DECREASE OF BOUND SIALIC ACID AND INHIBITOR IN
CHORIOALLANTOIC MEMBRANES INFECTED WITH
INFLUENZA VIRUS

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ABSTRACT

Tess, Bernard R. (University of Illinois at the Medical Center, Chicago) and J. Emerson Kempff. Decrease of bound sialic acid and inhibitor in chorioallantoic membranes infected with influenza virus. J. Bacteriol. 86:239-245. 1963.—During the adsorption period after the inoculation of chick embryos with the PR8 strain of influenza A virus, bound sialic acid (BSIA) in the chorioallantoic membranes decreased, presumably as a result of cleavage of surface glycoprotein by the viral neuraminidase. A further decrease occurred between 1 and 6 hr after infection, indicating that cleavage of the surface glycoprotein was continuing, and that the enzyme may have begun to act upon intracellular glycoprotein. Decreased levels of BSIA continued until 48 hr after infection, possibly as a result of continued intracellular enzyme activity plus destruction of glycoprotein as virus was released from the surface of the cells. BSIA began to increase 48 hr after infection, presumably because of regeneration of glycoprotein and its decreased destruction by the viral enzyme, since the process of infection was largely completed. Heat-stable hemagglutination inhibitor did not begin to decrease until 8 hr after infection; thereafter, the levels of BSIA and inhibitor appeared to be related.

Hirst (1942) demonstrated that the influenza virus is adsorbed to receptors on red blood cells (RBC) with resulting hemagglutination. The virus may subsequently elute because of enzymatic activity. Soluble substances analogous to these receptors have been found to combine with heat-inactivated or indicator virus and to inhibit hemagglutination. Therefore, these and related materials from other sources are also referred to as inhibitors.

The literature on this subject has been summarized by Burnet (1950) and Gottschalk (1950). Inhibitors have been found in a variety of animal tissues and fluids; some have been partially purified and shown to be glycoproteins. Gottschalk and Lind (1949) showed that the viral enzyme is a neuraminidase which attacks glycoprotein at the glycosidic linkage between sialic acid (SiA) and N-acetylgalactosamine, liberating free sialic acid (FSiA). SiA is the designation used for acetylated derivatives of neuraminic acid. SiA is also released on incubating mucoid substances of chorioallantoic membrane (CAM) with neuraminidase of viral origin (Laučkova, 1959). Mayron et al. (1961) isolated viral neuraminidase from influenza virus, and suggested that the hemagglutinin and enzyme of the virus are separate entities.

Observations of experimental infections suggest that the viral enzyme combines with and cleaves the glycoprotein of the cell surface before penetration into the cell (Stone, 1947), and possibly, in the case of Sendai virus, at the time of penetration (Zhdanov and Bukrinshaya, 1962). It has also been postulated that the viral enzyme may be important in the release of the infective virus from the cell surface (Cairns and Mason, 1953).

Scheisenger and Karr (1956), studying the fluctuations of influenza virus inhibitor in the CAM of chick embryos, found that the viral enzyme was probably active intracellularly during the period of virus synthesis; this finding confirmed an earlier suggestion of Edney and Isaacs (1950).

We therefore postulated that the results of measurements of bound sialic acid (BSIA) and

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inhibitor levels might suggest how glycoproteins or inhibitors function at various stages of infection, and help to clarify the role of neuraminidase.

**Materials and Methods**

**Stock virus.** Influenza virus PR8 strain was obtained from the Naval Medical Research Unit No. 4, Great Lakes, Ill., and reidentified by means of the Salk (1944) hemagglutination-inhibition (HI) test. The virus was propagated in the allantoic sacs of 11-day-old chick embryos from White Leghorn hens. Freshly harvested bacteria-free chorioallantoic fluids (CAF) having 10^4.9 EID_{50} and 10^{1.4} hemagglutination (HA) units per ml were stored at −54°C.

**Seed virus.** The stock virus was diluted 1,000-fold in 0.1 m phosphate-buffered saline (PBS) at pH 7.4, and 0.1 ml was inoculated into the allantoic sacs of chick embryos. After incubation at 37°C for 48 hr, the CAF were collected, and the virus was partially purified and concentrated by four cycles of adsorption and elution from guinea pig RBC, according to the method of Sheffield, Smith, and Belyavin (1954). After centrifugation at 110,800 × g for 30 min, the viral pellets were resuspended to one-fourth the initial volume in PBS. The virus had an EID_{50} of 10^9/ml for chick embryos and 10^{1.1} HA units/ml; its EID_{50}:HA ratio was 10^{4.7}:1.

**Assays of infectious virus.** Serial tenfold dilutions of CAF and extracts of CAM were made in PBS containing 200 μg of streptomycin and 200 units of penicillin/ml. Infectivity titrations were made in chick embryos by the method of Lauffer, Carnelly, and MacDonald (1948) or by the method of in vitro CAM culture as described by Fazekas de St. Groth and White (1958). Although we found the latter method only one-tenth as sensitive, it was economical, rapid, and gave a satisfactory correlation with SIa levels.

**Preparation of membranes and fluids for assay of SIa.** Groups of 12 to 16 11-day-old chick embryos were inoculated with 0.1 ml of a 10^-3 dilution of purified seed virus and incubated with uninoculated controls at 37°C for intervals from 0 to 72 hr.

In certain experiments, indicator virus was used as a control; it was prepared by heating at 50°C for 30 min at which time we postulated the viral enzyme was destroyed, since it failed to elute after adsorption to chicken RBC. After incubation, the eggs were chilled at −26°C for 5 min to depress metabolism as quickly as possible, and placed at 0 to 4°C overnight. The CAF and CAM were then collected in the same sequence as inoculated, and stored at 0 to 4°C. The CAF were centrifuged at 252 × g for 30 min, and 40-ml volumes were then lyophilized by means of a VirTis freeze-drying apparatus. After further drying over CaCl₂, the residues were weighed and stored at 0 to 4°C. Before use, the samples were reconstituted with PBS to approximately one-fourth the original volume.

The CAM were prepared by washing with PBS, mincing at 0°C in a Servall “Omni-Mixer” at 12,500 rev/min for 5 min, and then centrifuging at 1062 × g for 15 min. The supernatant fluid was considered “membrane extract” and consisted of tissue juices and whatever PBS remained from the washing process.

Infective CAF and membrane extracts were titered for viral hemagglutinin, and also for infectivity. After heating at 70°C for 30 min, or 100°C for 5 min, to inactivate the hemagglutinin, they were titered for hemagglutination inhibitor according to Hardy and Horsfall’s (1948) adaptation of the Salk (1944) HI test in which doubling dilutions are used. We also tried to obtain greater accuracy by using fractional dilutions according to Horsfall and Tamm (1953). However, the end points using either method were approximately the same.

**Assay for SIa.** The CAF and membrane extracts were assayed for FSIA and BSIA by the thiorbarbituric acid method of Warren (1959). (2-Thiobarbituric acid, obtained from Eastman Kodak Co., Rochester, N.Y., was recrystallized from hot water before use.) Unless otherwise mentioned, total SIa content was determined on samples which had been hydrolyzed with equal volumes of 16% trichloroacetic acid solution at 80°C for 1 hr (final concentration of trichloroacetic acid was, therefore, 8%). The standard used in the test was N-acetylserylaminic acid, a product of polymerized colominic acid (kindly supplied by M. E. Rafelson, Jr., Dept. of Biochemistry, University of Illinois at the Medical Center).

The standard error of the test for SIa was 1.1% based on ten determinations daily on 3 successive days. All assays on tissue extracts and fluids were made in duplicate, and differences between duplicates did not exceed 3 μg/ml. Samples were prepared so that they had approxi-
mately 1 µg of nitrogen/ml, according to the method of Lanni, Dillon, and Beard (1950). SiA content was expressed in µg/ml of original sample.

In our preliminary experiments, we experienced considerable difficulty with chromophores (possibly deoxyribose compounds) which had maximal absorbance at 532 mµ; however, we found that, with extraction of CAM samples with isoamyl alcohol followed by cyclohexane (Warren, 1959), we were able to obtain a curve with the same absorbance ratio of 549 to 532 µ as that seen with samples of pure N-acetylneuraminic acid. A major peak occurred at 549 µ with a minor one at 532 µ. Our assay procedures resulted in a loss of 15% of the SiA; that this loss was consistent was shown by adding known quantities of N-acetylneuraminic acid to normal membrane extracts and measuring SiA recovery.

Levels of neuraminidase in tissues and fluids. The presence of neuraminidase in tissues and fluids was determined by the method of Ada and Lind (1961). Purified N-acetylneuraminic acid (82 µg) was added to 0.5 ml of the sample and incubated at 37°C for 48 hr, then FSiA was determined. Results were expressed as µg of SiA yield per ml per hr.

RESULTS

Comparison of yields of SiA after digestion with trichloroacetic acid or sulfuric acid. Various investigators have found that the yield of SiA from tissues and fluids depends on the hydrolytic method used to free the BSiA. Svennerholm (1958) used 0.1 N H₂SO₄ for 60 min at 80°C; however, Dieche (personal communication) considered this treatment too drastic and recommended 10% trichloroacetic acid at 80 or 90°C for 1 hr. On comparing yields of BSiA from membranes treated with 0.05, 0.1, or 0.5 N H₂SO₄, or with 5, 10, or 15% trichloroacetic acid at temperatures from 70 to 100°C and intervals up to 120 min, it was found that 5 or 10% trichloroacetic acid at uniformity more effective than H₂SO₄, and that results with 5 or 10% trichloroacetic acid were nearly the same; maximal yields were obtained at 80°C for 60 min. In subsequent experiments, we used 8% trichloroacetic acid.

SiA and inhibitor levels of infected CAM. To determine what changes occurred in BSiA and inhibitor levels, extracts were prepared from membranes collected at intervals from the time of inoculation to 72 hr (Fig. 1). In five experiments, the BSiA dropped from an average of 41.2 to 30.4 µg/ml in 1 hr, although no decrease in inhibitor was noted. Any virus detected during the first 3 hr after inoculation was presumed to be that adsorbed from the inoculum (Henle, 1953).

After inoculation (3 hr), the virus titer of the CAM began to increase and reached a level of 10³⁻² ID₅₀ in 18 hr as measured by the method of Fazekas de St. Groth and White (1958), or 10⁷⁻² EID₅₀ (Fig. 2). The BSiA continued its downward trend to a low of 10.5 µg/ml in 48 hr, followed by a rise to 16.5 µg at 72 hr (Fig. 4). The amount
of FSIA in normal and infected membranes was negligible. Inhibitor levels of the CAM began to decrease at 8 hr, but fluctuated upward to $10^{3.1}$ units at 18 hr, decreased, and then gradually increased until the end of the experiment. These fluctuations may to some extent reflect errors due to difficulty in determining end points in the HI test. Inhibitor and BSIA in CAM from normal eggs (Fig. 1 and 3), and those inoculated with indicator virus, showed definite increases.

As seen in Fig. 4, between 6 and 24 hr there were small variations in the BSIA of the CAM, rather marked fluctuations in the inhibitor levels of the CAM, and the inhibitor level of the infective CAF was diminished (Fig. 3). As seen in Fig. 1, levels of FSIA in the infective CAF were considerably above normal and fluctuated between 6 and 24 hr; the levels then increased until the end of the experiment. The levels of FSI A in the CAF may have reflected breakdown of glycoprotein in membranes and CAF.

Our results on hemagglutinin titers yielded less information than infectivity measurements, since infectious virus appears earlier than hemagglutinating virus (Henle and Henle, 1944), and because smaller amounts of virus can be detected in the test for infectivity.

Neuraminidase content of normal embryo membranes and fluids. It was believed that some of the supposed viral enzyme activity might have been due to the neuraminidase present in normal fluids or extracts (Ada and Lind, 1961). Accordingly, CAM extracts and CAF from embryos 11 to 14 days old were tested for the liberation of SI A after incubation with N-acetyleneuraminic acid, as described in Materials and Methods. There was no FSIA in the CAM extracts until 15 days, at which time $1.0 \mu g$ of SI A per ml per hr was liberated. These findings are in agreement with those of Ada and Lind (1961) and Rafelson (personal communication). Since, during the 72 hr of our experiment, there was no measureable neuraminidase activity attributable to the host, it was concluded that enzyme activity was that of the virus, although it is possible that the infected membrane might produce more neuraminidase.

**Discussion**

Our main hypothesis was that a better understanding of the function of viral neuraminidase could be gained by observing changes in levels of BSIA and inhibitor in extracts of infected CAM. Edney and Isaacs (1950), Schlesinger and Karr (1956), and others obtained significant results based on this assumption. Kelly, Greiff, and Anderson (1962) questioned the value of studying changes in SI A and inhibitor in CAM, and we agree that the procedure would be of dubious value if only small changes were observed; however, when the observed changes are reproducible and greater than the experimental error of the tests, we believe they are significant.

Our seed virus was partially purified to remove as much inhibitor as possible. This procedure reduced the EM/PHA ratio from $10^{4.9}$ to $10^{1.7}.1$. On the basis of Henle's (1933) data, we calculated that our inoculum contained 1 HA unit...
per 10 entodermal cells of the CAM and one \\( \text{ID}_50 \) per 1,000 cells.

One of the difficulties in establishing a relationship between BSiA and inhibitor is that measurements of these substances can be regarded as only approximate because of difficulties in the methods of assay. HI end points are difficult to determine, and it is quite possible that inhibitor may be present in varying degrees of aggregation or bound to membrane fragments which may mask it.

Other points are that there may be more than one inhibitor present, and that some glycoproteins which yield SIA after exposure to neuraminidase are not inhibitory (Gottschalk, 1960). Finally, there are inhibitors which are not acted upon by neuraminidase (Belyavin and Cohen, 1962).

We believe that only tentative conclusions concerning the relationship of SIA to inhibitors can be reached until purified glycoproteins and other inhibitors are isolated from CAM and assayed accurately for both inhibitory activity and BSiA, before and after exposure to partially purified viral neuraminidase. Nevertheless, it would seem that the relationship shown between the two in this study was significant because of what is known of the action of viral neuraminidase on heat-stable glycoprotein inhibitor with the resulting liberation of SIA. Similar conclusions were reached by Kelly et al. (1962).

The decrease in BSiA in infected membranes during the adsorption period was unexpected in view of the reports of Edney and Isaacs (1950), Schlesinger and Karr (1956), and our confirmation of their finding that inhibitor did not decrease during this period. However, the decrease of BSiA in the CAM and the increase of FSiA in the CAF strongly suggest that some glycoprotein was split by neuraminidase before the levels of inhibitor showed a decrease in the HI test, or, less likely, that the glycoprotein had no inhibitory activity. It is also possible that some SIA may be liberated on penetration of the cell membrane. Cleavage of glycoprotein presumably occurs after the initial reversible adsorption; virus is then eluted from nonsusceptible cells and penetrates susceptible cells (Zhdanov and Bukrinskyaya, 1962).

There were several possibilities to consider in the interpretation of the decrease of inhibitor and BSiA in the CAM during the period of virus synthesis: namely, that viral enzyme of the inoculum, although continuing to act on surface receptors, was starting to act on intracellular receptors (Edney and Isaacs, 1950); that metabolism of glycoprotein or inhibitor serves a purpose in virus synthesis; that the virus was released only after enzymatic action; or that some combination of these occurred. Schlesinger and Karr (1956), using de-embryonated eggs, found that the periodicity of inhibitor breakdown and regeneration was in close agreement with the duration of primary and secondary cycles of viral multiplication, and not with viral release. Although our data did not reveal a definite sequential relationship between inhibitor levels of the CAM and periodicity of viral multiplication, it was nevertheless clear that the fluctuation of CAM inhibitor was reflected in the variations of FSiA of CAF during a period of intense viral synthesis. It seems most likely that during this period the enzyme was acting on glycoprotein on the cell surface and perhaps also on intracellular receptors. However, we have no evidence to exclude the possibility that glycoprotein or inhibitor metabolism may be involved in the process of virus synthesis.

The observation that inhibitor and BSiA levels of the membranes began to increase 24 and 48 hr, respectively, after infection may be explained by the facts that the processes of virus synthesis and release were probably diminished, and that inhibitor or glycoprotein was being regenerated more rapidly than it was being inactivated.

It may be postulated that the increases and fluctuations of FSiA in CAF were due to enzymatic disruption of the glycoprotein of CAM and its liberation into CAF. However, part may have been due to viral action on the inhibitor already present in the CAF or other parts of the embryo. Some of the periodic decreases of FSiA may have been due to the action of an aldolase (Comb and Roseman, 1958). The rather marked increases in FSiA we observed between 24 and 72 hr were also reported by Kelly et al. (1962). These workers did not measure SIA at intervals of less than 12 hr and none earlier than 12 hr after inoculation, so no further comparisons can be made. We are now using de-embryonated eggs to focus attention on changes in CAM.

Our findings are in marked contrast to those of Noll, Aoyagi, and Orlando (1961), who found little change in SIA levels in the CAF and CAM.
during the first 9.5 hr of infection with the Lee strain of the influenza B virus. However, the Lee strain may be low in enzymatic activity. If measurements had been made for longer periods, or had their samples been more concentrated, changes in Sia might have been found.

Detection of glycoprotein by staining procedures (Kelly et al., 1962) and measurements of Sia and inhibitor levels may suggest a relationship between these and various phases of the infectious cycle. However, this information does not tell us the function of neuraminidase, glycoprotein, and inhibitors, particularly during the period of virus synthesis. Experiments now in progress may resolve some of these problems.

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LITERATURE CITED


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