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SIALIDASE ACTIVITY OF CHICK AND MOUSE EMBRYO TISSUE CULTURE CELLS INFECTED WITH MYXOVIRUSES AND THE EFFECT OF ACTINOMYCIN D ON THIS ACTIVITY

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Summary. — In chick embryo tissue culture infected with influenza A/WSN virus, two rises in sialidase activity were observed: the first rise took place 2 hours after infection, while the second one began after 4 hours and reached a maximum by the 7th hour. Actinomycin D in a concentration of 0.5 μg/ml added simultaneously with virus completely inhibited the early rise in sialidase activity and reduced by 65% the enzymatic activity at the 7th hour of infection. Introduction of the antibiotic at 4 hours reduced the "late" activity by 30%. The second rise in sialidase activity somewhat preceded the accumulation of haemagglutinins in this system. The formation of sialidase activity in chick embryo cells infected with Newcastle disease virus (NDV) was characterised by only "late" rise which was unaffected by actinomycin D. In mouse embryo tissue culture cells infected with A/WSN virus, which support influenza virus reproduction without the concomitant production of haemagglutinins, no sialidase activity was observed. Homogenates of chick embryos possessed sialidase activity while in their cultured cells as well as in mouse embryos the activity was not detectable.

Introduction

Sialidase, the only enzyme present in myxoviruses, has been the subject of numerous investigations concerning its function in the process of viral reproduction. Some suggestions were offered on the role of the enzyme in the early phases of virus-cell interaction: adsorption, elution and penetration (Hirst, 1948; Rubin, 1957; Zhdanov, 1961; Tess and Kempf, 1962). On the other hand, Hoyle (1954) suggested, and Seto and Rott (1966) experimentally proved, that sialidase is essential for the process of virion formation within cellular membranes and its release from the cell. The occurrence of sialidase in normal animal tissues and changes of its activity under some conditions made it possible to assume that the enzyme is of cellular origin and is incorporated into virus particles as a host cell component. However, the investigation by Seto and Rott (1966) showed that the virus sialidase possessed type and strain specificity.

Our previous investigation (Orlova and Eremkina, 1968) showed that influenza A/WSN virus, which produces in chick embryo cell cultures haemagglutinin formation, is reproducing in mouse embryo cell cultures without haemagglutinin formation. It was of interest, therefore, to study sialidase synthesis in these two cell systems. For comparison, chick embryo cell cultures infected with NDV were also studied, sialidase activity of which has recently been investigated by Lipkind and Tsvetkova (1967).

Materials and Methods

Influenza A/WSN and NDV/H were used. The methods of maintenance of the viruses, their titration and the preparation of tissue cultures have been described (Orlova *et al.*, 1967). For investigation of the sialidase activity, 3-day cultures of chick embryo cells and 5-day cultures of mouse embryo cells, both grown in 500-ml flasks, were used. Cell layers were infected with influenza A/WSN virus at a multiplicity of 10 to 15 plaque forming units (PFU) per cell, or with NDV/H at a multiplicity of 100 PFU/cell. The time of addition of viruses is reffered to as zero time. After 1 hour at 37° C the virus was removed and the cells washed with warm Hanks' solution; the cultures were supplied with warm medium 199 supplemented with 2% of bovine serum and incubated at 37° C. At 1-hour intervals, one flask each was removed, the supernatant decanted and the cells washed with cool 0.1 M phosphate buffer (pH 6,2). The cells collected by scraping in 6 ml of the same buffer were vigorously pipetted to and fro. The cell suspensions were then disrupted by freezing and thawing in the dry ice-acetone-water system (Ada, 1963). The homogenates prepared in this way were stored at −10° C. Each sample was assayed for infectivity, haemagglutinin content, sialidase activity and total nitrogen.

Sialidase activity was assayed using 0.6 ml of the homogenate in 0.1 M phosphate buffer, pH 6.2, and 0.6 ml of ovomucin preparation as the substrate. Parallel with test mixtures, control tubes, containing homogenates of uninfected and infected cells without the substrate, were incubated. The incubation was carried out in a water bath for 1 hour at 37° C.

The ovomucin used as the substrate was prepared according to Gottschalk and Lind (1949) with slight modifications as follows. White from 20 eggs was thoroughly freed from halases, cooled to 0°−2° C and poured at constant mixing into 1.6 l of cooled distilled water. The sediment formed was collected by centrifugation (6000 to 8000 rev/min) dissolved in a minimal volume of 10% NaCl solution and added to 20 volumes of cooled distilled water. The sediment obtained was dissolved in a minimal volume of 5% NaCl solution and dialyzed overnight against 1.8% NaCl solution and for another day against 1% NaCl solution. The yield was 100 to 150 ml of ovomucin preparation, which was pipetted to produce a homogeneous solution. One ml of the solution contained from 1.0 to 2.0 micromoles of neuraminic acid (NANA) if measured after 1 hour's hydrolysis with 0.1 N H_2SO_4. The substrate prepared in this way was stored at 4° C and could be used for 1 to 2 months.

The sialidase activity was measured by increase of NANA content in the incubation mixture as compared with the control containing the substrate alone. The amount of NANA was determined using the thiobarbituric acid method of Warren (1959). The intensity of colour of the chromogen was measured in an SF-4A spectrophotometer at 549 nm. The specificity of the chromogen was checked by measurements at 532 nm which were considerably lower then those at 549 nm. The enzymatic activity was expressed in micrograms of NANA per 1 mg of total nitrogen of the homogenate. The total nitrogen determinations were made by the Conway (1957) microdiffusion technique.

Results

Fig. 1 shows the results of determination of sialidase activity in homogenates of chick and mouse embryo cell cultures infected with A/WSN influenza virus as well as the curve of accumulation of haemagglutinins in the chick cells. It is seen that, after adsorption of the virus (1 hour of infection), the sialidase activity of infected chick homogenates was equal to 50 μg

NANA/mg N. At 2 hours of infection the amount of liberated NANA increased to 100 and in some experiments even to 160 μg/mg N, which indicated an increase in sialidase activity of the homogenates. Then a decrease was observed, followed by a progressive rise of sialidase activity beginning from the 4th hour. By 7 hours the amount of free NANA in the incubation mixtures reached 250 μg/mg N. Control mixtures, containing homogenates of normal and virus-infected cells with no added substrate, did not show any increase in the amount of free NANA. Beginning from the 4th hour haemagglutinin appeared in the virus-infected chick embryo cells and its maximum level was reached by the 7th or 8th hour of infection.

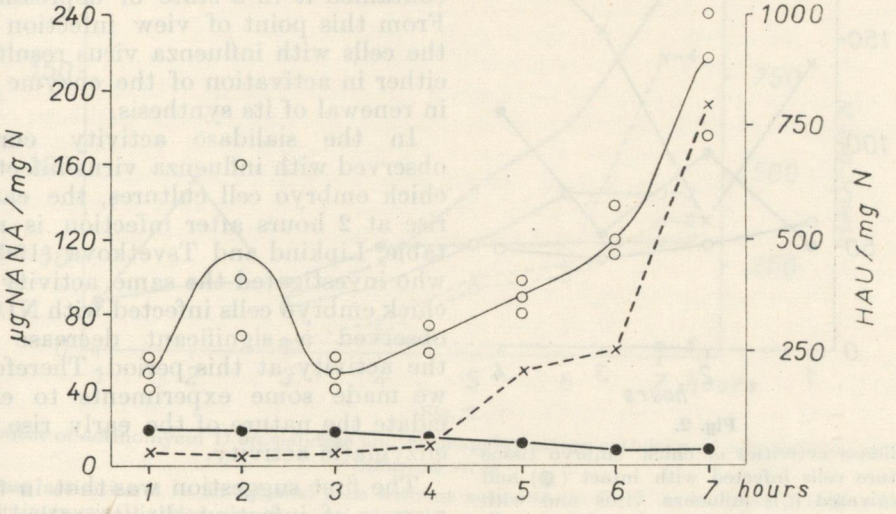

Fig. 1.

Sialidase and haemagglutinating activities of chick and mouse embryo tissue culture cells infected with influenza A/WSN virus

○ and ●: Sialidase activities in chick embryo (○) and mouse embryo (●) tissue culture cells
×: Haemagglutinating activity in chick embryo tissue culture cells (in units per mg N)

In homogenates of infected mouse embryo cell cultures the sialidase activity was about 10 μg/mg N and did not change over the entire period of observation. Nor was there any increase in haemagglutinin content. The uninfected controls of both chick and mouse cell cultures possessed no detectable sialidase activity.

Based on these results we decided to check the sialidase activity of tissues from which the cells used for cultivation were derived. For this purpose homogenates of chick and mouse embryo bodies were prepared. Besides, homogenates of lungs and brains from adult mice, i.e. of tissues capable to support multiplication of influenza A/WSN virus, were included in the experiment. The chick embryo homogenate possessed active sialidase (60 μg NANA/mg N). The mouse embryo homogenate contained no active sialidase.

while the homogenates of adult mouse lungs and brains were comparatively active (83 and 25 μg NANA/mg N, respectively).

Thus we found that mouse embryos possessed no sialidase and that cells obtained from them did not synthesize the enzyme either in the process of in vitro cultivation or after infection with influenza virus. As distinct from them, chick embryos synthesized sialidase in the course of normal development, while culture cells derived from them possessed no active sialidase. It may be assumed that chick embryo cells either ceased to produce the enzyme during in vitro cultivation or contained it in a state of depression. From this point of view infection of the cells with influenza virus resulted either in activation of the enzyme or in renewal of its synthesis.

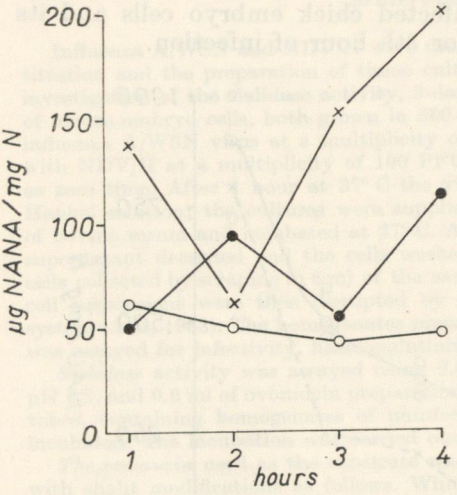

Fig. 2.

Sialidase activities of chick embryo tissue culture cells infected with intact (●) and inactivated (○) influenza virus and with NDV (×)

In the sialidase activity curve observed with influenza virus-infected chick embryo cell cultures, the early rise at 2 hours after infection is notable. Lipkind and Tsvetkova (1967), who investigated the same activity in chick embryo cells infected with NDV, observed a significant decrease of the activity at this period. Therefore we made some experiments to elucidate the nature of the early rise in enzymatic activity.

The first suggestion was that in the process of infection disintegration of influenza virus into subunits took place with resulting increase in apparent sialidase activity. To test this suggestion, influenza A/WSN virus, which had been irradiated with 800 000 r of gamma-rays in a Co^{60} installation, was used. Such irradiation resulted in a drop of infectivity titre from 10^7 to 10^2 PFU/ml, while haemagglutinating and sialidase activities remained unchanged. Chick embryo cell cultures were infected with such virus. Besides, cells infected with intact NDV were included in the experiment for comparison. The results are shown in Fig. 2. The enzymatic activity of chick embryo cell cultures infected with intact influenza virus increased at 2 hours, while after addition of the irradiated virus there was no rise in activity of the enzyme. Cultures infected with NDV revealed a decrease in the enzyme activity at 2 hours, followed by its rise. Thus, the early increase in sialidase activity in infected chick embryo culture cells was characteristic of influenza A/WSN virus only and was not connected with disintegration of the virus particles into subunits. The increase was induced by intact RNA which had penetrated the cell.

It is known that virus reproduction is sensitive to actinomycin D. This

fact is connected by some authors with participation of the cellular genome in the process of replication of viral RNA. To elucidate further the nature of the early rise in sialidase activity of infected cells, we carried out experiments in which actinomycin D was added in concentrations inhibiting development of influenza virus (0.5 μg/ml). Since the action of the antibiotic is greatly dependent on time of addition (the actinomycin-sensitive phase of viral development lasts for 3 hours), we used it immediately after adsorption and 4 hours after infection. The results of the experiments are shown in Fig. 3.

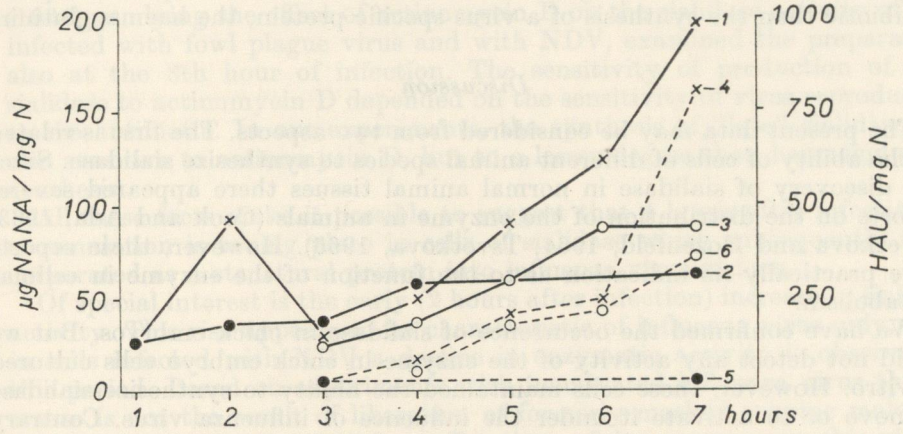

Fig. 3.

The effect of actinomycin D on sialidase and haemagglutinating activities of cells infected with influenza virus

Sialidase activities: 1 — in infected cells without actinomycin D; 2 — in infected cells with actinomycin D added simultaneously with virus; 3 — in infected cells with actinomycin D added at 4 hours after infection.

Haemagglutinating activities: 4 — in infected cells without actinomycin D; 5 — in infected cells with actinomycin D added simultaneously with virus; 6 — in infected cells with actinomycin D added at 4 hours after infection.

Mean values from 5 experiments.

The sialidase activity of control cells subjected to influenza virus alone showed the usual two rises. The first one appeared at 2 hours and the second began at 4 hours of infection. Maximal accumulation of the enzyme (192 μg NANA/mg N) was observed at 7 hours. Slight increase in haemagglutinins in infected cells could be noted as early as 4 hours after infection. By 7 hours the accumulation of haemagglutinins was rather high (815 HAU/mg N).

Introduction of actinomycin D immediately after adsorption almost completely removed the early rise in sialidase activity. However, subsequently, beginning from the 4th hour of infection, the enzymatic activity increased and by the end of the observation period its level reached 35% of that in untreated infected control cells. It should be noted that the antibiotic concentration used was sufficient to completely inhibit the production of hae-

magglutinins. The addition of actinomycin D at 4 hours of infection also produced a decrease in enzymatic activity, which by 7 hours reached 45% of the level found in untreated infected controls. A decline in haemagglutinin formation was also noted in this case, the haemagglutinin titre reaching 25% of the controls.

Sialidase synthesis in cells infected with NDV appeared to be completely resistant to actinomycin D in the concentrations used.

Thus we found that the early rise of sialidase activity in infected chick embryo cell cultures is sensitive to actinomycin D and that the formation of the "late" sialidase during influenza virus infection is less affected by the antibiotic than the synthesis of a virus-specific protein, the haemagglutinin.

Discussion

The present data may be considered from two aspects. The first is related to the ability of cells of different animal species to synthesize sialidase. Since the discovery of sialidase in normal animal tissues there appeared several reports on the distribution of the enzyme in animals (Cook and Ada, 1963; Tsvetkova and Rozenfeld, 1964; Tsvetkova, 1965). However, these reports gave practically no indication as to the function of the enzyme in cellular metabolism.

We have confirmed the occurrence of sialidase in chick embryos. But we could not detect any activity of the enzyme in chick embryo cells cultured in vitro. However, these cells maintained the ability to synthesize sialidase de novo or to activate it under the influence of influenza virus. Contrary to this, mouse embryos possessed no active sialidase. Nor did mouse embryo cells cultured in vitro contain any active enzyme. Moreover, infection of the cells with influenza virus did not result in sialidase formation in them. It should be emphasized that the appearance of active enzyme in chick cells after infection with influenza virus was accompanied by subsequent production of haemagglutinin, while mouse cells were lacking this latter process as well. An impression might appear that mouse cells do not at all interact with influenza virus. However, such an impression would be false, since we showed earlier (Orlova and Eremkina, 1968) that influenza A/WSN virus was replicated in mouse embryo tissue culture cells with an intensity equal to that for chick embryo cells, that the virus could be successfully passaged in mouse cells and that in all these cases virus reproduction was not accompanied by haemagglutinin formation.

Thus, the data obtained permit it to suggest a possible correlation between the ability of cells to produce sialidase in normal conditions and to bring about its appearance during influenza virus infection.

The apparent relation between abilities of cells to produce sialidase and haemagglutinin is also of interest. Although synthesis of both proteins is induced by the virus, the role of the host cell in their production is great.

Another aspect of the present study is concerned with the dynamics of appearance of sialidase in chick embryo cells under the effect of influenza virus. It was found that the sialidase activity exhibited two rises. The first

rise had a maximum at 2 hours after infection and the second started at the 4th hour and went on till the 7th hour. This "late" increase in enzymatic activity slightly preceded in time the accumulation of haemagglutinin. It may be assumed that this "late" sialidase is incorporated into virions. The latter suggestion is based primarily on the fact that it is exactly during this period that the synthesis of viral structural proteins is taking place. Also, the identity of sialidase appearing at the late phase of infection with the enzyme of the mature virus was proved by Seto and Rott (1966). In their experiments the antiserum against sialidase from purified mature virus inhibited the process of formation of influenza virus. Rott and Scholtissek (1964), studying the effect of actinomycin D on the sialidase activity of cells infected with fowl plague virus and with NDV, examined the preparations also at the 8th hour of infection. The sensitivity of production of such sialidase to actinomycin D depended on the sensitivity of virus reproduction to the antibiotic. In our experiments, the synthesis of "late" sialidase was also sensitive to actinomycin D, but to a lesser degree than haemagglutinin synthesis.

All these facts make it possible to suggest that 4 hours after infection an accumulation is taking place in cells of sialidase which subsequently is incorporated as a structural protein into influenza virus virions.

Of special interest is the early (2 hours after infection) increase in sialidase activity. This rise appears to be characteristic of influenza virus only, since in cells infected with NDV a decline in enzymatic activity is observed at this period. Based on experiments with inactivated influenza virus this increase is not the result of liberation of formerly masked enzyme molecules during disintegration of virions. By time of its appearance this rise in sialidase activity coincides or is only little later than the reported early increase in RNA synthesis (Orlova *et al.*, 1968), while its sensitivity to actinomycin D indicates that DNA-dependent RNA synthesis is involved.

The information available at present does not permit a definite answer as to the origin of this "early" enzyme and to its function. There is ground to believe however, that the "early" sialidase is probably different from the "late" enzyme and may have different properties and function.

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