has been described before (5), it seemed reasonable to expect even more pronounced effects in the CAM in which viral reproduction was unrestricted.

In preliminary experiments, membranes from eggs inoculated 40 to 48 hours previously with various strains of influenza virus were harvested and assayed for HI contents. This time interval was chosen because viral proliferation should be at or past its peak, depending on the infecting dose, so that maximal amounts of newly produced virus would be present either in the CAM or in the allantoic fluid. The results of HI titrations on such specimens were surprisingly variable. A few random examples are summarized in

TABLE I

HI Titers of CAM Harvested 40 to 48 Hours after Inoculation of Various Strains of Influenza Virus

Experiment	Infecting strain	Dose (ID_{50})	HI activity against 3 HA units indicator strain (per cent of normal CAM)			
			PR8	WS	Melb	Lee
1	WS	10^3	26	38	32	191
	PR8	10^3	19	35	21	174
2	PR8	10^3	63	—	—	130
	LEE	10^3	121	—	—	121
3	Melb	10^3	—	—	1	22
4	WS	10^1	—	32	—	123
		10^3	—	59	—	117
		10^5	—	55	—	125
		10^7	—	46	—	174
		10^9	—	87	—	174

Table I. A striking finding was that titers against H-Lee were either normal or slightly increased in membranes with significantly reduced activity against PR8, WS, or Melb indicator viruses. Even in an experiment in which Melb-HI was extremely low (Experiment 3), Lee-HI was reduced proportionately much less. In the preceding paper it was shown that Lee-HI behaved similarly when active viruses acted on normal CAM extracts *in vitro* (1).

Another finding of interest is indicated under Experiment 4 in Table I. It is seen that WS-HI titers of CAM from eggs inoculated with 10^9 ID_{50} of WS virus were normal, while those after inoculation of smaller doses were decreased.

The irregularity of these results seemed to be at variance with findings reported by others (6, 7) to the effect that infection with influenza virus

induced a marked drop in HI content of the CAM concurrently with the first cycle of viral multiplication. It was thought therefore that such losses, if they had occurred early in infection, had been compensated for by the allantoic cells.

Groups of eggs were infected with either 10^8 or 10^2 ID_{50} of PR8 virus. The larger dose was sufficient to infect all susceptible cells (8), and with the smaller dose it was expected that virus would increase at a rate leading to involvement of all cells between 15 and 20 hours after inoculation. Accordingly, membranes were harvested from 4 eggs of each group at 1, 2, 3, 4, 5, 6½, 17, 24, 41, and 65 hours after inoculation. The CAM were ground up, portions of each suspension incubated with RDE and titrated for virus (HA), and the remainders boiled for 10 minutes, centrifuged, and tested for HI.

The results of the titrations are depicted in Fig. 1. It is seen that in eggs infected with 10^8 ID_{50} the initial rapid rise in virus concentration was associated with a precipitous drop in HI titer. At 65 hours, when the virus concentration in the CAM had decreased by 97 per cent, the inhibitory titer had returned to normal, although HA titers remained undiminished in allantoic fluid. In contrast, eggs inoculated with 10^2 ID_{50} showed normal HI titers in membranes harvested from 1 to 17 hours after inoculation, but then a drop occurred coincident with a rise in HA between 17 and 24 hours after inoculation. At 65 hours, when virus present in the CAM was still at peak, the HI titer remained low.

In this experiment, as in all others, the variations in HI titer revealed by densitometric HI titrations were also found by the pattern method. In the light of findings discussed previously (1), these similarities were taken to indicate that the fluctuations were quantitative in nature.

The outcome seemed to point to an association of HI decrease with periods of active viral multiplication, followed by restoration of normal HI activity after virus production had ceased. The findings at 65 hours in eggs inoculated with 10^8 ID_{50} indicated that the mere presence in the allantoic cavity of very large amounts of enzymatically active virus did not prevent restoration. In order to relate the fate of HI more precisely to phases of viral multiplication, membranes and allantoic fluids of eggs inoculated with different amounts of virus were harvested at hourly intervals and assayed for virus and HI in the manner described before.

The procedure in all these experiments was the same: Allantoic fluids and membranes were harvested and kept either individually or in pools of from 3 to 5 per sample. Allantoic fluids were stored in the refrigerator, pending HA titrations. CAM were placed in lusteroid tubes immersed in dry ice–alcohol mixtures and stored in dry ice boxes. When suspensions were prepared, the frozen membranes were partially thawed, weighed, and ground up in ice-cold saline solution. A portion of each suspension was immediately transferred to a boiling water bath for 10 minutes or to a 65°C. water bath for 30 minutes. After cooling, the heated suspensions were centrifuged for 10 minutes at 8,000 to 10,000 R.P.M. in a Sorvall

angle centrifuge. To the unheated remainder of each suspension, 1/10 volume of RDE was added, and the mixture incubated for 1 hour at 37°C. after which it was titrated for HA.

Infection with Large Doses of Virus.—The events following inoculation of undiluted, PR8-infected allantoic fluid ($10^{8.7}$ ID_{50}) are illustrated in Fig. 2.

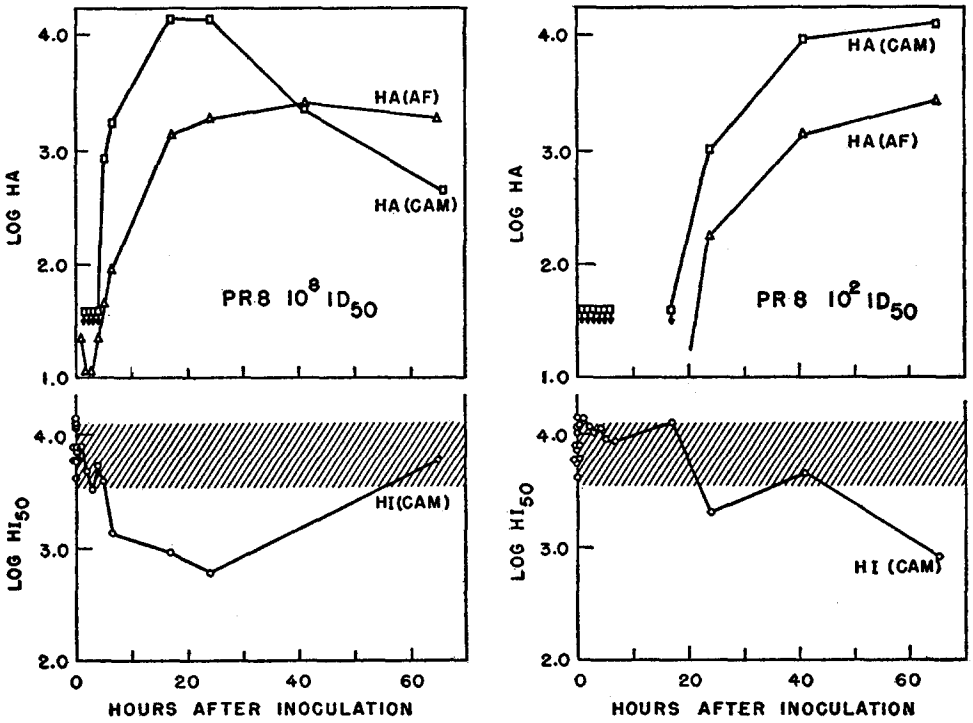

FIG. 1. Viral multiplication and liberation and associated changes in inhibitory titer of CAM extracts after intraallantoic inoculation of 10^8 or 10^2 ID_{50} of PR8 virus. Each point represents a pool of four allantoic fluids or membranes.

Note.—No allowance has been made for the fact that HA titers are expressed as HA units per milliliter in the case of allantoic fluid, per gram wet weight in the case of CAM. For this reason, titers in the CAM appear to be unduly high. HA titrations on CAM after incubation of suspensions with RDE. Arrows under symbols indicate titers less than the lowest dilution tested. Shaded area indicates normal mean HI titer ± 2 × standard error (see text and reference 1). Symbols on the ordinate (time 0) represent titers of *normal* CAM extracts prepared as part of this experiment.

The impression appears inescapable that the curve plotted from HI titers is a mirror image of the HA titers in the CAM. At least two consecutive phases, one lasting 10 hours, the second about 6½, are discernible, in which rises in virus were associated with significant falls in inhibitory activity. During stationary periods in viral growth, the HI retained a low plateau or, indeed,

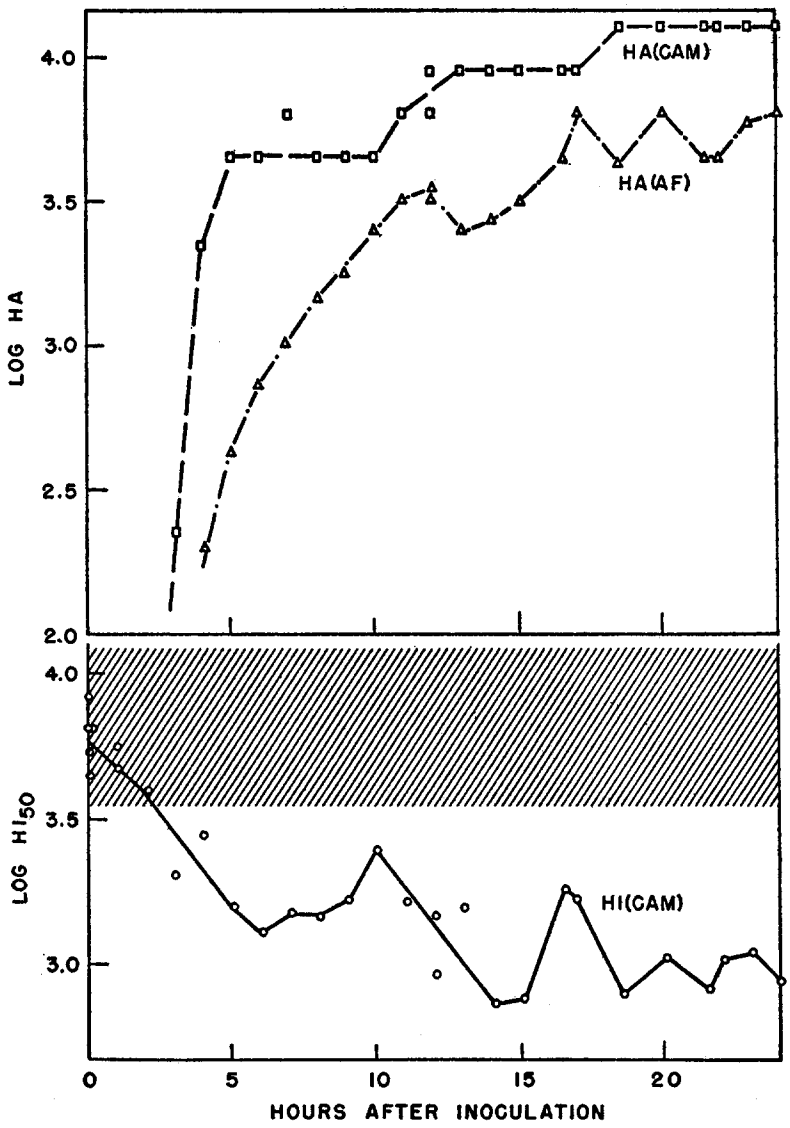

FIG. 2. Viral multiplication and liberation and associated changes in inhibitory titer of CAM extracts. Intraallantoic inoculum $10^{8.7}$ ID_{50} of PR8 virus. Each point represents a pool of 3 to 5 CAM or the mean titer of 3 to 5 individual allantoic fluids.

See Note under Fig. 1.

underwent slight increases. It is especially noteworthy that release of virus from the CAM into the allantoic fluid between 5 and 10 hours after inoculation was not associated with a decrease in HI titer.

Again, plotting of densitometric titration curves (Fig. 3) revealed parallel slopes for normal and infected membranes, even when the position of the curves was significantly displaced. This was another way of demonstrating the quantitative, rather than qualitative, nature of the viral effect on the

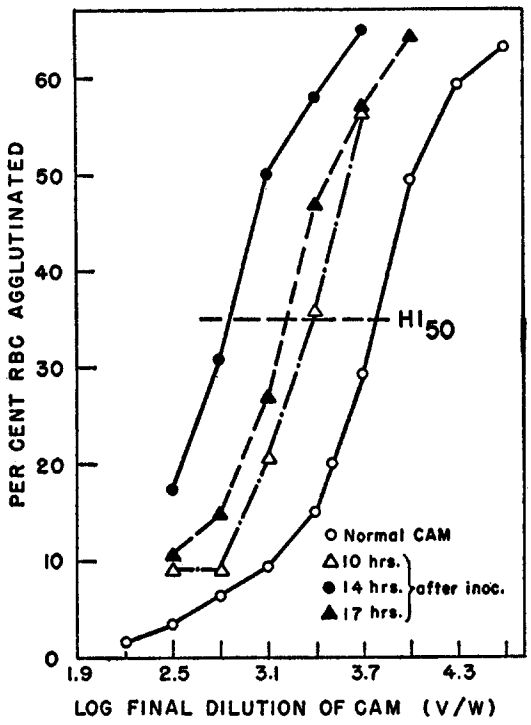

FIG. 3. Densitometric HI titration curves on selected CAM extracts from experiment depicted in Fig. 2, to show parallelism of slopes.

inhibitory material (1). Because of this parallelism, it was possible to utilize an arbitrary end-point (HI_{50}) as an expression of the absolute amount of HI present in membrane extracts. In Figs. 1 and 2, as in the figures to follow, the range of normal mean titers $\left(M \pm 2 \times \frac{\sigma}{\sqrt{N}}\right)$ calculated for each set of circumstances from the data presented in the preceding paper (1) has been indicated. As mentioned before, any deviation in excess of twice the standard error of the normal means was considered as significant. It should be stressed

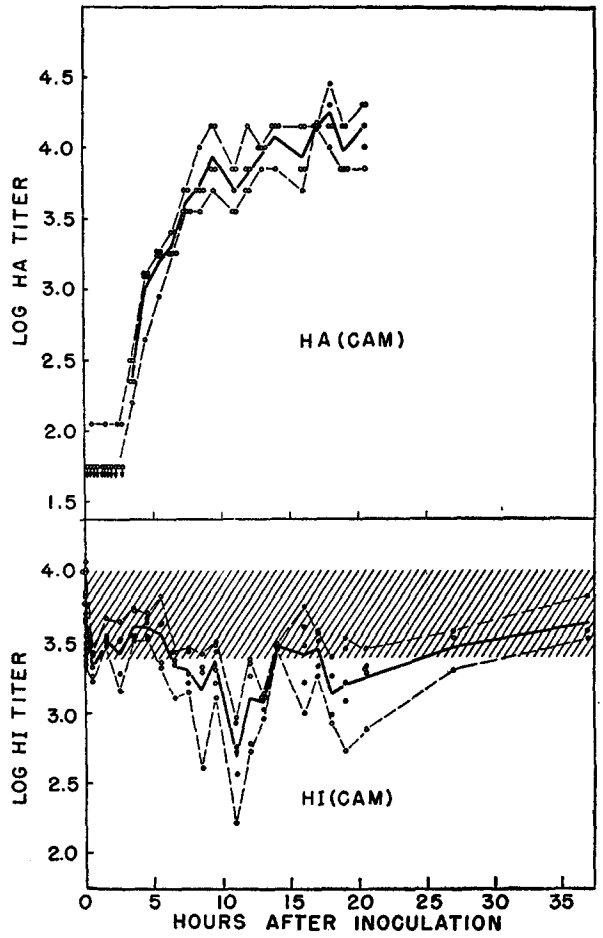

FIG. 4. Viral multiplication and associated changes in inhibitory titer of CAM extracts. Intraallantoic inoculum $10^{8.5}$ ID_{50} of Melb virus. Each point represents an individual CAM. Broken lines connect maximum or minimum, solid lines mean titers.

See Note under Fig. 1.

again that the significance of decreased titers was strengthened by the fact that inhibitory activity against PR8 or other type A strains rarely if ever exceeded the normal maximum.

A criticism which could be levelled against the experiment shown in Fig. 2 would be that each point on the curve was determined with a single pool of CAM. However, when membranes were harvested from individual eggs, the results showed very similar trends. This is illustrated in Fig. 4. In this experiment, the infecting dose was 0.2 ml. of undiluted Melb-

infected allantoic fluid, again enough (about $10^{8.5}$ ID_{50}) to saturate all infectible cells. Three to five individual membranes were harvested at each interval along with the corresponding allantoic fluids.

It is clear from Fig. 4 that progressive diminution in maximum, minimum, or mean inhibitory titer again coincided with the initial rise in hemagglutinin,

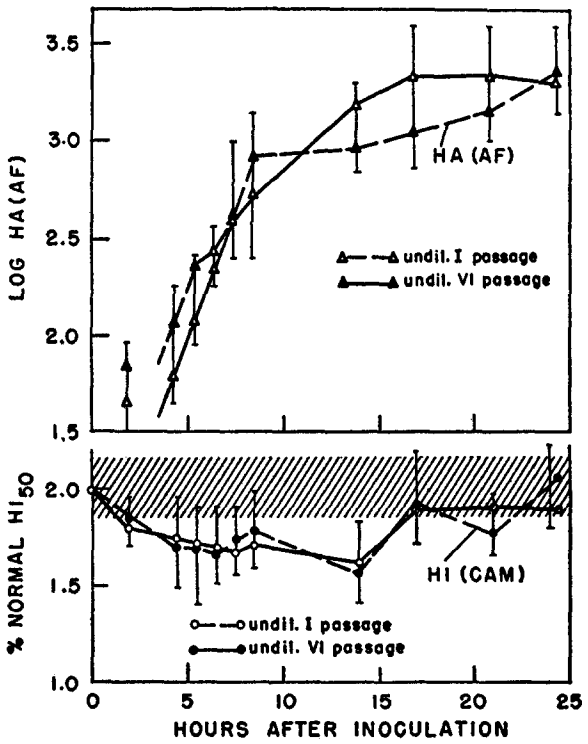

FIG. 5. Liberation of virus into allantoic fluid and associated changes in inhibitory titer of CAM extracts. Fluids and tissues derived from eggs yielding "normal" Lee (ID_{50}/HA ratio 5.72 log, "undiluted I passage") or "incomplete" Lee (ID_{50}/HA ratio 3.23 log, "undiluted VI passage"). Intraallantoic inoculum 10^8 ID_{50} in each case. The range of titers obtained at each point with 8 individual fluids or CAM extracts is indicated by vertical bars. Symbols represent means of 4 samples derived from each of the two experiments.

and that this was followed by rapid return into the normal range between 11 and 14 hours after inoculation. Thereafter, some individual membranes showed a secondary drop in titer, others did not. The mean HI titer underwent a suggestive second drop at 18 hours and remained below normal for at least 2 to 3 hours. Titers at 27 and 37 hours were in the normal range.

In keeping with the observation that active Lee virus is less effective than type A strains in inactivating HI derived from the CAM (1), changes in

inhibitory titer in infection with this strain were relatively elusive. Nevertheless, it is apparent from the experiment depicted in Fig. 5 that an initial downward trend in HI titer was reversed during or after release of virus into the allantoic fluid.

Inhibitor Breakdown and Production of "Incomplete" Lee Virus.—The possibility was considered that production and release of "incomplete" virus as observed by von Magnus (9) might be associated with more permanent or far reaching impairment of inhibitory activity than that of "normal" virus. This thought was prompted by these considerations: (*a*) Non-infectious ("incomplete") virus production has been shown to occur either in organs or

TABLE II

Production of "Incomplete" Lee Virus by Serial Undiluted Passages

Experiment	Passage	Period of incubation	Viral titers in allantoic fluid		
			ID_{50}/ml.	HA/ml.	ID_{50}/HA
		hrs.	*log*	*log*	*log*
1	Standard*	40	9.2	3.75	5.45
	Undiluted I	14	9.7	3.45	6.25
	" II	14	9.1	3.75	5.36
	" III	14	9.1	3.45	5.65
	" IV	14	9.0	3.45	5.55
	" V	14	9.0	3.45	5.55
	" VI	14	7.5	3.45	4.05
2	Undiluted I	8½	8.7	2.74‡	5.96
	" I	14	8.9	3.18‡	5.72
	" VI	8½	6.7	2.92‡	3.78
	" VI	14	6.2	2.97‡	3.23

* Eggs inoculated with 10^{-6} diluted stock virus.

‡ Mean HA titers of 4 allantoic fluids.

cells not ordinarily thought of as secretory in nature which therefore may not be geared to rapid restoration of mucoid material (5, 10, 11), or in the CAM and in mouse lung under the impact of overwhelming doses of virus (9, 12); (*b*) massive infection, like treatment with hyaluronidase, has been shown to induce increased permeability of the allantoic epithelium to isotopes (13); (*c*) breakdown of a mucoid outer layer of the cells without adequate regeneration could explain the release of immature or abnormal viral particles.

Lee virus lent itself to a study of this problem particularly well because of its demonstrated weak enzymatic activity against CAM inhibitors under ordinary conditions and because it requires about six serial undiluted passages before the yield changes from high to low ID_{50}/HA ratios (14). Confirmation of the latter finding with our strain Lee is demonstrated in Table II. On the

other hand, it is clear from Fig. 5 that the effect on inhibitory substrate was indistinguishable in membranes yielding "normal" and "incomplete" Lee virus (Passages I and VI). Indeed, the two curves were so closely similar as to lend added significance to changes in mean HI titers which might otherwise be considered as trivial.

Infection with Small Doses of Virus.—The observations described in the preceding sections led to the working hypothesis that inactivation of inhibitory substrate was associated in some way with the intracellular phases of viral activity. A test of this hypothesis was the time at which a reduction in substrate became demonstrable in membranes infected with small doses of virus.

Since HI assays were done on portions of the entire CAM, a significant decrease in titer should not be detectable unless more than one-half the total cell population contributing to the substrate pool was involved by the virus. The most reliable counts indicate that the entire allantoic membrane contains about 1.8×10^7 epithelial cells (8). Therefore presence in the allantoic cavity of $10^{7.5}$ ID_{50} should be enough to infect all or the great majority of all susceptible cells. This would correspond to an average of 20 HA units, in keeping with the average ID_{50}/HA ratio of about $10^{6.2}$ (15). This amount of virus in an average volume of 7 ml. of allantoic fluid would represent approximately 3 HA units per ml. of allantoic fluid. Hence all cells can be assumed to be infected when detectable amounts of hemagglutinin first appear in the allantoic fluid.

It had already been demonstrated (see Fig. 1) that in membranes of eggs infected with less than saturating doses of virus a significant decrease in HI was not demonstrable until measurable amounts of HA appeared in the fluid. A more detailed experiment is illustrated in Fig. 6. Here, eggs were infected with 10^8 ID_{50} of PR8 virus. Hemagglutinin titrations indicated that after 18 hours saturating amounts of virus had been liberated. From this time on, inhibitory titers against PR8 indicator virus underwent at least three consecutive phases of decrease and increase, each lasting about 5½ hours. The periodicity was demonstrated by the densitometric as well as the pattern technique. Activity against H-Lee, determined with the same sets of dilutions as the PR8 titrations, showed less striking fluctuations and almost no over-all downward trend. This difference in the behavior against the two indicator strains was in agreement with observations already reported.

Effect of Metaperiodate on Inactivation of HI in Infected Eggs.—It has been reported that KIO_4, in a certain range of low molar concentrations, modifies virus receptors on red cells and tissue cells in such a way that they are resistant to inactivation by influenza viruses or RDE (16, 17). Attachment of virus to such modified receptors is said to be irreversible. Fazekas de St. Groth found that pretreatment with KIO_4 failed to reduce the capacity of the allantoic epithelium to support multiplication of influenza virus, and he postulated on this basis that the virus could infect cells "through modified receptors" without being able to exert its enzymatic action (17).

In a series of experiments carried out *in ovo* and in tissue culture (18) it was found that treatment of the allantoic membrane with KIO_4 failed to mitigate the breakdown of substrate associated with infection. On the other hand, it was found—in agreement with observations recently reported by Fazekas de St. Groth (19)—that the infectious titer of virus recovered from KIO_4-

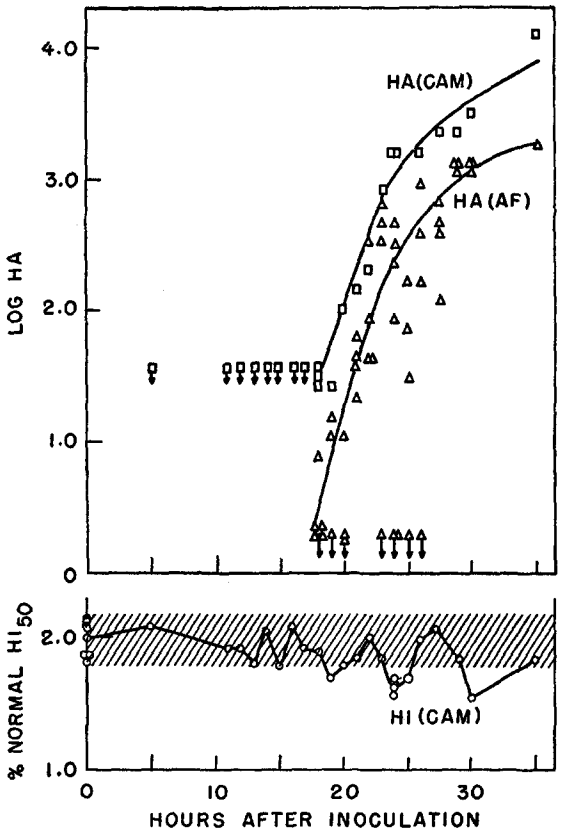

FIG. 6. Viral multiplication and liberation and associated changes in inhibitory titer of CAM extracts. Intraallantoic inoculum 10^3 ID_{50} of PR8 virus. Each symbol represents a pool of 4 CAMS or an individual allantoic fluid.

See Note under Fig. 1.

treated eggs was extremely low. In our hands, this was true even when the periodate was mixed with glycerol or glucose *before* inoculation into the allantoic cavity.[2]

[2] Fazekas de St. Groth has recently suggested that metaperiodate-treated eggs produce "incomplete" virus even after inoculation of small doses and of strains of influenza virus which ordinarily do not give rise to such non-infectious progeny (19). A simple refinement of the technique described by this author has revealed, however, that the yield from such

One of the phenomena seen in eggs treated with periodate or reduced periodate was a general slowing-down of the infectious process. As a result, the cyclic nature of viral multiplication as well as of HI breakdown appeared to be accentuated. This is illustrated by the curves depicted in Fig. 7. In contrast to viral growth curves obtained with PR8 in the CAM and allantoic fluid of normal eggs (see Fig. 2), those shown here were characterized by extended plateaus or, indeed, by a net loss in HA preceding a secondary rise which did not commence until 16 to 20 hours after inoculation. During the stationary or negative phases, the level of inhibitory activity showed a definite increase which was reversed when more virus was produced. Infectivity titrations were

eggs is different from that described by von Magnus. Instead of harvesting virus at a single time interval after inoculation, additional samples were taken from eggs at a point when viral multiplication could first be expected to be at or near peak. The results after inoculation of $10^{9.5}$ or $10^{4.5}$ ID_{50} of PR8 virus into KIO_4-treated or untreated eggs are shown in the following table:—

Group	Treatment	Dose of PR8 inoculated ID_{50}	Time of harvest	Titers*			ID_{50}/HA after undiluted subpassage (16 hr. harvest)
				ID_{50}/ml.	HA/ml.	ID_{50}/HA	
			hrs.	*log*	*log*	*log*	
A	None	$10^{9.5}$	8	8.41	3.00	5.41	—
			21	8.70	3.60	5.10	3.42
B	1 ml. 0.01 M KIO_4‡	$10^{9.5}$	8	7.78	2.10	5.68	—
			21	7.52	3.00	4.52	—
C	None	$10^{4.5}$	21	9.36	3.00	6.36	—
			45	9.45	3.75	5.70	—
D	1 ml. 0.01 M KIO_4‡	$10^{4.5}$	21	8.20	1.50	6.70	—
			45	8.44	3.45	4.99	5.04

* All titrations were carried out immediately upon harvest of the allantoic fluids.

‡ KIO_4 followed 1 hour later by 0.25 ml. of 0.1 M glycerol, given to treated as well as untreated eggs. Virus inoculated 10 minutes after glycerol.

It is clear that (*a*) the rate of viral multiplication was slower in KIO_4-treated than in untreated eggs; (*b*) the relative loss in infectivity between the two periods of harvest was significantly greater in the two KIO_4-treated than in the corresponding control groups; (*c*) the 21 hour harvest from eggs in group A and the 45 hour harvest from group D had comparable titers and ID_{50}/HA ratios, yet on further undiluted subpassage they behaved differently: the group A inoculum yielded "incomplete" virus of the low ratio expected of a second undiluted passage (9), while the group D inoculum gave the type of yield expected from a first undiluted passage (see 21 hour harvest from group A). This would indicate that the non-infectious hemagglutinin contained in the group D inoculum was not of the "von Magnus type."

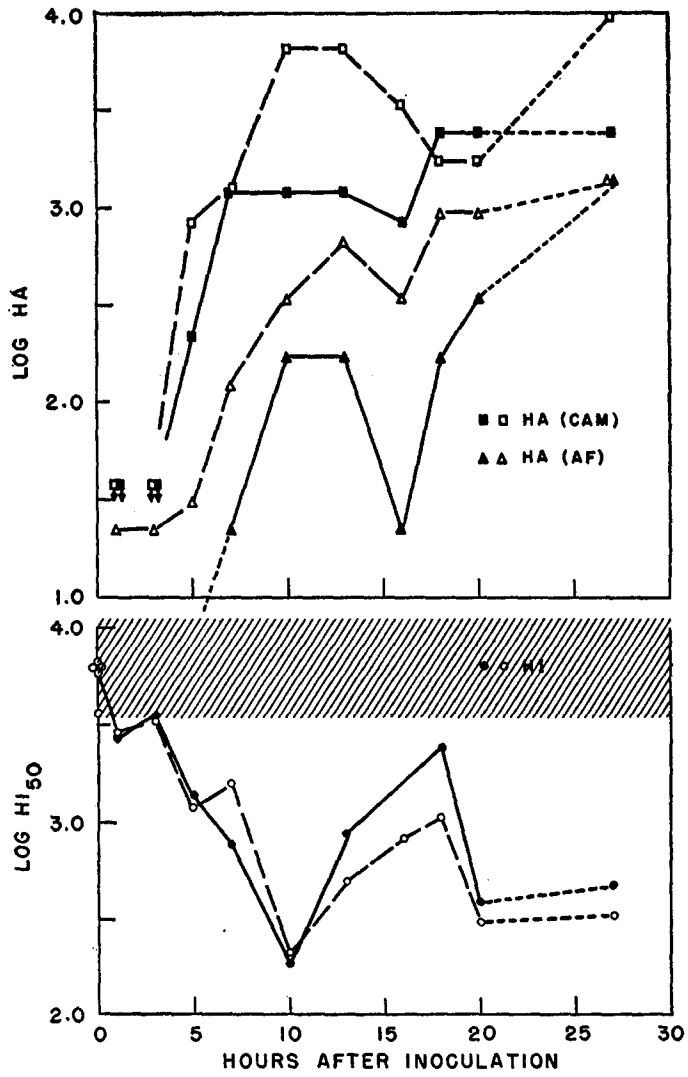

FIG. 7. Viral multiplication and liberation and associated changes in inhibitory titer of CAM extracts. Effect of pretreatment of eggs with metaperiodate. Intraallantoic inoculum $10^{8.5}$ ID_{50} of PR8 virus. One-half hour before infection, eggs received either 0.5 ml. of M/100 KIO_4 followed 15 minutes later by 0.5 ml. of 5 per cent glucose (closed symbols), or a mixture of M/100 KIO_4 and 5 per cent glucose in two doses of 0.5 ml. each 15 minutes apart (open symbols). Each symbol represents titer obtained with a pool of 5 allantoic fluids or membranes.

See Note under Fig. 1.

done on unheated aliquots of the same CAM suspensions after they had been stored at 2°C. for no more than 24 hours. None of them contained more than $10^{6.2}$ to $10^{6.4}$ ID_{50} per gm. of tissue. Such low titers in a first undiluted passage would suggest the presence in the suspensions of an inactivating agent. Perhaps the inactivation of virus and the over-all retardation of the infectious process is in some way related to the release of formaldehyde as a reaction product of periodate and reducing sugars (20).

DISCUSSION

When this work was started a few years ago (18), it was thought that the chief function of the viral enzyme was to facilitate the entrance of viral particles into the host cell (21), and it was expected that the rate of substrate breakdown would prove to be a simple function of virus concentration in the tissue and in the surrounding medium. Early findings in mouse brain infected with influenza virus were in line with this assumption since inactivation of substrate (hemagglutination inhibitors or HI) was progressive, and restoration of normal HI activity very slow (3, 4). These results were also consistent with observations by Cairns (22) to the effect that restoration of normal amounts of soluble HI in mouse brain after intracerebral inoculation of RDE (receptor-destroying enzyme of *V. cholerae*) required periods in excess of 2 weeks.

That the situation was fundamentally different in the case of the CAM became apparent from work discussed in the preceding paper (1) which showed that the level of mucoid substrate contained in the intact allantoic membrane was stable under the action of extraneous RDE. It was detectably lowered only when the tissue was maintained under unfavorable conditions. It was postulated that the allantoic cells had a highly developed capacity of maintaining or restoring their normal supply of inhibitory mucoprotein. This postulate was borne out by the finding, reported in the present paper, that membranes in which viral multiplication had apparently ceased but which were still exposed to maximal amounts of enzymatically active virus in the allantoic fluid yielded normal or almost normal amounts of soluble HI.

More detailed analysis of the fate of HI in relation to the viral growth process has revealed that there exists a definite temporal relationship between viral multiplication and decrease in HI activity. As might be expected, a significant decrease in HI can be demonstrated only when all infectable cells of the allantoic membrane are involved in the infectious process. Once virus has attained a concentration in the egg at which this condition is fulfilled, the inactivation of HI is demonstrable as a discontinuous process in which periods of diminution are followed by stationary periods or by partial restoration of normal HI levels. A striking feature of this periodicity is that it is in close agreement with early observations on the duration of viral growth cycles. Cairns has reported, in close agreement with the earlier data of Freymann *et al.*

(23), that the "average liberation time" lasted 7½ to 9 hours for the first viral growth cycle, 4½ to 7 hours for subsequent cycles (24). The curve shown above for HI titers of CAM after inoculation of $10^{8.7}$ ID_{50} of PR8 virus (Fig. 2) suggests an initial step of 10 hours' duration, followed by a second step lasting 6½ hours. In eggs inoculated with smaller doses of virus, not enough cells were infected initially to affect the HI titer during the first one or two growth cycles, but thereafter three consecutive discrete phases were discernible at 5½ hour intervals.

Even more pronounced periodicity of HI breakdown and restoration was found in KIO_4-treated eggs in which the entire infectious process appeared to be retarded. Thus, significant regeneration of normal HI activity occurred before a second growth cycle began.

The suggestion that breakdown of substrate occurs stepwise in association with periods of viral reproduction poses a dilemma in the light of recent data indicating that *liberation* of influenza virus from allantoic cells is not a cyclic but a continuous process (25). It must be recognized that it would be difficult to discern discrete cycles of virus production once all cells are involved in the process. If one assumes that virus is produced at a constant rate, either continuously or in cycles, the total amount produced initially by all cells could, at best, be doubled during a subsequent period of comparable duration (2). As pointed out by Henle *et al.* (25), subsequent 0.3 log-fold increments would then occur successively later. Since this increase in virus in the tissue is countered by release into the allantoic fluid, it may not at all be recognizable as such. Nevertheless, consecutive cycles of viral multiplication in the CAM have been observed before, not only for influenza virus (26), but also for Newcastle disease virus (27). In a paper appearing while this is being written, Horsfall (28) presents two graphs showing the number of viral particles per allantoic cell after inoculation of saturating doses of Lee virus (1.5×10^7 and 1.5×10^8 infectious particles, respectively). Both curves show secondary rises in infectious titers after a definite dip. Because it is difficult to standardize infectivity or even hemagglutinin titrations to a point where two-fold increments could be relied upon as significant, it has been helpful to fall back on the curves obtained with periodate-treated membranes which show quite clearly that under these conditions virus production in the CAM does proceed in cycles.

Moreover, the finding that the inhibitory titer of CAM remained stationary or actually increased during periods of active liberation of virus from allantoic cells seems to indicate that breakdown of substrate was not prominently associated with the release mechanism but rather with the period during which new viral particles were produced in the cells.

In this respect, then, the periodic breakdown and partial restoration of inhibitory mucoprotein appears to be a more sensitive indicator of the cyclic

nature of the infectious process than direct titration of viral activities. The data presented above do not permit any conclusion concerning the role which the enzyme-substrate interaction may play in the viral growth process. One can speculate that it is in some way involved in the synthesis of new viral material, and that this phase of viral replication takes place in the mucoid outer layer of the cell (29), under the control of viral genetic material which has penetrated into the cytoplasm itself. This process could be envisaged as akin to the production of specific soluble polysaccharides in the capsule of *Diplococcus pneumoniae* under the influence of transforming principle. Consecutive cycles could then be explained as due to the reentry of viral genetic material into the cytoplasm from the mucoid layer, without assuming that reinfecting viral particles have to first be liberated into the allantoic fluid. In this way, the shorter duration of secondary cycles could be explained, since reinfecting viral particles would not have to traverse the entire mucoid layer of the cells.

SUMMARY

The fate of heat-stable inhibitors (HI) of hemagglutination by influenza viruses in the infected chorioallantoic membrane has been studied. The amount of soluble HI extractable from the normal CAM is taken as a measure of the total mucoprotein substrate derived from the allantoic cells.

It has been shown that a decrease in HI is demonstrable when all or nearly all cells of the allantois are involved in the infectious process. Once this condition is fulfilled, viral multiplication in the CAM is associated with stepwise breakdown and partial restoration of HI. The periodicity of these steps is in close agreement with earlier reports by others on the duration of primary and secondary cycles of viral multiplication. Periodicity of viral multiplication and of HI breakdown with subsequent restoration is particularly pronounced in eggs pretreated with metaperiodate or with mixtures of periodate and glucose or glycerol.

The stepwise nature of the HI breakdown indicates that it is in some way related to intracellular phases of viral reproduction, and that individual cells produce virus in cycles rather than continuously. A possible mechanism of this process is proposed.

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