INFLUENZA VIRUS AND ITS MUCOPROTEIN SUBSTRATE IN THE CHORIOALLANTOIC MEMBRANE OF THE CHICK EMBRYO

I. Characterization and Quantitative Assay of Soluble Substrate and Studies on Its Relation to Allantoic Cells

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The discovery of enzymatic activity associated with influenza viruses (1) has stimulated speculation concerning its possible role in infection of cells with these agents.

It has been suggested that the enzymes promote penetration of viral particles into cells (2), or that access of virus to susceptible cells is facilitated by enzymatic breakdown of an extracellular barrier of mucoid material (3). The force of these hypotheses is weakened to some extent by the results of interference experiments (4) and of genetic studies (5) which suggest that heat-inactivated viral particles are capable of penetrating into cells even though they have lost their enzymatic activity (6, 7).

As alternatives, breakdown of mucoprotein substrate may be thought of as in some way related to viral reproduction or to the emergence of newly produced viral particles from infected cells (8). As far as is known, only enzymatically active virus is capable of reproducing and of initiating multiplicity reactivation (9) or reactivation of inactive viral particles (5). The body of circumstantial evidence supports a purely teleological argument that the viral enzyme system, having persisted in the evolution of many strains of influenza and other "myxoviruses" (10), must have some function essential to the continuity of the species.

The breakdown of soluble substrate (hemagglutination inhibitors of the "Francis type" (11)) or of red cell receptors by active viruses has been the subject of intensive study in several laboratories (12). So has the nature of inhibitory mucoproteins derived from various sources (13). By comparison, work on the fate of mucoid substrate in infected tissues has been scant (14–17) and, as far as we can see, no serious effort has been made to ascertain the topical or functional relationship of substrate to cells.

In approaching a systematic study of this problem, we were guided by these basic assumptions: (*a*) that the mucoprotein substrate in which we were

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interested was a normal constituent of the host tissue; (*b*) that normally the cells did not produce or liberate enzyme-like substances capable of modifying the substrate; (*c*) that infection with influenza viruses "triggered" the cells to produce such enzymes along with other components incorporated in newly synthesized virus.

To facilitate interpretation of findings in infected tissues, it was necessary to define the substrate, to establish standards for quantitative measurements, and to gain insight into the way in which the substrate fitted into the economy of normal tissues. Experiments in these directions will be described in this paper. The substrate was represented by heat-stable hemagglutination inhibitors (HI) which could be extracted from tissues and which were susceptible to *in vitro* inactivation by active influenza viruses or receptor-destroying enzyme of *Vibrio cholerae* (18). The work was carried out chiefly on the chorioallantoic membrane (CAM) of chick embryos, but reference will also be made to a few experiments on mouse brain.

Materials and Methods

This section covers work reported in this as well as the following paper (19).

Virus Strains.—The PR8, WS, and Melb strains of influenza virus, type A, and the Lee strain of type B were propagated in the allantoic cavity of 11-day-old chick embryos. Eggs were inoculated with 0.2 ml. of 10^{-6} diluted seed virus, incubated for 40 to 44 hours, and the allantoic fluid (AF) was harvested either immediately or after overnight chilling at 4°C. Only freshly harvested virus was used in experiments.

Indicator Viruses.—For use in HI titrations and in studies on adsorption of heat-inactivated virus, infected allantoic fluids were dialyzed in the cold against two changes of 0.1 M phosphate buffer pH 7. A rocking dialyzer was used. After dialysis, the fluids were heated for 40 minutes at 56°C. In the case of Melb virus, heating was done for 1 hour after addition of citrate and borate buffer, according to the method of Stone (20). Such treatment preserved the HA titers undiminished but entirely eliminated the capacity of the viruses to elute spontaneously from red cells. The prefix H- will be used to identify indicator strains. For convenience, the activity of the extracts against different indicator viruses will be referred to as PR8-, WS-, Melb-, or Lee-HI.

Receptor-Destroying Enzyme (RDE) of V. cholerae.—Cholera vibrio strain 35A3 (Inaba) was originally obtained from the National Institutes of Health, Bethesda. Berkefeld N or Seitz filtrates of 16- to 18-hour cultures in beef heart infusion broth served as source of RDE. Occasionally, such filtrates were concentrated by pervaporation and dialysis, as described by Hirst (21).

Virus Titrations.—Infectivity titrations were done by preparing 10-fold serial dilutions of test material in buffered glucosol (22). 0.2 ml. of each appropriate dilution was inoculated into 4 to 6 eggs. Titers are expressed as 50 per cent infective doses (ID_{50}) per milliliter or per gram of tissue.

Hemagglutinin (HA) titrations were done by the pattern method, employing 0.5 ml. of each 2-fold dilution of virus and an equal volume of 0.5 per cent red cell suspension. Dilutions and cell suspensions were made in saline containing 2 per cent sodium citrate (citrate saline). The tests were read as soon as cells in control tubes had settled in a clearly demarcated button. Agglutination was read as either complete or partial, with partial agglutina-

tion usually limited to a single tube. The end-point was either the highest dilution giving complete agglutination or the geometric mean between the highest dilution giving partial agglutination and the next lower dilution. Titers are expressed as the log of the reciprocal of the final dilution (after addition of RBC) of the test material.

RDE Titrations.—The method of Burnet and Stone (18) proved to be unsuitable because our Melb strain failed to give stable agglutination patterns at 37°C. For this reason, tests were set up with 4 units of Melb and PR8 *indicator* viruses. RDE dilutions and red cells in calcium-borate buffer were incubated for 30 minutes at 37°C., the virus diluted in citrate saline was added, and the test was read after overnight incubation in the refrigerator. Titers of crude filtrates by this method averaged 1:256 against both strains of virus.

Deembryonated Eggs.—Shells of 13-day-old eggs were scored with an electric drill around the entire circumference about midway between the two poles. The two halves were separated with fine scissors cutting through shell membrane and chorioallantois. All contents, except a smooth single layer of CAM, fell away from the portion containing the air sac without further cutting. This portion was washed out with several changes of Tyrode's solution or buffered glucosol (22) before use. Shells so prepared were loosely covered with Esmarch dishes and were incubated at 36°C. on a tray maintained in slow see-saw motion. Each shell contained 5 to 8 ml. of liquid, and care was taken that only that portion continuously bathed in liquid was harvested. Each shell yielded about 0.3 gm. of CAM (wet weight).

Preparation of Tissue Extracts for HI Assays.—CAM were washed with large volumes of cold saline or buffered glucosol while still adhering to the shell membranes. After excess fluid was drained off, the membranes were removed and either ground up immediately or first frozen in dry ice-alcohol mixture and stored in the frozen state. Grinding was done in the cold, under addition of 9 volumes (V/W) of chilled saline, either by hand in an all glass TenBroeck mill or mechanically in VirTis homogenizers. The resulting suspensions were heated and centrifuged as described below.

Assays of HI Activity.—Inhibitory activity of tissue extracts was measured by two techniques. The densitometric method of Hirst and Pickels (23) determined the 50 per cent HI titer (HI_{50}); *i.e.*, that dilution of tissue extract which reduced by one-half the number of red cells agglutinated by a fixed amount of indicator virus. Titers are expressed in terms of tissue wet weight, considering the initial extract as 10 per cent W/V (24).

The pattern technique (25) was used to determine either the HI titers of extracts in presence of a fixed amount of virus—basically similar to the densitometric method—or the inhibitory capacity; *i.e.*, the number of HA units of indicator virus inhibited by a given dilution of tissue extract.

Data to be presented below will indicate to what extent results obtained by these techniques were comparable.

EXPERIMENTAL

Characteristics of Hemagglutination Inhibitors (HI) Extracted from CAM Tissue

Because so much work had been done in various laboratories on the purification and characterization of HI from numerous biological sources, it did not seem necessary to go through laborious attempts at purification of the inhibitory material from CAM, as long as its essential behavior permitted it to be classified as analogous to soluble HI from normal urine (26), allantoic fluid (27, 28), egg white (29, 30), and other sources. The following criteria

were set up to satisfy this requirement: (*a*) heat stability, (*b*) precipitability by one or several of the methods employed in fractionation of other HI, (*c*) progressive inactivation by either active influenza viruses or by RDE.

Heat Stability.—Crude CAM suspensions in saline or similar suspensions which had been clarified by centrifugation at 10,000 R.P.M. for 20 minutes were heated at 65°C. for 30 min-

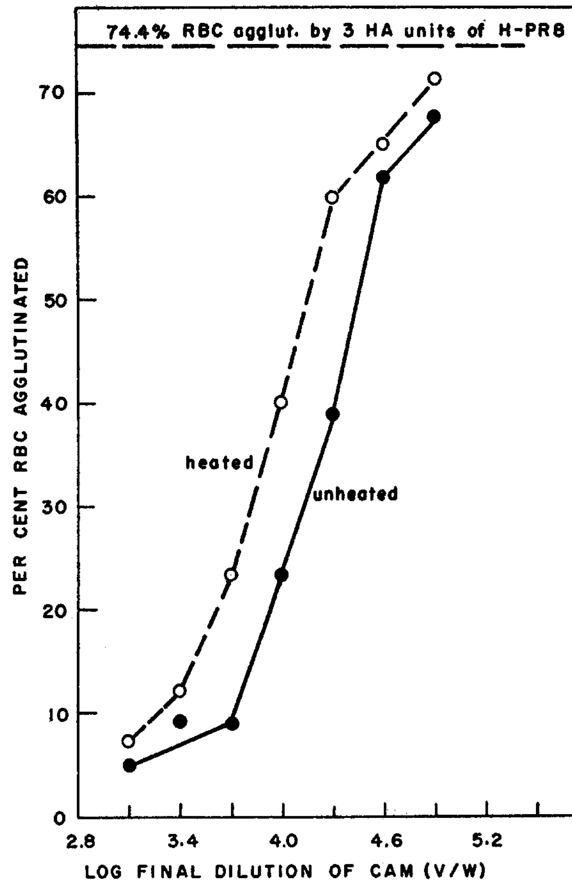

FIG. 1. Effect of heating (65°C. for 30 minutes) on slope of PR8-HI titration curve.

utes or in a boiling water bath for 10 minutes. The resulting precipitates were spun out at 10,000 R.P.M. for 10 minutes, and the clear supernatants of heated and unheated portions were titrated for HI activity.

Heating in either manner resulted in an average loss of 50 per cent of the inhibitory titer. As shown in Fig. 1, the slope of titration curves remained unaltered after heating, suggesting that the reduction in titer was not associated with significant qualitative changes in the inhibitory material (see

below). Moreover, when CAM extract was prepared in salt-free 0.25 M sucrose, a medium in which heating after preliminary centrifugation produced no visible precipitate, no loss of HI activity was encountered. Hence, HI *per se* appeared to be heat-stable.

Partial Purification.—Although several methods described by other authors worked well in our hands when applied to the materials for which they were elaborated, they failed in the case of CAM-HI. Thus, fractionation of egg white with phosphate buffers according to Lanni *et al.* (30) gave good yields, but HI precipitated from CAM extracts with 0.1 M KH_2PO_4 did not redissolve in 0.06 M phosphate buffer at pH 7.2. On the other hand, no HI could be precipitated from CAM extracts with cold distilled water as described for ovomucin by Gottschalk and Lind (29).

Tamm and Horsfall (26) described the precipitation of inhibitory mucoprotein from normal human urine by 0.58 M sodium chloride. Accordingly, suspensions of CAM in saline were heated at 65°C. for 30 minutes and centrifuged. The pH of the supernatant was adjusted to 6.5 by addition of N/1 HCl. Then NaCl was added in the cold to a concentration of 0.6 M, and the suspension was kept in the cold. Precipitates were centrifuged out after 1, 2, 3, and 6 days and extracted with distilled water. When tested for HI, the supernatants contained undiminished activity throughout, and none was recovered from the precipitates. Similar results were obtained when precipitation with 0.6 M NaCl was carried out at pH 4, 6, 7, 8, or 10.

CAM suspensions were subjected to fractionation with cold ethanol. Preliminary tests indicated that ethanol at a concentration of 60 per cent (V/V) precipitated 90 per cent or more of the HI activity. Almost no activity could be extracted from precipitates with unbuffered distilled water, but satisfactory extraction of alcohol-precipitated HI from the CAM occurred at neutral or alkaline pH. Precipitation was independent of pH (range 4 to 10) or salt concentration (0.15 to 0.75 M NaCl).

In order to determine the least amount of ethanol required to precipitate the major proportion of HI, aliquots of heated, centrifuged CAM suspension were treated in the cold with 20 to 70 per cent ethanol which had been cooled to −50°C. Precipitates were extracted with M/20 borate buffer pH 7.2. Titrations of HI activity in the various fractions by the pattern and densitometric methods gave seemingly discrepant results, as shown in Table I. An explanation of this difference was found in the plotting of densitometric readings presented in Fig. 2. It is evident that the slopes of titration curves obtained with extracts of 20, 30, or 40 per cent alcohol precipitates were steeper than those with the starting material or with the yields from higher alcohol concentrations. The convergence of the 30 and 40 per cent curves with the control curve in the zone of complete inhibition was obviously reflected in the results of pattern tests (see Table I), which, in effect, determined complete inhibition of visible pattern formation rather than partial inhibition

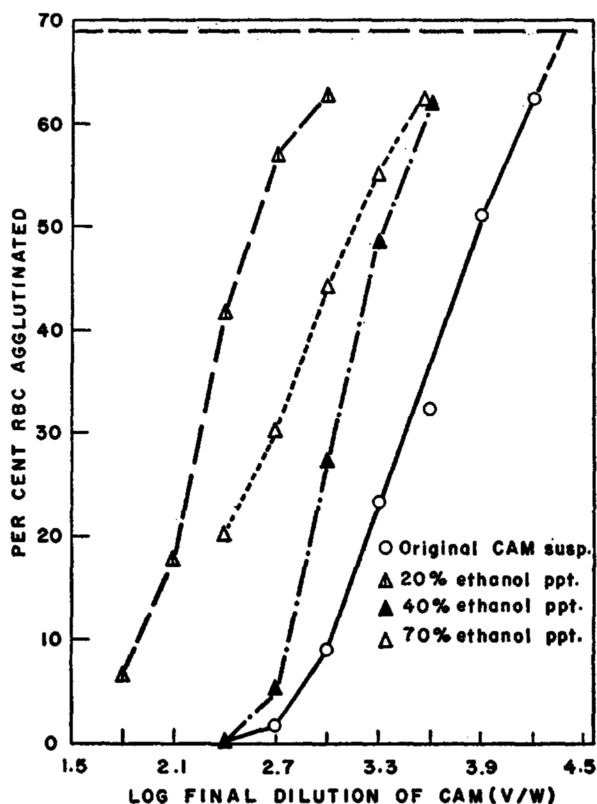

FIG. 2. Shift in slope of PR8-HI titration curves after precipitation with ethanol. Note: The curve obtained with 30 per cent ethanol precipitate was almost identical with the 40 per cent curve and has been omitted for the sake of clarity. Similarly, the 50 and 60 per cent curves fell close to the 70 per cent curve shown.

TABLE I

Yield of HI from Ethanol Precipitates of CAM Extract: Comparison of Pattern and Densitometric Titers

Experiment No.	Concentration of ethanol	Fraction	Per cent of original HI activity recovered	
			Pattern test	Densitometric test (50 per cent HI end-point)
	per cent (V/V)			
1	20	Extracted precipitate	n.t.*	4.6
	30	" "	100.0	30.0
	40	" "	50.0	29.0
	50	" "	6.4	27.0
	60	" "	6.4	21.0
	70	" "	6.4	15.0
2	30	Non-precipitated residue	50.0	n.t.*
		Extracted precipitate	50.0	26.0
	45	Non-precipitated residue	<2.0	44.0
		Extracted precipitate	100.0	51.0

* n.t., not tested.

This interpretation was borne out by experiments in which not only the redissolved precipitates but also the non-precipitated residues in the alcohol-containing fractions were titrated. This is illustrated in Table I (Experiment 2). At 45 per cent concentration of alcohol, no HI capable of complete inhibition (pattern test) was detectable in the alcohol fraction, and extraction of

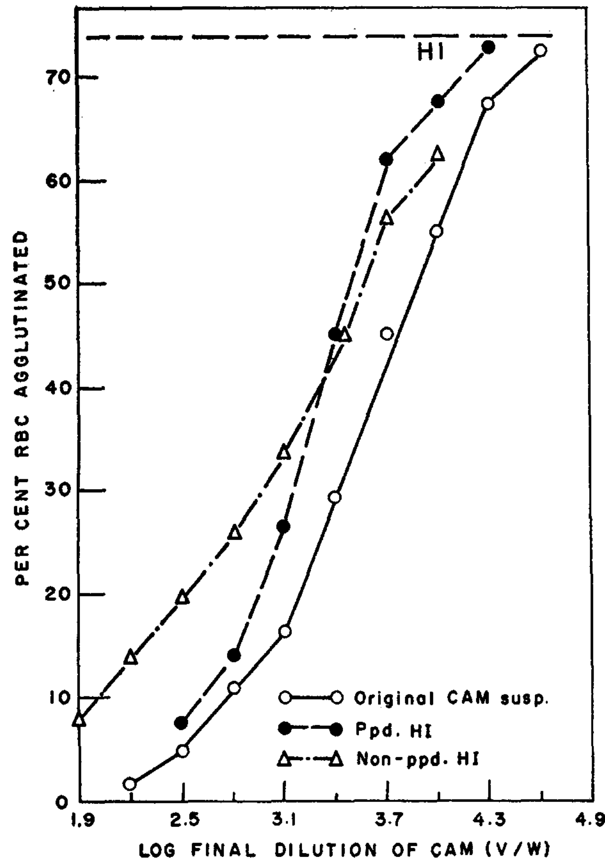

FIG. 3. Precipitation of PR8-HI with 45 per cent ethanol. Titration curves for extracted precipitate and non-precipitated fraction.

the precipitate yielded 100 per cent of the original activity. In contrast, the densitometric test revealed a loss of only 56 per cent of the original activity in the non-precipitated fraction, and of this 91 per cent was recovered from the precipitate. In keeping with these figures, the densitometric readings plotted in Fig. 3 reveal that the curve obtained with precipitate is steeper than that with the starting material, while the non-precipitated fraction gives a flatter curve.

With alcohol in concentrations in excess of 45 per cent, no significant amounts of HI remained detectable in the non-precipitated fractions. Nevertheless, the precipitates had almost no activity when tested by the pattern method (Table I, Experiment 1), although the densitometric tests revealed

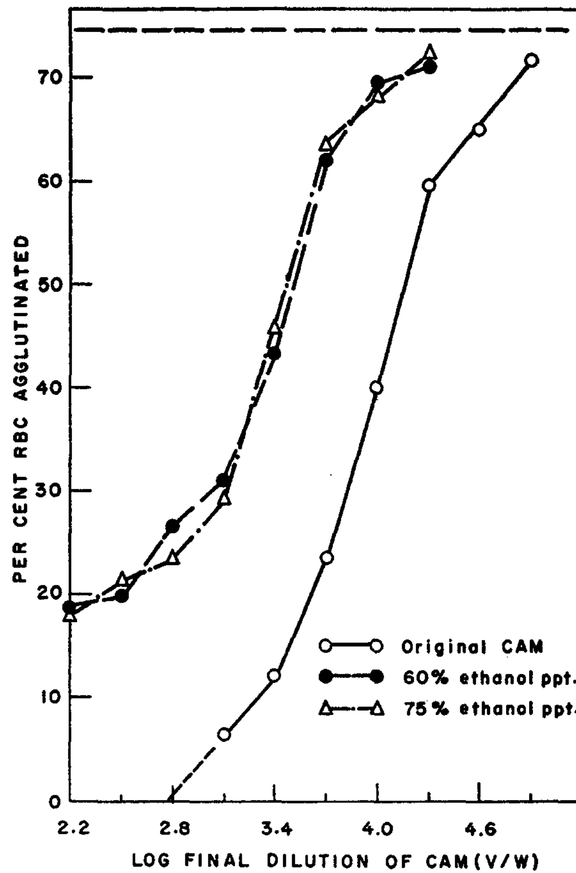

FIG. 4. Shift in slope of PR8-HI titration curves after precipitation with 60 or 75 per cent ethanol.

significant amounts in the zone of partial inhibition. Again, plotting of titration curves offered an explanation for this discrepancy in that, even at high concentration, HI precipitated with 60 and 75 per cent alcohol was capable of only partial inhibition. Hence, the tendency of the curves in Fig. 4 to level off in the range corresponding to agglutination of 20 per cent of the red cells (about 70 per cent inhibition).

These findings suggested that alcohol in relatively high concentration in-

duced a qualitative change in the nature of the inhibitory material. The available data do not permit a definitive interpretation of this change. The low over-all yield could be due either to denaturation or to poor solubility. The change in the titration curve, *i.e.* the predominance of "weak" over "strong" inhibitors (31), may likewise be caused by denaturation or may be an expression of greater solubility of smaller, less efficient inhibitor molecules. The latter interpretation would presuppose initial inhomogeneity of the inhibitory material. This assumption is favored in view of the results obtained with precipitates at low concentrations of alcohol. The relatively high yield under these circumstances of the more efficient HI, *i.e.* those molecules capable of completely inhibiting agglutination of red cells, suggests preferential precipitation of such elements out of a heterogeneous population.

This view would be in line with electron microscopic observations on HI derived from egg white and urine which have shown them to be filaments of varying length (32, 33). In both cases, heating seems to increase heterogeneity, as revealed by sedimentation studies on urinary mucoprotein and on semipurified egg white inhibitors reported by Tamm, Bugher, and Horsfall (34) and by Sharp *et al.* (35). Although heating did not in itself alter the slope of titration curves of CAM extracts (see Fig. 1), the possibility has not been excluded that the heterogeneity revealed after ethanol precipitation may have been increased by the preliminary heating to which the extracts used in the preceding experiments were subjected.

In order to compare the inhibitory material from CAM with similar materials isolated from other organs, rather than from body fluids, an attempt was made to apply the method used successfully by McCrea for the preparation of mucoid inhibitor from sheep salivary gland (36). Experiments were done in parallel on mouse brain and on CAM with contrasting results.

Whole mouse brains were dehydrated for 3 months in several changes of absolute alcohol. After removal from alcohol, the tissue was minced and dried. 126 gm. of tissue was ground up in 378 ml. of distilled water. The suspension was brought to 80°C. for 2 minutes, cooled to room temperature, and shaken for 5 hours. After addition of a small amount of chloroform, the suspension was kept overnight at 4°C. This was followed by centrifugation, first at low speed, then at 8,000 R.P.M. in a Sorval angle head. The resulting sediments were pooled and reextracted with distilled water as above. The supernatants from the two extractions were lyophilized, yielding 2.7 gm. of white powder (2.14 per cent of the tissue weight). This preparation, dissolved in 50 volumes of distilled water, was subjected to fractionation in the cold with ethanol.

The results, shown in Table II, revealed highest HI activity in the fraction precipitated with 30 to 40 per cent ethanol, somewhat less in the 40 to 60 per cent fraction. The combined activity of these two fractions amounted to 270 per cent of that of the resuspended powder, a discrepancy probably due to incomplete dispersion of the latter.

When the same method was applied to the CAM, entirely negative results were obtained. No activity remained extractable from membrane tissue after prolonged dehydration with absolute alcohol. Since it was known from experiments already described that ethanol in concentrations in excess of 50 per cent either denatured HI extracted from the CAM or rendered it insoluble, the failure to recover any HI from membranes after treatment with absolute alcohol was not surprising. The difference in behavior of HI from mouse brain and CAM could be interpreted as due to chemical differences or to the fact that HI in the CAM was located near the surface, while in the case of mouse brain or salivary gland it may have been situated so that it was protected from the direct action of alcohol.

TABLE II

Ethanol Fractionation of Lee-HI from Mouse Brain after Dehydration with Absolute Alcohol

	Total volume	HI titer (log) of sample* per ml.	Total HI (log) in fraction‡
	ml.		
Crude extract§	81.0	2.50	4.41
<30 per cent ethanol precipitate	7.9	1.75	2.65
30–40 " " " "	15.1	3.55	4.73
40–60 " " " "	11.2	3.25	4.30
60–82 " " " "	9.5	<1.00	1.98

* Titrations by pattern method.

‡ HI titer × volume.

§ Crude extract: centrifuged suspension of lyophilized powder (see text).

Effect of Active Influenza Virus and RDE on HI Activity of CAM Extracts.—The specific identification of the inhibitory material rested on the demonstration that it was inactivated *in vitro* under the action of active influenza virus or of the receptor-destroying enzyme of *V. cholerea.*

In a typical experiment, a 20 per cent suspension of CAM's was centrifuged at 10,000 R.P.M. for 10 minutes. The supernatant was diluted 16-fold in calcium borate buffer (20) containing penicillin 100 units/ml. and streptomycin 50 mg./ml. To 4 ml. aliquots of this extract were added equal volumes of freshly harvested PR8- or Lee-infected allantoic fluid or of RDE, each diluted serially in 5-fold steps with calcium borate buffer. These mixtures and a control mixture containing only CAM extract and buffer, were incubated at 37°C. and sampled at 1, 2, 3, and 4 hours. Each sample was immersed in a 65°C. water bath for 30 minutes and centrifuged at 10,000 R.P.M. for 10 minutes. All supernatants were titrated for HI.

The rates of HI breakdown by the three agents are depicted in Fig. 5. It should be noted that the titers of untreated CAM extract remained unchanged throughout the period of incubation. Stability of HI in crude CAM

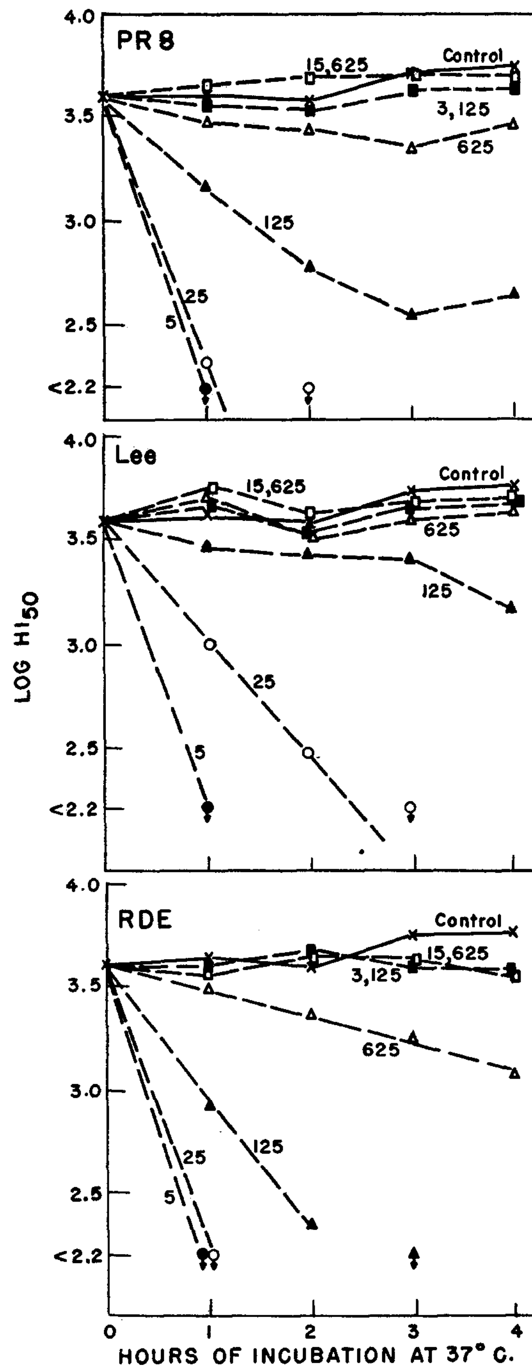

FIG. 5. Rate of inactivation of PR8-HI on incubation at 37°C. with varying amounts of active PR8 or Lee virus or RDE. Numbers indicate reciprocals of dilutions of the macti-vating agents.

extracts under various conditions of storage and incubation was confirmed repeatedly and indicated that normal membranes did not contain measurable amounts of enzymatic activity similar to that associated with influenza viruses or RDE. RDE and PR8 were nearly equivalent in enzymatic activity, RDE being slightly superior in rate and completeness of action. Lee had

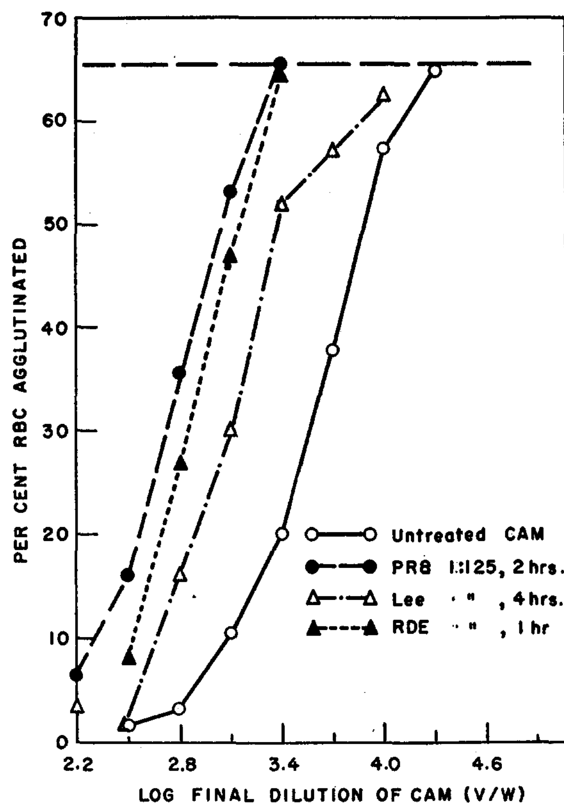

FIG. 6. Slope of titration curves of PR8-HI partially inactivated by incubation with active PR8 or Lee virus or RDE.

about one-fifth the activity of RDE. Of special significance was the finding, illustrated in Fig. 6, that the slope of HI titration curves was not significantly altered by viral enzymatic degradation. This fact indicated that the decrease in titer was quantitative; *i.e.*, that HI molecules, regardless of any innate heterogeneity, were equally susceptible to enzymatic action.

Under similar conditions, activity against H-Lee was found to be more resistant to inactivation by active virus and RDE than PR8-HI. Indeed, under some conditions, the Lee-HI titer was higher after treatment than before (Table III). Although these apparent increases were of an order which made

it hazardous to assess their significance (see below), similar suggestive results were obtained in infected eggs (19). Moreover, in the case of mouse brain, where Lee-HI activity is nine times more potent than inhibition of PR8 or other type A indicator strains, the latter showed a relative increase after short incubation with active viruses or in early stages of infection. No explanation for this effect can be offered, other than to suggest that perhaps untreated extracts contain some HI in aggregates (as suggested by others (37)), and that apparent increases in HI activity such as described represent a manifestation

TABLE III

In vitro Effect of Active PR8, Lee, or RDE on PR8- and Lee-HI Titer of CAM Extract after 1 Hour at 37°C.

Treatment		HI_{50} (per cent of untreated CAM) *vs.* heated	
Inactivating agent	Dilution	PR8	Lee
PR8	1:1	<15	67
	1:5	<15	97
	1:25	<15	68
	1:125	<30	126
	1:625	62	145
	1:3125	81	160
Lee	1:1	30	55
	1:5	<30	43
	1:25	44	82
	1:125	74	99
	1:625	110	200
	1:3125	128	228
RDE	1:1	<15	<30
	1:5	<15	<30
	1:25	<15	31
	1:125	36	72
	1:625	91	113

of disaggregation detectable only in a narrow borderline zone of early enzymatic activity.

No further attempts at purification and characterization of the inhibitory material from CAM were undertaken. It was felt that the demonstration of heat stability, the behavior on fractionation with alcohol, and the inactivation by active virus and by RDE sufficed to classify it with inhibitors from other sources which have been identified as mucoprotein in nature.

Reproducibility of HI Titration End-Points

In view of the fact that the ultimate aim of these investigations was to study the changes in tissue substrate associated with infection, it was impor-

tant to establish criteria by which to assess the significance of such changes. Once it had been demonstrated that the action of active virus and of RDE induced no major change in the slope of titration curves, it seemed permissible to use the HI_{50} values as a gauge of quantitative differences between normal and infected membranes. Tables IV and V present, in summary, an analysis of the titers obtained in comparable control titrations on individual or pooled

TABLE IV

Geometric Means and Standard Deviations of Hemagglutination Inhibition Titers Determined Densitometrically for Individual CAM Extracts with PR8 Indicator Virus

Experiment	No. of membranes	Age of embryos	HI_{50} (log)		
			Range	Geometric mean	Standard deviation
		days			
A	12	11–12	3.54–4.04	3.84	0.150
B	18	11–12	3.23–4.07	3.68	0.256
C	12	11	3.50–3.93	3.71	0.143
Total.......	42	11–12	3.23–4.07	3.73	0.209

TABLE V

Geometric Means and Standard Deviations of Hemagglutination Inhibition Titers Determined Densitometrically for Extracts of CAM Pools with PR8 and Lee Indicator Viruses

Indicator virus	HA units	No. of pools tested	Age of embryos	HI_{50} (log)				
				Range	Mean		Standard deviation	
			days					
PR8 (8 experiments)	2.5–3.0	15	11	3.62–3.89	3.78	3.81	0.083	0.136
	2.5–3.0	15	12–13	3.53–4.07	3.84		0.171	
Lee (9 experiments)	2.5–2.7	18	11–14	3.05–3.48	3.25		0.131	
	3.0–4.0	25	11–14	2.60–3.32	2.89		0.200	

normal CAM extracts. As would be expected, the scatter of values was greater for individual specimens than for membrane pools. Moreover, the data on pools were combined from eight (PR8-HI) or nine (Lee-HI) experiments, and the variations encountered with several pools in any single experiment were always smaller than those given in Table V. Mean titers or titers of pools in experiments to be described below and in reference 19 were considered to deviate significantly from the norm when they differed from the appropriate control mean by more than twice the standard error $\left(2 \times \frac{\sigma}{\sqrt{N}}\right)$.

It will be shown in the following paper (19) that deviations of this order of magnitude, at least in the case of PR8-HI, were invariably in the direction of decrease, and this tended to enhance their significance.

The mean titers given in Table V show that PR8-HI exceeded Lee-HI activity by a factor of 3- to 8-fold. On the other hand, Melb- and WS-HI titers were consistently of the same order of magnitude as PR8-HI titers.

Relation of HI Activity to Cells of Allantoic Epithelium

It has been demonstrated by others (14, 15) that multiplication of influenza virus in the allantoic cavity may be associated with a precipitous drop in the amount of HI extractable from the CAM. This has been confirmed in investigations reported in the following paper (19). Such a drop in the inhibitory activity makes it compelling to assume that its main source is the allantoic epithelium, inasmuch as viral multiplication after intraallantoic inoculation

TABLE VI

In Ovo Substitution of HI-Free for Normal Allantoic Fluid. Compensatory Release of HI into Fluid

Sample	HA units of H-PR8 inhibited
Original AF	22
Immediately after substitution	2
1 hr. " "	5
4.5 hr. " "	13

appears to be restricted to this layer of the CAM (38). Moreover, the presence of HI in allantoic fluid of normal chick embryos and its increase with advancing age (27, 28) suggest that it is a secretory product of the cells lining the allantoic cavity. That this is so can be demonstrated directly.

The allantoic cavities of 13-day-old embryos were drained of allantoic fluid (7 to 10.5 ml per egg) and flushed with three consecutive changes of warm physiological saline solution in volumes equivalent to the AF removed. Allantoic fluid which had previously been freed of HI by treatment with RDE and heated at 65°C. for 30 minutes was then substituted for the original fluid. The eggs were replaced in the incubator and tapped at varying time intervals.

Table VI shows the results of HI tests on some of the samples, and it is clear that loss in HI after substitution was compensated for progressively. That the release was not due to a simple washing off of a surface coat of mucoid material but rather to active secretion was suggested by the following considerations: (*a*) the amount of HI extractable from the CAM was not detectably lowered by substitution of HI-free fluid for HI-rich allantoic fluid either *in ovo* or in deembryonated eggs, although HI was in both cases re-

leased into the fluid; (*b*) intensive washing of the intact CAM with copious amounts of saline solution failed to reduce the HI titer; (*c*) treatment with

TABLE VII

Effect of RDE on HI of CAM under Various Conditions of Incubation

Group	Treatment	Period of incubation at 36°C. after treatment	Densitometric HI titer (log HI_{50})*	
			PR8	Lee
		hrs.		
A	Normal eggs 11-day-old	—	3.86	3.37
	" " 12-day-old	—	3.94	3.43
B	Inoculated with 0.8 ml. glucosol	3½	3.80	3.26
		23	3.72	3.23
C	Inoculated with 0.8 ml. RDE	1½	3.66	3.11
		5	3.55	3.14
		23	3.63	3.48
D	Chilled overnight, then at 36°C. for 2 hrs., then inoculated with 0.8 ml. glucosol	1	3.74	3.04
		4	3.68	3.09
E	Chilled overnight, then at 36°C. for 2 hrs., then inoculated with 0.8 ml. of RDE	1	3.77	3.18
		4	3.53	3.11
		24	4.08	3.52
F	Whole CAM suspended in 22.5 ml. (10 *V*) of 10 per cent RDE in glucosol. At stated intervals, ⅒ volume of 30 per cent Na citrate added and entire contents homogenized.	2	3.34	2.81
		4	3.31	2.76
G	Whole CAM ground up in 22.5 ml. of 10 per cent RDE in glucosol, incubated for stated periods, then ⅒ volume of 30 per cent citrate added	2	<1.60	<1.60
		4	<1.60	<1.60

Each sample was a pool of 5 CAM's. At the end of incubation, each pool was ground up in 10 volumes of 3 per cent citrate saline in VirTis homogenizer. All suspensions heated at 65°C. for 30 minutes, then centrifuged at 10,000 R.P.M. for 10 minutes.

* Normal range (from Table V): PR8-HI = 3.81 ± 0.27; Lee-HI = 3.25 ± 0.26.

RDE likewise failed to lower the HI titer except when the membrane was maintained in an unphysiological milieu *in vitro* or after deembryonation.

The latter point is illustrated by the data in Table VII. It is shown that the HI of the intact membrane was protected from inactivation by RDE even when the embryos had previously been killed by chilling (Group E). On the other hand, it is evident that the amount of RDE inoculated was capable of inactivating in 2 hours all of the soluble HI in CAM extract (group G). Sig-

nificant decrease in HI occurred also when the entire CAM was suspended in glucosol and treated with RDE (group F). Similar changes were noted when RDE was permitted to act on the allantoic epithelium of deembryonated eggs. When, however, RDE was removed from deembryonated membranes and washed out, restitution of HI activity was almost completed within 2 hours.

The fact that all or most of the HI contained in the CAM escaped the action from without of RDE indicated either that all except a minute proportion

TABLE VIII

Effect of RDE on Adsorption of Active or Heat-Inactivated Virus in the Allantoic Cavity of Intact Eggs

Experiment No.	Virus	Control HA titer*	First inoculum‡	Mean HA titers of allantoic fluids sampled at time intervals after inoc. of virus					
				5 min.	1 hr.	2 hrs.	3 hrs.	6½ hrs.	20 hrs.
		log							
1	Active Melb	1.60	None	1.52	—	1.15	—	—	—
			RDE	1.57	—	1.51	—	—	—
2	Heated PR8	1.75	Broth	—	1.72	1.73	1.77	—	1.80
			RDE	—	1.33	<0.85	<0.85	—	<0.85
3	Heated PR8	1.75	Broth	—	—	1.70	—	1.75	—
			RDE	—	—	1.40	—	0.45	—

Mean titers calculated from individual titrations on AF's of 10 (Experiment 1), 15 (Experiment 2), or 3 (Experiment 3) eggs each. At stated intervals, 0.5 ml. of AF was removed from each egg. To each sample of fluid obtained from control eggs, 1/10 volume of RDE was added to release HA from soluble inhibitors, and then all samples, including those from RDE-treated eggs, were incubated at 37°C. for 60 to 90 minutes.

* Control titer: Estimated on the basis of dilution of inoculum by average volume of AF.

‡ Experiment 1: 1.0 ml. of RDE broth filtrate followed 1 hour later by virus.

Experiments 2 and 3: 0.5 ml. of RDE concentrated by pervaporation or of broth, followed ½ hour later by virus.

was located intracellularly or that breakdown was balanced by regeneration. In this connection, it was readily confirmed that RDE, in the concentrations used, was capable of reducing the virus-adsorbing capacity of the allantoic epithelium.

In experiments closely patterned after those of Stone (39), it was found that RDE completely inhibited viral multiplication in 72 per cent of the eggs inoculated with 20 ID_{50} of Melb strain virus. In the remainder viral proliferation was significantly delayed. As shown in the upper portion of Table VIII, adsorption of large amounts of active virus was likewise delayed in the presence of RDE. That this effect may require the synergetic action of active

virus is clear from findings recorded in Experiments 2 and 3 of Table VIII: adsorption of heat-inactivated, enzymically inert PR8 was not prevented but, in contrast, promoted in the presence of the *V. cholerae* enzyme. In absence of RDE, heated virus obviously combined with soluble HI in the allantoic fluid and could therefore not attach itself to the lining membrane. The action of RDE, on the other hand, destroyed the soluble inhibitor but failed to abolish the receptor function of the membrane surface. This situation was reversed in deembryonated eggs, as shown in Table IX. Here, after removal of soluble

TABLE IX

Effect of RDE on Adsorption of Heat-Inactivated PR8 Virus in Deembryonated Eggs

Experiment No.	Control HA titer*	Treatment‡	Mean HA titers of fluids sampled at intervals after addition of virus			
			1 hr.	2 hrs.	3 hrs.	20 hrs.
1	1.75	Broth	—	1.67	1.74	1.15
		RDE	—	1.55	1.52	1.65
2	2.10	Broth	1.72	—	—	1.46
		RDE	2.10	—	—	1.91
		RDE for 2 hrs., then replaced by broth	2.00	—	—	1.46

To each fluid sample obtained from broth-treated eggs, $\frac{1}{10}$ volume of RDE was added, and then all samples, including those from RDE-treated eggs, were incubated for 90 minutes at 37°C.

* Control titer: Aliquots of fluids used to inoculate deembryonated eggs were incubated *in vitro* for 20 hours and then titrated for HA.

‡ Experiment 1. RDE filtrate or broth were inoculated simultaneously with virus. Total volume inoculated was 5.0 ml. of serum ultrafiltrate–Tyrode's solution containing $\frac{1}{10}$ volume each of broth or RDE and virus. 6 eggs per group.

Experiment 2. Mixture of 5 ml. of Tyrode's solution plus 3 ml. of RDE concentrate (or broth) in each egg. After 2 hours, 0.8 ml. was removed and replaced by virus. 4 eggs per group.

HI from the fluid medium, measurable adsorption of heated virus occurred in absence, but not in presence of RDE. Moreover, it is shown that continued presence of RDE was required to prevent adsorption. When the enzyme was removed after 2 hours, the adsorptive capacity of the membrane was restored.

These findings, consistent with the data on HI contents of the CAM after treatment with RDE, were interpreted as evidence that, under physiological conditions (*i.e. in ovo*), enzyme acting from without was incapable of interfering with cellular homeostasis. The consideration that in intact eggs the soluble HI in allantoic fluid could effectively block the action of RDE on cell-bound substrate seemed irrelevant in view of its relatively low concentration.

It appeared that enzymatic depletion of substrate was demonstrably effective only under conditions which rendered it abnormally accessible to enzyme or slowed down its regeneration.

DISCUSSION

The work reported in this paper was done because it was felt that the effects of viral enzymatic activity in allantoic membranes infected with influenza viruses could not be measured without having on hand data on the nature of the substrate and its relation to normal cellular function. Titrations of heat-stable hemagglutination inhibitors extracted from the tissue were used as an index of its total substrate supply.

Although no attempt was made to isolate the inhibitory material from CAM tissue in pure form, its properties, to the extent that they were characterized, were consistent with the conclusion that it was closely related to similar materials derived from various body fluids or organs, which have been identified as mucoproteins. Difficulties encountered in applying certain methods successfully used in the isolation of soluble inhibitory mucoproteins from normal human urine (26) or egg white (29, 30) were undoubtedly due to the fact that the CAM extracts used were crude mixtures of tissue components. It has been observed repeatedly that the relative stability of such substances under the influence of heat or various fractionation procedures or their solubility after precipitation varied with different degrees of purity of the starting materials (29, 30, 40). Thus, heating of whole human plasma to 100°C. for 30 minutes resulted in 75 per cent loss of HI activity, while similar heating of the HI-rich IV-4 fraction prepared by Cohn *et al.* (41) brought about either no change, or a significant increase, or a loss in activity depending on dilution and pH (40). Similarly, soluble HI from the CAM suffered a 50 per cent decrease when heated in presence of salt, but no loss when the extract was prepared with isotonic sucrose solution in which heating did not produce visible precipitation of proteins.

Of more immediate biological significance was the finding that prolonged exposure of entire CAM to absolute alcohol eliminated from them all extractable HI. This was contrary to McCrea's experience with sheep salivary gland (36) and our own, reported above, with mouse brain. This may suggest differences in susceptibility to alcohol or reflect the different localization of HI in these organs. Salivary gland and mouse brain are complex parenchymatous organs, and it is possible that on dehydration with absolute alcohol, mucoprotein located intra- or intercellularly may be protected from the direct action of alcohol by coagula forming peripherally. In the case of the CAM, the mucoid layer of allantoic cells is more directly exposed to the denaturing effect of alcohol.

That ethanol in high concentration may have such an effect was shown in

experiments involving fractionation of soluble HI in CAM extracts. It was found that precipitates with ethanol in concentrations in excess of 45 per cent yielded no HI capable of *complete* inhibition of hemagglutination. As a result, the slopes of HI titration curves were less steep than those obtained with the starting material and, instead of reaching the level of complete inhibition, they tended to level off in the range of 70 per cent inhibition. On the other hand, ethanol in concentration of 30 to 45 per cent gave precipitates which yielded HI of relatively increased efficiency and homogeneity: under optimal conditions, such yields were quantitative in the range of complete inhibition, partial in the zone of intermediate inhibition.

If HI from the CAM, like those from egg white (32, 35) or urine (33, 34), are asymmetric molecules of variable length, and if it is assumed that efficiency of inhibition increases with the average length of the molecules, the above findings would fall into a logical pattern. One would expect that alcohol in low concentration would precipitate preferentially the longest filaments, leaving the smaller ones behind. This expectation is borne out by the flattened slope of titration curves obtained with non-precipitated HI left in the alcohol-containing residues. On the other hand, poor over-all yield and reduced efficiency after treatment with more concentrated alcohol could then be explained as due to either partial denaturation or reduced solubility of precipitated inhibitors.

These findings underline the fact that the slope of densitometric HI titration curves is a sensitive indicator of the relative homogeneity and efficiency of soluble HI. Qualitative changes in the inhibitory material may escape detection when arbitrary end-points are chosen to express HI activity. This point was stressed earlier by Lanni *et al.* (31) and was reaffirmed in the present study by the apparent discrepancies between 50 per cent HI titers calculated from densitometric readings and end-points of inhibition of visible pattern formation.

The detection of qualitative changes in the composition of HI solutions is of particular importance when it comes to the study of HI degraded by either active virus or RDE. Thus, Lanni *et al.* (31) found that the extent of conversion of "efficient" to "weak" inhibitors by viral enzyme acting on egg white varied with the strain of active virus acting on the substrate as well as with the indicator strain used for testing: PR8-HI titration curves were displaced but retained parallel slopes under the action of either PR8, Lee, or Swine, while the same treatment induced marked conversion of Lee- and particularly Swine-HI. Soluble PR8- and Lee-inhibitors from CAM tissue retained titration curves of unaltered slopes after progressive degradation by PR8, Lee, or RDE. Even under the conditions already discussed, in which active virus induced an increase in Lee-HI activity in the CAM or type A-HI in mouse brain, the slopes of titration curves did not change. It will be shown in the

following paper (19) that PR8-HI was equally stable qualitatively under conditions created by infection of the allantois with influenza viruses, even when the absolute amount of extractable HI was subject to considerable fluctuation. This circumstance facilitated quantitative interpretation of the results obtained.

One of the basic premises which guided this investigation was the rather obvious one that the substrate under consideration was a normal constituent of the tissue and that as such it had to fit in some manner into the general economy of the cells making up the chorioallantoic membrane. It may be postulated that inhibitors in allantoic fluid and in various body fluids represent cast-off "receptors."

Certain lines of evidence, particularly studies on the effects of RDE and of metaperiodate (42) on the receptors of the allantoic epithelium had—perhaps inadvertently—stimulated a somewhat simplified image of the tissue surface as a fixed membrane studded with receptor molecules. Such a concept would have the agglutinable red cell as a plausible model. It has been shown that the red cell can be readily freed of receptors by active viruses (1) or by RDE (20) acting from without, and that hemagglutination inhibitors can be extracted from normal but not from virus-treated red cells (43). *In vivo* regeneration of receptors on guinea pig red cells, as far as it can be determined, is an exceedingly slow process, requiring a period of 3 weeks (44). Studies on the regeneration of receptors and inhibitors in the respiratory tract of mice (17) and Stone's evidence on the restoration of adsorptive capacity of the allantois after treatment with RDE (39) have provided some evidence indicating that the analogy between red cells and secretory tissue cells is untenable when carried beyond the phase of initial attachment of viral particles. The difference was apparent to Hirst in his initial observation on cellular receptors, when he noted that virus failed to elute from cells of the respiratory tract of the living ferret (2).

Some of the work reported in this paper bears further on this contrast. It seems to be a perfectly reasonable approach to look upon the relationship of virus to cellular substrate as fortuitous, at least from the point of view of the cell. That is to say, that the cell is geared to the production and secretion of mucoid material, some of which happens to be a substrate for "myxoviruses." Thus, the reaction of the cells to enzymatic attack becomes a function of their ability to maintain homeostatic conditions under stress. Apparently, this ability remains unchallenged under the action of enzyme from without as long as the cells are exposed to a physiological milieu. Although RDE is capable of "browsing" on the cellular surface and of temporarily competing with *effective* adsorption of active virus, it does not, in the CAM of intact eggs, induce a lowering of the total extractable inhibitory substrate. Nor does it prevent the uptake by the allantoic epithelium of viral particles which have lost their own enzymatic activity as a result of heating. This finding, documented above, indicates that stable attachment of such viral particles to

surface receptors is not apt to be broken before engulfment ("viropexis"? (45)) of the receptor-virus complex has been effected. The fact that in the deembryonated egg treatment with RDE leads to a demonstrable reduction in extractable HI and also prevents adsorption of heat-inactivated virus suggests that the altered physiological state of the cells or the change in the surrounding fluid medium has made the mucoid outer layer of the cells more readily penetrable to the enzyme. It has been shown that the allantoic cells tend to release inhibitory mucoprotein after removal of the amount normally present in allantoic fluid. Under unfavorable conditions, such as apparently exist in the excised membrane or in the deembryonated egg, it is possible that the continued presence of RDE during the compensatory release of HI will induce a chain reaction of release and breakdown in which the capacity of the cell to maintain its normal share of HI is eventually overwhelmed. That this capacity is remarkably stable in the intact egg will be shown in the following paper which deals with the fate of HI in the infected allantoic membrane (19).

SUMMARY

As a preliminary to a study of the fate of mucoprotein substrate in tissues infected with influenza virus, some characteristics of soluble hemagglutination inhibitors (HI) extracted from chorioallantoic membranes (CAM) have been investigated. The inhibitory material was found to be heat-stable, precipitable with cold ethanol, and subject to progressive inactivation by active viruses or by receptor-destroying enzyme (RDE).

Certain changes in the slope of titration curves obtained with precipitated and non-precipitated fractions upon alcohol fractionation suggested that the HI in heated CAM extracts was heterogeneous. Alcohol in low concentration precipitated preferentially the more efficient (longer?) HI leaving the "weak" component in the non-precipitated fraction. With higher concentrations of alcohol, "strong" HI were converted to "weak" ones, either by denaturation or as result of reduced solubility. These changes in slope of titration curves were reflected in significant discrepancies when densitometric titers (HI_{50}) of fractions were compared with their titers in pattern tests.

The action of active viruses on HI, on the other hand, did not induce qualitative changes in the composition of the inhibitory principle: Titration curves retained parallel slopes even when their position was markedly displaced from that of control curves. In its essential properties, the HI from the CAM appeared to be similar to inhibitors isolated from various other biological sources which have been identified as mucoprotein in nature.

Standards for reproducibility of inhibitory titers obtained by the densitometric method of Hirst and Pickels have been presented.

The relation of HI to the allantoic epithelium has been analyzed. It has been concluded that the HI is a normal constituent and secretion product of

these cells. Under physiological conditions, *i.e.* in the intact egg, the HI contained in the mucoid outer layer of allantoic cells appears to be protected from enzymatic action from without, although adsorption of viral particles may be temporarily impeded. In deembryonated eggs, or in excised membranes, a reduction in total inhibitory substrate as a result of the action of RDE has been observed. It has been shown in experiments on adsorption of active or heat-inactivated virus on the allantoic membrane that prevention of adsorption by RDE may require the synergistic action of the active viral enzyme itself.

It has been concluded that the maintenance or restoration of a normal supply of mucoprotein substrate is a function of the ability of allantoic cells to maintain homeostatic conditions under stress.

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