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Metabolic Requirements for the Development of Hemadsorption Activity and Virus Formation in BHK-21 Cells Infected with Newcastle Disease Virus

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Differential effect of various metabolic inhibitors on the development of hemadsorption activity and virus formation in cells infected with Newcastle disease virus (NDV) was investigated. It was found that, in BHK-21 cells infected with NDV, cycloheximide did not prevent the development of hemadsorption activity, whereas protein synthesis and virus formation by the cell were rapidly inhibited by the drug. When the drug was added to the culture at 4.5 h after infection or later, hemadsorption activity of the cell continued to develop normally for about 1 h. Similar increase in hemadsorption activity was found in cells which were treated with anti-NDV serum (to neutralize their hemadsorption activity) and then washed and incubated with cycloheximide. However, when cells were treated with the drug early in the infection (1.5 or 3.0 h), they did not show any detectable hemadsorption reaction throughout the infection. In contrast to cycloheximide, iodoacetate added to the culture together with sodium azide inhibited completely both the development of hemadsorption activity and the formation of progeny virus. These results suggest that the change of cell surface to become hemadsorptive may depend upon the energy generating system but not upon de novo synthesis of protein, whereas production of infectious virus may require continuous synthesis of protein.

Erythrocytes are adsorbed to the surface of myxo- or paramyxovirus-infected cells (1, 4, 15). It is likely that the hemadsorptive areas of plasma membrane contain viral envelope proteins. They may be precursors to the modified membrane which becomes the envelope of the virion (2). Thus, the change of the cell surface to become hemadsorptive would be one of the characteristic steps of maturation of myxo- and paramyxoviruses. However, many details of this process are yet obscure.

During studies on the replication of Newcastle disease virus (NDV) in various cells, it was found that BHK-21 cells exhibited more extensive hemadsorption reaction than other cell cultures examined. In the present communication, the effect of various metabolic inhibitors on the modification of the cell membrane and on the subsequent virion formation in BHK-21 cells will be described, and evidences will be presented which suggest that the modification of the cell surface to adsorb red blood cells may depend upon the function of the energy generating system but not upon active protein synthesis, although continuous virus production may require continuous synthesis of protein.

MATERIALS AND METHODS

Cell culture and media. A continuous line of BHK-21 cells was used in this study. Cells were grown in Eagle minimum essential medium supplemented with 5% calf serum and 10% tryptose phosphate broth. The same medium, in which the concentration of serum was reduced to 1%, was satisfactory for maintenance of the cells and was used for virus growth.

Virus and virus assay. Miyadera strain of NDV was used throughout this study. The method for infectivity assay of the virus using chicken embryo cell monolayers was described previously (10).

Hemagglutinin titration. Serial twofold dilutions of virus in a 0.25-ml volume of phosphate-buffered saline (PBS) were made in plastic trays. Chicken or guinea pig erythrocytes were added as a 0.025-ml drop of a 5% suspension. Titers were read by the pattern method after 35 min at 4 C.

Neuraminidase assay. Neuraminidase assay was performed with a fetuin substrate by a modification of the thiobarbituric acid method of

Warren (8, 9). Enzyme activity was measured by extinction readings taken from the part of the slope where they vary linearly with the enzyme concentration. Extracts of uninfected BHK-21 cells did not contain measurable quantities of free *N*-acetyl-neuraminic acid or other color-forming substances.

Quantitative hemadsorption test. Tests for the capacity of infected cells to adsorb red blood cells to their surface were performed as follows. The culture medium was removed, and the monolayer (10^6 cells) was washed with PBS. Two milliliters of a 2% suspension of guinea pig erythrocytes in PBS was added, and the cells were left at 4 C for 30 min. After unadsorbed erythrocytes were removed by washing with PBS, the monolayer adsorbing red blood cells was dissolved in 1 ml of 0.5 N NH_4OH. Hemadsorption activity of the cells was expressed by the optical density read at 540 nm against a blank tube prepared from non-infected cells treated similarly.

Infection and sampling of NDV. Monolayers of BHK-21 cells were infected with NDV at an input multiplicity of 15 plaque-forming units (PFU) per cell. After incubation at 37 C for 40 min to allow for virus penetration, the cultures were treated with anti-NDV serum diluted to 1:100 with maintenance medium for 20 min, and then washed 5 times with Hanks solution. At various times during further incubation in fresh medium at 36 C, the culture medium and cells were harvested separately and assayed for viral activities. To examine cell-associated viral activities, monolayer cultures were washed with PBS three times, and the cells were harvested by scraping off and suspending the cells in 2 ml of PBS. The cell suspension thus obtained (10^6 cells/ml) was treated with an ultrasonic oscillator (Tominaga, type UR, 168 W, 20 kc/s) for 20 s before titrations for viral infectivity, hemagglutinating, and neuraminidase activities.

Antiserum. Antiserum against NDV was obtained by immunizing rabbits with NDV grown in the chicken embryos and purified by differential centrifugation followed by centrifugation through a 10 to 40% sucrose gradient. This serum showed a hemagglutination inhibition (HI) titer of 1:1280.

Chemicals. Cycloheximide was purchased from Sigma Chemical Co. Monoiodoacetate, *p*-chloromercuribenzoate (PCMB), sodium azide, and potassium cyanide were purchased from Katayama Chemical Co. ^{3}H-leucine (specific activity, 270 mCi/mmol) was obtained from Daiichi Kagaku Co., Ltd.

RESULTS

Developmental sequence of hemadsorption, hemagglutinating, and neuraminidase activities of BHK-21 cells infected with NDV. In a preliminary experiment, it was found that the hemadsorption reaction by NDV-infected BHK-21 cells was more extensive than the reaction by the other cell cultures examined (unpublished data). Therefore, the developmental sequence of hemadsorption, hemagglutinating, and neuraminidase activities in BHK-21 cells was examined during a single growth cycle of NDV in them. Monolayer cultures of BHK-21 cells were infected with NDV and allowed to stand for 40 min at 37 C and treated with anti-NDV serum diluted to 1:100 for 20 min to neutralize unadsorbed virus. The cultures were then washed five times with Hanks solution and incubated with maintenance medium at 36 C. At various times, hemadsorption activity and cell-associated hemagglutinating and neuraminidase activities were examined, and the results are shown in Fig. 1. As shown in the figure, hemadsorption activity of the cell was first detected between 4.5 and 5.0 h after infection and increased linearly until the 8th-h. The cell-associated hemagglutinating and neuraminidase activities became detectable coincidentally somewhat later (5.0 or 5.5 h) than the appearance of hemadsorption activity. These activities increased in parallel thereafter.

Modification of cell surface to adsorb red blood cells in the presence of cycloheximide. Various concentrations of cycloheximide were

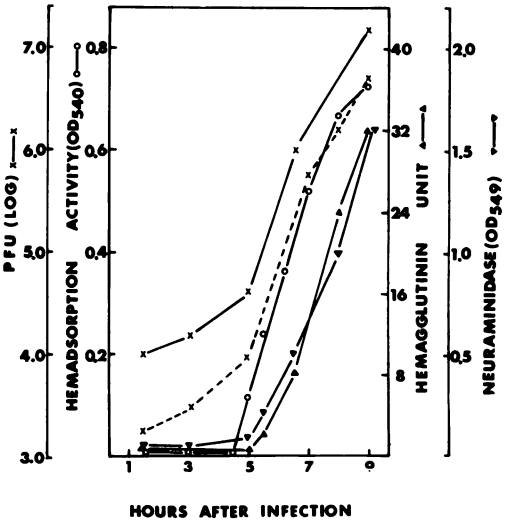

FIG. 1. *Development of cell-associated viral activities during single cycle growth of NDV in BHK-21 cells. Monolayer cultures of BHK-21 cells were infected with NDV and incubated. One series of duplicate set of cultures served as assays of cell-associated hemagglutinating* (△), *neuraminidase* (▽) *activities, and cell-associated infectivity* (×). *Infectivity of each culture fluid was shown in dashed line* (×). *The other duplicate set of cultures was used for assay of the capacity of the cells to adsorb red cells, i.e., hemadsorption activity* (○).

added to infected cultures at 6.5 h after infection, when the cells showed a moderate hemadsorption. The subsequent increase in hemadsorption activity was measured during incubation for 90 min. As shown in Table 1, cycloheximide could not inhibit the increase in hemadsorption activity even at a concentration of 100 μg/ml, whereas protein synthesis of the cells was rapidly and strongly inhibited. The maximum level of the inhibition of protein synthesis was about 93% (Table 2). Then, the effect of cycloheximide (100 μg/ml) added at various times during a single growth cycle of NDV was examined. As shown in Fig. 2, when the drug was added at each 4.5, 5.5, and 6.5 h after infection, hemadsorption activity continued to increase at a normal rate for approximately 45, 60, and 90 min, respectively. However, the culture treated earlier (1.5 or 3.0 h) did not exhibit any hemadsorption reaction throughout the infection.

TABLE 1. *Effect of cycloheximide on hemadsorption activity of NDV-infected BHK-21 cells*[a]

Concentration of cycloheximide (μg/ml)	Time of incubation (min)	Hemadsorption activity[b]
0	90	0.670
30	90	0.700
60	90	0.710
100	90	0.680
Control[c]	0	0.385

[a] Various concentrations of cycloheximide were added to NDV-infected BHK-21 cells at 6.5 h after infection. After 90 min of further incubation at 36 C, hemadsorption activity of the cells was measured as described in Materials and Methods.

[b] Absorbance at 540 nm.

[c] Hemadsorption activity at 6.5 h after infection.

TABLE 2. *Inhibition of protein synthesis in BHK-21 cells infected with NDV*[a]

Cycloheximide treatment	Trichloroacetic acid-precipitable radioactivity (counts/min) at labeling periods		
	10 min	30 min	60 min
−	14,250[b]	32,682	53,066
+	860 (94.1%)[c]	2,125 (93.2%)	3,658 (93.2%)

[a] BHK-21 cells were infected with NDV, and, after incubation for 6.5 h at 36 C, 4 μCi of ^{3}H-leucine per ml was added to them simultaneously with (+) or without (−) 100 μg/ml of cycloheximide. After further incubation for designated periods, the cells were suspended in PBS, washed with 5% trichloroacetic acid, and finally dissolved in 1 N NH_4OH.

[b] A sample (0.1 ml) was determined in an Aloka scintillation spectrophotometer.

[c] Number in parentheses indicate the percentage of inhibition.

These results suggest that the modification of the cell surface to adsorb red blood cells could develop in the absence of active protein synthesis and its intensity or duration might depend upon the amount of viral precursors which had been synthesized and accumulated within the cell by the time the drug was added. The synthesis of viral precursor proteins which would be responsible for the alteration of cell surface seemed to start between 1.5 and 3.0 h after infection.

Effect of treatment with anti-viral serum of infected cells on the development of hemadsorption reaction in the presence or absence of cycloheximide. Since antibody

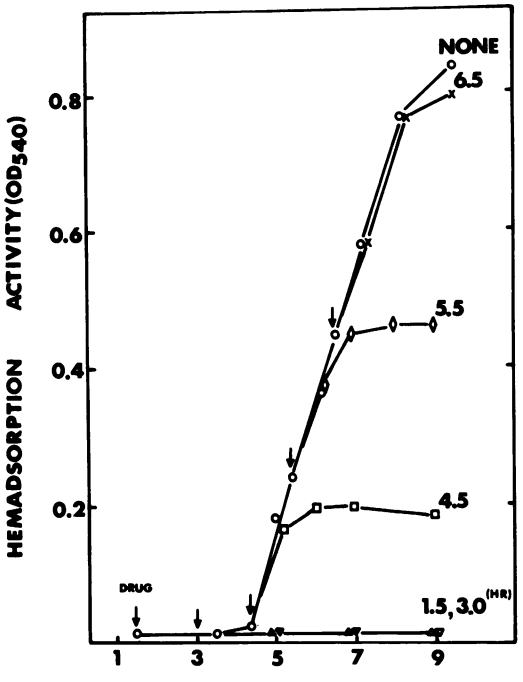

FIG. 2. *Effect of cycloheximide added at various times after infection on hemadsorption activity. Cycloheximide was added to the culture at 1.5 (△), 3.0 (▽), 4.5 (□), 5.5 (◇), and 6.5 (×) h after infection to give a final concentration of 100 μg/ml, and subsequent hemadsorption activity was measured. Hemadsorption activity in control cultures (○) is also shown.*

does not presumably enter living cells, anti-NDV serum added to infected culture during virus growth may react with viral antigens only at the cell surface. Monolayer cultures of BHK-21 cells were infected with NDV, and at 6.5 h after infection, the culture medium was replaced with a medium containing 1% of anti-NDV serum (HI titer, 1:1280). After incubation for 20 min at 36 C, the cells were washed 5 times with ice-cold Hanks solution, and incubated further in a fresh medium with or without 100 μg of cycloheximide per ml. As shown in Fig. 3, the hemadsorption activity, once neutralized by the antiserum, increased again even in the presence of cycloheximide as rapidly as in control cells, and reached a maximum within 90 min. Cell-associated hemagglutinating and neuraminidase activities were also measured under similar conditions. These activities did not increase in parallel with the increase of hemadsorption activity, although a slight but definite increase in hemagglutinating activity was observed.

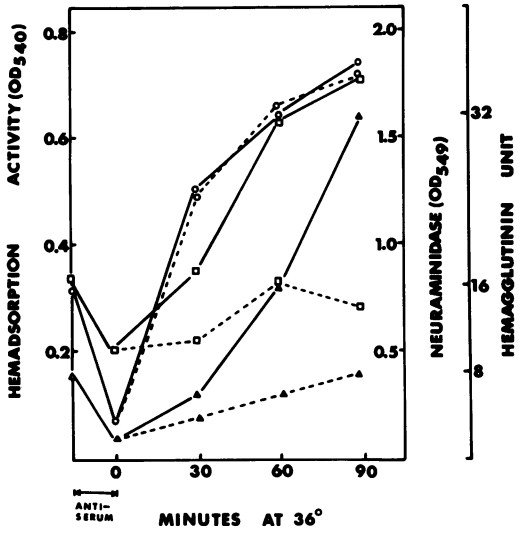

FIG. 3. *Anti-NDV serum treatment and subsequent development of cell-associated viral activities in the presence or absence of 100 μg of cycloheximide per ml. BHK-21 cells infected with NDV were treated with anti-NDV serum diluted to 1:100 at 6.5 h after infection for 20 min. The cultures were then washed five times with Hanks solution and incubated further in the presence* (-------) *or absence* (——) *of 100 μg of cycloheximide per ml. At various times during incubation, hemadsorption activity* (○), *cell-associated hemagglutinating activity* (△), *and neuraminidase activity* (□) *were measured as described in Materials and Methods. Assay of hemagglutinating activity was performed by using both chicken and guinea pig erythrocytes, but none of significant difference in titer was observed. In this figure, hemagglutinating activity assayed by using chicken erythrocytes was shown.*

These results suggested that viral proteins, which had been synthesized but never exposed to antiserum because of their presence within the cell, might be incorporated into the cell membrane even in the absence of active protein synthesis, thus modifying the membrane to become hemadsorptive.

The experimental result which indicated that, in the presence of cycloheximide, the cell-associated hemagglutinin activity did not increase in parallel with the increase in hemadsorption activity was rather difficult to explain. If we hypothesize that the hemadsorption reaction and the cell-associated hemagglutinin activities are the different ways of manifesting the same phenomenon, i.e., incorporation of hemagglutinin protein into the cell membrane, then the cell-associated hemagglutinin should increase with the increase in hemadsorption activity. In this case, such hemagglutinins would be the fragments of modified cell membrane produced by the disruption of infected cells by sonic treatment. However, there may be several plausible explanations for the difference between the increase of hemagglutinating activity and that of the hemadsorption activity. For example, there is a possibility that this may be a result of the difference in sensitivity of the method used. As shown in Fig. 1, hemadsorption appears to be a more sensitive index than cell-associated hemagglutinin. When few and very limited areas of cell membrane are modified to react with red blood cells, and if these areas are arranged at a suitable distance, such a minor change of the cell membrane would be sufficient to adsorb a single red blood cell. When such cells were sonically treated, however, the amount of resulting hemagglutinin would still be insufficient to increase the hemagglutinin titer. There may be several alternative explanations, and further study will be required to clarify this problem.

Requirement of energy for virus-induced modification of cell surface. Some biological processes involving a cellular membrane such as phagocytosis and pinocytosis are known to require energy (12, 14). The effect of inhibitors of energy metabolism on the process of hemadsorption was therefore examined. BHK-21 cells infected with NDV were pulse-treated with anti-NDV serum as described in the above section and then treated with various inhibitors. As shown in Table 3, simultaneous addition of iodoacetate (2 mM) and sodium azide (5 to 10 mM) markedly inhibited the process of cells to become hemadsorptive, although each of the drugs had only a slight or no inhibitory effect when added alone.

TABLE 3. *Hemadsorption activity after incubation with various inhibitors*[a]

Inhibitors	Concentration (mM)	Hemadsorption	
		Activity[b]	Inhibition (%)
None		0.490	
Iodoacetate	5	0.496	0
	2	0.480	2.4
PCMB	0.2	0.520	0
	0.1	0.490	0
Sodium azide	20	0.400	21.3
	10	0.420	16.6
Potassium cyanide	10	0.335	26.7
	5	0.418	17.1
Iodoacetate plus sodium azide	5[c], 10[d]	0.068	100
	5, 5	0.060	100
	2, 10	0.060	100
	2, 5	0.080	97.2
Iodoacetate plus potassium cyanide	2, 10	0.090	94.8
Cycloheximide	100 μg/ml	0.470	4.7
Control[b]		0.068	

[a] BHK-21 cells infected with NDV were pulse-treated with anti-NDV serum at 6.0 h after infection, as described in the text. The cells were incubated further for 90 min at 36 C in the presence of various inhibitors, and then assayed for hemadsorption activity.

[b] Absorbance at 540 nm.

[c] Concentration of iodoacetate.

[d] Concentration of sodium azide.

[e] Cultures similarly infected and treated with antiserum were allowed to stand for 90 min at 4 C and then were assayed for hemadsorption activity.

Cell-associated hemagglutinating and neuraminidase activities were measured under similar conditions. These activities did not increase at all during incubation for 90 min in the presence of both drugs. Further, such a combination of drugs was found not to interfere with the hemagglutinating and neuraminidase activities of virions or hemadsorption activity of cells already emerged.

It has been shown that azide inhibits synthesis of adenosine 5'-triphosphate (ATP) by blocking cytochrome oxidase, which is also inhibited by cyanide. The effect of cyanide was then examined. As can be seen in Table 3, potassium cyanide added together with iodoacetate was found to prevent similarly the development of hemadsorption activity. On the other hand, iodoacetate, known as a general sulfhydryl (SH) modifying reagent, has been shown to inhibit phosphoglyceraldehyde dehydrogenase and to block production of ATP. SH modifying reagent alone, however, does not seem to block the step of hemadsorption, since *p*-chloromercuribenzoate was not inhibitory at all.

It is therefore likely that the inhibition of modification of the cell membrane by the combined treatment with sodium azide and iodoacetate may be due to inhibition of ATP synthesis, and that some compensative event would occur when one of the pathways of ATP synthesis is blocked by the respective inhibitor.

The effect of sodium azide and iodoacetate was reversible. These drugs were added to the culture at the 6th h of infection, and after incubation for 90 min with the inhibitors the medium was removed and the cells were washed three times with Hanks solution and then incubated in a fresh medium without inhibitors. As can be seen in Fig. 4, the increase in hemadsorption activity started again soon after the removal of the drug.

Effect of metabolic inhibitors on virus formation. It has been reported that complete progeny virus may be formed by budding of an area of plasma membrane modified to adsorb erythrocytes and then further modified to become a viral envelope (2). Therefore, we examined the effect of various inhibitors on virion formation. NDV-infected BHK-21 cells were pulse-treated with anti-NDV serum at 6.5 h after infection and incubated further in a fresh medium containing various inhibitors at 36 C for 90 min. Virus titers in the culture fluid and hemadsorption activity of the cells were then examined. Prior to assay, the culture fluids were dialyzed against fresh medium to avoid the effect of the drugs on the virus titer. As shown in Table 4, in cultures incubated with cycloheximide (100 μg/ml), progeny virus formation was significantly inhibited in contrast with hemadsorption reaction. A similar result was obtained in a kinetic study (Table 5) indicating that, although virion formation occurred in the presence of cycloheximide, the rate was much reduced as compared with that of the increase in hemadsorption activity.

These results suggest that virion formation may require continuous synthesis of protein. This would occur after the modification of the cell membrane became hemadsorptive. Iodoacetate and sodium azide completely inhibited the modification of cell membrane and progeny virus formation (Tables 4 and 5). The rapidity of inhibition of virus growth shown in these experiments suggests that the modification of the cell membrane has to occur continuously to support continuous production of the virus.

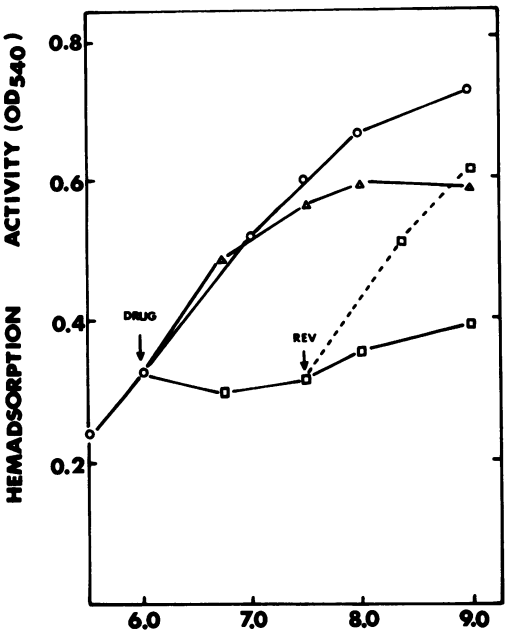

FIG. 4. *Effect of various inhibitors added at 6.0 h after infection on subsequent hemadsorption activity. At 6.0 h after infection, 2 mM of iodoacetate and 10 mM of sodium azide (□) or 100 μg/ml of cycloheximide (△) was added to the culture medium, and hemadsorption activity of the cells was measured at various times. When the culture medium of iodoacetate and sodium azide-treated cells was replaced with fresh medium without inhibitors at 7.5 h after infection, the effect of the drugs could be reversed, and rapid increase in hemadsorption activity was observed (□---□). Hemadsorption activity of untreated cells (○) was also shown.*

TABLE 4. *Hemadsorption activity and virus titer after incubation with various inhibitors*[a]

Inhibitors	Concentration (mM)	Activities	
		Hemadsorption activity[b]	Virus titer[c]
None		0.490	60.1
Cycloheximide	100 μg/ml	0.475	12.1
Iodoacetate plus sodium azide	2[d], 5[e]	0.100	4.10
	2, 10	0.080	3.66
Control[f]		0.080	3.90

[a] BHK-21 cells infected with NDV were pulse-treated with anti-NDV serum at 6.0 h after infection as described in the text, incubated further at 36 C for 90 min in the presence of various inhibitors, and then assayed for hemadsorption activity and virus titer in culture fluid.

[b] Absorbance at 540 nm.

[c] PFU/ml × 10^{-3}.

[d] Concentration of iodoacetate.

[e] Concentration of sodium azide.

[f] Cells similarly infected and treated with antiserum were left at 4 C for 90 min.

DISCUSSION

The assembly of paramyxoviruses is a multistep process which may be common to most of the enveloped viruses. As one of the steps in virus assembly, there is a phenomenon known as hemadsorption reaction; erythrocytes are adsorbed specifically to the surface of infected cells. These areas of cell membrane may contain viral envelope proteins, and they may be precursors of the modified membrane which becomes the envelope of the virion. In the present study, inhibition of such modification of cell membrane was attempted by using several metabolic inhibitors to clarify the mechanisms of this phenomenon.

In BHK-21 cells infected with NDV, the modification of the cell surface to adsorb red blood cells continued to develop normally for a certain period in the presence of cycloheximide when the drug was added at a later stage of infection. The membrane modification was also found in cells which were treated with anti-NDV serum to block viral antigens, which had already appeared at the cell surface, and then were incubated with cycloheximide. The intensity or duration of the development of such modification of the membrane was found to depend upon the time of addition of the drug. Therefore, it is likely that viral precursor proteins already synthesized and accumulated within the cell may perhaps be incorporated into the plasma membrane and modify it to become hemadsorptive. This step may proceed even in the absence of active protein synthesis. The synthesis of viral precursor proteins which are needed for the modification of the cell membrane seems to start between 1.5 and 3.0 h after infection, since any detectable hemadsorption could not be observed when the drugs were added at 1.5 or 3.0 h. It is not known, however, which kind of viral protein is essential to modify the membrane to become hemadsorptive. The finding that, in NDV-infected HeLa cells, an arginine-dependent protein other than the hemagglutinin, neuraminidase, or nucleocapsid protein, may be essential for the membrane to become hemadsorptive is of special interest in this connection (Iinuma et al., personal communication).

A combination of inhibitors of glycolysis and oxidative phosphorylation, on the other hand,

TABLE 5. *Effect of various inhibitors on the development of hemadsorption activity and virion formation*[a]

Activities	Inhibitors	Time of incubation (min)			
		0	30	60	90
Hemadsorption[b]	None	0.060	0.500	0.640	0.740
	Cycloheximide[c]		0.495	0.650	0.720
	Iodoacetate plus sodium azide[d]		0.068	0.060	0.080
Cell-associated virus titer[e]	None	13.7	45.0	88.5	174
	Cycloheximide		28.0	39.0	40.3
	Iodoacetate plus sodium azide		15.0	ND[f]	15.6
Released virus titer[e]	None	0.84	3.10	6.70	21.0
	Cycloheximide		2.04	2.73	4.02
	Iodoacetate plus sodium azide		0.88	ND	0.84

[a] BHK-21 cells infected with NDV were pulse-treated with anti-NDV serum at 6.5 h after infection as described in the text and then incubated further with various inhibitors. At various times during incubation, culture medium and cells were harvested separately and assayed for infectivity. The culture medium was dialyzed against fresh medium before titration. In companion cultures infected and treated similarly, hemadsorption activity was measured.

[b] Absorbance at 540 nm.

[c] 100 μg/ml.

[d] 2 mM iodoacetate and 10 mM sodium azide.

[e] PFU/ml × 10^{-5}.

[f] Not done.

was shown to rapidly inhibit the development of hemadsorption, suggesting that energy is required for this step to proceed. However, the respective inhibitors, iodoacetate or sodium azide, had little or no inhibitory effect when added alone. A possible explanation is that, since ATP is synthesized by oxidative phosphorylation and by glycolysis, blocking of one of the pathways may result in a compensation by each other. The growth of several enveloped viruses has been reported to be effectively prevented by such a combination of iodoacetate and sodium azide (16) or by other inhibitors of energy metabolism (5, 11). The existence of active processes which depend upon energy metabolism has been suggested in some phenomena related to biological membranes. Incorporation of polypeptides of influenza virus into the smooth membrane of HeLa cells has been shown to require energy (17). Tan et al. (13) have reported that the transport and release of interferon from the site of its synthesis into extracellular phase is an active process inhibited by cyanide or SH-modifying reagents but not by cycloheximide. The impression gained from the results of these investigations is that certain biological processes including virus-induced modifications of cell membranes may depend upon the functioning of the energy generating system but not upon de novo synthesis of protein.

Although the modification of the cell surface to adsorb red blood cells may be one of the essential steps in virus maturation and was found not to be inhibited by cycloheximide, the formation of a virion was significantly reduced by the drug, suggesting that continuous synthesis of protein may be required for the formation of a virion. The hemadsorptive areas of the membrane of myxo- or paramyxovirus-infected cells contain viral proteins but are not always altered to be morphologically similar to the viral envelope (2–4). On the one hand, it has been proposed that there is a protein of unknown function, other than the already known structural components of the virion, which is essential for virus maturation (6). These authors have suggested that this protein is labile and requires constant synthesis. Therefore, it may be possible to assume that the membrane modified in the presence of cycloheximide may lack some such essential protein for virus maturation. In this connection, the role of a structural protein of influenza virus (protein 7) which was found in a relatively small amount within the cell and which was detected predominantly in the modified plasma membrane is of special interest (7).

Studies on the morphological appearance or polyacrylamide gel electrophoretic analysis of the membrane modified in the presence of cycloheximide may shed some light on this problem.

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