BIOCHEMICAL BASIS FOR ALTERATIONS IN STRUCTURE AND FUNCTION OF HeLa CELLS INFECTED WITH NEWCASTLE DISEASE VIRUS*

By E. FREDERICK WHEELOCK,‡ M.D., AND IGOR TAMM, M.D.

(From The Rockefeller Institute)

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In a previous communication (1), the biological aspects of Newcastle disease virus (NDV)-HeLa cell interaction were described, and a systematic analysis presented of relationships between phases in the virus reproductive sequence and aspects of the cellular response to infection. It was demonstrated that the time course of events in virus-cell interaction depends on the multiplicity of the infecting virus.

Results of experiments on viral interference raised the possibility that in NDV-infected cells, metabolic deficits develop which are proportional to the amount of virus multiplication that has taken place (1). In the present investigation the nature and extent of virus-induced metabolic alterations were explored by studying the biosynthetic capacities of NDV-infected HeLa cells. The incorporation of tritiated thymidine into DNA, of uridine and cytidine into RNA, and of amino acids into protein was determined at frequent intervals after infection of HeLa cells with NDV at a virus/cell multiplicity of 500:1. The autoradiographic technique was used and the grains counted over entire cells or nuclei.

In this communication, early and marked inhibition of incorporation of precursors into DNA and protein of infected cells will be described. Continued incorporation, at a decreasing rate, of uridine into RNA will also be described. The inhibitory effects of NDV infection on DNA synthesis and mitosis will be compared with the effects of 5-fluoro-2-deoxyuridine (FUDR)[1] on these processes. The results of the biochemical studies are integrated with the biological observations on virus-cell interaction (1), and possible causal relationships between events are discussed.

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‡ Present address: Departments of Preventive Medicine and Medicine, School of Medicine, Western Reserve University, and the University Hospitals, Cleveland.

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Materials and Methods

The materials and experimental procedures outlined in preceding communications (1, 2) were used in the performance of these studies.

In brief, the experimental arrangement consisted of monolayer cultures of HeLa cells on coverslips. A modified procedure was used in the preparation of coverslip cultures in that after planting of cells, the Leighton tubes were incubated for 3 hours, whereupon an additional volume of growth medium was added to each tube. The cultures were used the following day; each consisted of an incomplete monolayer composed of approximately 200,000 cells. As indicated below, in certain experiments complete monolayers composed of approximately 400,000 cells were used.

The allantoic fluid seed of NDV contained 2.9×10^9 CIU (cell-infecting units) per ml. In all virus experiments the virus/cell multiplicity, *i.e.* the ratio of infective virus particles inoculated to the number of cells in culture, was 500:1. The multiplicity of adsorption under the conditions employed was 90:1; *i.e.*, 90 particles adsorbed per cell.

Autoradiographic Technique.—Tritiated nucleosides or amino acids were used. Infected or uninfected coverslip cultures of HeLa cells were washed four times in phosphate buffered saline (PBS) and placed in Leighton tubes which contained labeled precursor in 1 ml volumes of protein-free Eagle's medium. The tubes were gassed with 5 per cent CO_2 in air, stoppered, and incubated at 37°C. After appropriate intervals, groups of coverslips were removed, washed four to six times in PBS, placed in medium lacking labeled precursor and incubated for appropriate periods thereafter. When incubation was completed, coverslip cultures were fixed for 45 minutes in acetic acid:alcohol in proportions of 1:3 and washed twice in absolute ethanol. The coverslips were then dried in air, treated according to the procedures described below, and mounted on slides. Kodak stripping film AR10 was placed over the slides according to the procedure of Doniach and Pelc (3), and the preparations incubated for 1 to 10 days. The film was then developed, the cells stained through the emulsion with hematoxylin or the May-Greenwald stain, and the grains counted either on 4 x 5 inch photomicrographs taken at a magnification of 1000, or on slides examined under the light microscope.

Cultures were exposed to tritiated precursors for 30 or 45 minutes. Grain counts recorded at completion of experiments are plotted at the midpoints of the incubation periods with tritiated precursors.

Incorporation of tritiated precursors into the substances studied may take place by processes other than true synthesis. However, when incorporation ceases, it can be confidently concluded that synthesis has also ceased.

Incorporation of Tritiated Thymidine into DNA.—To determine whether grains in the autoradiographs of cultures which had been incubated with tritiated thymidine were due to labeling of DNA, the fixed cells were treated with deoxyribonuclease to remove DNA, or with 2 per cent perchloric acid to remove unincorporated thymidine.

Uninfected cultures were incubated with tritiated thymidine, 1 μc per ml, for ½ hour at 37°C, and then with unlabeled thymidine, 0.2 mg per ml, for an additional ½ hour. After incubation, the cultures were washed, fixed, and dried.

The coverslips were then divided in half, and one-half treated with deoxyribonuclease for 1 hour at 37°C followed by a second fixation in acetic alcohol. The concentration of the enzyme was 30 μg per ml of PBS. The other half was not treated. The treated and untreated halves of the coverslips were then processed for autoradiography and examined microscopically. It was found that deoxyribonuclease treatment removed all labeled material from fixed cells.

Fixed cells treated with 2 per cent perchloric acid at 2°C for 40 minutes, followed by 4 washes in water, gave as many grains as untreated cells, thus indicating that all thymidine which had not been incorporated into DNA had been extracted by the acetic alcohol fixative.

Incorporation of Tritiated Leucine, Methionine, and Phenylalanine into Proteins.—To determine whether labeling of cells which had been incubated with tritiated amino acids and fixed in acetic alcohol was due to incorporation of the amino acids into proteins, the fixed cells were extracted to remove free amino acids, lipids, and nucleic acids.

Uninfected HeLa cells were incubated with tritiated leucine, methionine, or phenylalanine, 1 μc per ml, for ½ hour at 37°C. The medium consisted of protein-free Eagle's medium lacking in unlabeled leucine, methionine, or phenylalanine, respectively. After incubation with labeled amino acid, the cultures were incubated in complete protein-free Eagle's medium without labeled amino acids for an additional ½ hour. After incubation, the cultures were washed, fixed, and dried. The coverslips were then divided in half, and one part treated with 5 per cent trichloroacetic acid (TCA) at 90°C for 30 minutes, washed four times in water, and air-dried. This was followed by treatment for 30 minutes at 50°C with a mixture of absolute ethanol:ether:chloroform, in proportions of 2:1:1, and by washing with cold ether. The treated cultures were then air-dried. The other half of the culture was not treated. The treated and untreated halves of the coverslips were processed for autoradiography, examined, and the grains counted.

It was found that the number of grains over cells in treated and untreated cultures was comparable indicating that all of the amino acid which had not been incorporated into protein, had been removed by the acetic alcohol fixative.

Incorporation of Tritiated Uridine and Cytidine into RNA.—In studies on incorporation of tritiated uridine into RNA, it was important to prevent uptake of the label into DNA. Experiments were therefore carried out in which tritiated uridine was supplemented with other nucleosides which were unlabeled, and deoxyribonuclease and ribonuclease were used to identify the nucleic acids which became labeled.

Uninfected cultures were incubated with tritiated uridine, 1.5 μc per ml, for 45 minutes at 37°C. To some tubes no supplement was added, but others received either unlabeled thymidine or cytidine, 0.2 mg per ml. After incubation with tritiated uridine, the cultures were incubated for 15 minutes in the presence of the unlabeled supplement plus unlabeled uridine, 0.2 mg per ml, washed, fixed, and dried.

The coverslips were then divided into thirds and one-third treated with ribonuclease, 30 μg per ml of PBS, for 1 hour at 37°C, followed by a second fixation in acetic alcohol; another third was treated with deoxyribonuclease under the same conditions, and the last third was not treated. The treated and untreated thirds of the coverslips were then processed for autoradiography and examined microscopically.

It was found that, in the absence of supplements, tritiated uridine was incorporated into both RNA and DNA. When cytidine was used as supplement, incorporation of uridine into both DNA and RNA was inhibited. However, when thymidine was present, incorporation of uridine into RNA was marked, but that into DNA was inhibited. Therefore, in the experiments on RNA synthesis to be described below, tritiated uridine was supplemented with unlabeled thymidine.

In studies on the incorporation of tritiated cytidine into RNA, attempts to prevent uptake of the label into DNA by means of supplements were unsuccessful. Therefore, following fixation with acetic alcohol, cells were digested with DNAase to remove tritiated DNA. All tritium remaining after this treatment could then be removed from cells by additional digestion with RNAase.

It was found, furthermore, that during digestion with DNAase, large amounts of tritiated RNA were removed from cells owing to solubilization of the RNA in the aqueous medium. Since this loss of RNA could not be prevented, the tritiated RNA remaining in cells following digestion with DNAase represents some but not all of the originally labeled RNA.

EXPERIMENTAL

I. Biosynthetic Capacities of Infected Cells

Effect of NDV Multiplication on Cellular DNA Synthesis.—The ability of HeLa cells to synthesize DNA after infection with NDV was determined by measuring the incorporation of tritiated thymidine into DNA of infected cells.

Coverslip cultures were inoculated with 0.1 ml of NDV seed diluted 1:3 in protein-free Eagle's medium. The virus/cell multiplicity was 500:1. After a 1 hour adsorption period at 37°C, the cultures were washed 4 times in PBS and returned to growth medium for further incubation at 37°C. Uninfected control cultures were treated similarly, except that the inoculum contained no virus. At intervals after inoculation, a coverslip culture from each series was washed in PBS and placed in a fresh tube containing 1 ml of tritiated thymidine diluted

TABLE I

Incorporation of Tritiated Thymidine into DNA of NDV-Infected HeLa Cells

Experiment No.	Number of grains per nucleus*							
	Uninfected cells	Infected cells, hrs. after infection						
		½	1¼	2¼	3¼	4¼	5¼	6¼
1	144	147		153		36		0
2	99		88	96	63	16	0	

* 25 cells counted.

in protein-free Eagle's medium to give 0.05 μc per ml. The tubes were gassed with 5 per cent CO_2, stoppered, and incubated at 37°C for ½ hour. The coverslips were then washed 6 times in PBS and placed for ½ hour in protein-free Eagle's medium containing unlabeled thymidine, 0.4 mg per ml. They were then washed in PBS, fixed, and processed for autoradiography. Ten days later the slides were developed, stained with hematoxylin, and examined. Approximately 30 to 40 per cent of cells incorporated tritiated thymidine into DNA and grains over nuclei of these cells were counted. No grains were seen over the cytoplasm.

As can be seen in Table I, the nuclei of infected cells which were exposed to tritiated thymidine 1 or 2 hours after infection showed about as many grains as the nuclei of uninfected control cells. 3¼ hours after infection the number of nuclear grains was 64 per cent of that in uninfected controls, and at 4¼ hours, the number had decreased to 21 per cent. In the two experiments summarized in Table I no grains were seen in infected cells 5¼, 6¼, or 7¼ hours after infection. In one additional experiment, an occasional infected cell, examined 5 or 6 hours after infection, contained grains in numbers corresponding to less than 50 per cent of those seen in uninfected cells.

Effect of NDV Multiplication on Cellular Protein Synthesis.—The ability of NDV-infected HeLa cells to synthesize protein was determined by measuring

the incorporation of each of the following three tritiated amino acids: DL-leucine, DL-methionine and DL-phenylalanine.

Coverslip cultures of HeLa cells were infected with NDV at a multiplicity of 500:1. At hourly intervals after infection, groups of cultures were incubated for ½ hour in the presence of each of the three tritiated amino acids. The medium employed during exposure of cells to a tritiated amino acid did not contain the corresponding unlabeled amino acid, but did contain all other essential amino acids. After incubation in labeled medium, the cultures were washed in PBS, and incubated in the absence of tritiated compounds in complete protein-free Eagle's medium for an additional ½ hour. The cultures were then fixed and processed for autoradiography. The developed preparations were stained with the May-Greenwald stain, and the grains were counted.

TABLE II

Incorporation of Tritiated Amino Acids into Protein of NDV-Infected HeLa Cells

	Number of grains per cell*					
	Uninfected cells	Infected cells, hrs. after infection				
		1¼	2¼	3¼	4¼	5¼
Leucine						
Exp. 1	70	68	42	48	6	3
Exp. 2	33	34	37	27	7	2
Methionine						
Exp. 1	52	51	55	38	7	5
Exp. 2	78	58	74	79	13	3
Phenylalanine						
Exp. 1	35	21	29	20	2	1
Exp. 2	29	27	25	22	9	2

* 25 cells counted.

Two such experiments were performed and the results are summarized in Table II. There was no consistent decrease in incorporation of any of the three amino acids until 3¼ hours after infection. At 4¼ hours, an 80 to 90 per cent decrease was observed, and by 5¼ hours, incorporation was almost completely inhibited.

It should be mentioned that the grains were evenly distributed over the entire cell. There was no increased uptake of labeled amino acids at sites of virus antigen formation (1, 4) in the cytoplasm.

Effect of NDV Multiplication on Cellular RNA Synthesis.—Synthesis of RNA by NDV-infected HeLa cells was studied by determining the incorporation of tritiated uridine into RNA at intervals after infection.

Coverslip cultures of HeLa cells were inoculated with NDV at a multiplicity of 500:1, as described above. At 2 hour intervals following inoculation of virus, a coverslip was washed

four times in PBS and incubated for 45 minutes at 37°C with tritiated uridine, 1.5 μc per ml in protein-free Eagle's medium which was supplemented with unlabeled thymidine, 0.2 mg per ml. The coverslips were then washed six times in PBS, and incubated in the presence of unlabeled uridine and thymidine, 0.2 mg per ml of each, for an additional period of 15 minutes. After incubation the cultures were washed six times in PBS, fixed in acetic alcohol, and dried. The cultures were then treated with 2 per cent perchloric acid at 2°C for 1 hour, washed with cold 2 per cent perchloric acid, air-dried, and processed for autoradiographs. Exposure to film was for 5 days.

As can be seen in Table III, incorporation of tritiated uridine into RNA continued at a decreasing rate until at least 10⅜ hours after infection when a 40 per cent decrease in incorporation was noted. In these studies on uridine incorporation into RNA, many grains over the nucleoplasm were observed,

TABLE III

Incorporation of Tritiated Uridine into RNA of NDV-Infected HeLa cells

Experiment No.	Number of grains per cell*					
	Uninfected cells	Infected cells, hrs. after infection				
		2⅜	4⅜	6⅜	8⅜	10⅜
1	50	44	45	43	37	37
2	49	48	44	40	24	35

* 25 cells counted.

but nucleolar labeling was not pronounced. Only a small number of grains were observed over the cytoplasm.

Additional studies on the sites of RNA synthesis in NDV-infected cells were carried out with tritiated cytidine. The experimental procedures used were identical with those just described, with one exception: following fixation, cells were digested with DNAase, 30 μg per ml of PBS, for 1 hour at 37°C. The cells were then refixed in acetic alcohol, dried, and processed for autoradiographs.

In uninfected HeLa cells the label appeared predominantly over nucleoli with small amounts scattered throughout the nucleoplasm. Cells infected for 2 hours incorporated tritiated cytidine in a similar manner. At subsequent intervals, however, the label was more diffusely distributed over the nucleoplasm, and by the 8th hour, there was little if any nucleolar labeling of RNA in infected cells.

It should be pointed out that it is not possible to identify the intracellular site of viral RNA formation by this technique, for the tritiated precursors can be incorporated into both viral and cellular components, and no distinction between the two can be made. Furthermore, the total amount of viral biosynthesis is probably exceedingly small when compared to cellular biosynthesis,

and, thus, all detectable incorporation of precursors may have represented synthesis of cellular macromolecules only.

II. Experiments with a Metabolic Inhibitor of DNA Synthesis

It was reported previously (1) that the mitotic activity of HeLa cells infected with NDV at a multiplicity of 500:1 is markedly inhibited 7 to 8 hours after infection. The demonstration that inhibition of mitosis in NDV-infected cells is preceded by inhibition of DNA synthesis by 2 to 3 hours and the fact that the time courses of inhibition are approximately parallel, raised the possibility that inhibition of mitosis by NDV multiplication might be mediated through interference with DNA synthesis. To test this, 5-fluoro-2-deoxyuridine (FUDR) was used to inhibit DNA synthesis and mitosis, and the time relationship between inhibition of these two processes was compared to that observed in NDV-infected cells.

Effect of FUDR on DNA Synthesis.—There is evidence that FUDR inhibits DNA synthesis by preventing the methylation of deoxyuridine monophosphate to thymidine monophosphate (5). Therefore, tritiated deoxyuridine was used as a precursor of DNA. In addition, experiments were also done with tritiated cytidine and thymidine.

Coverslip cultures containing approximately 200,000 HeLa cells were inoculated with 10^{-7} M FUDR in protein-free Eagle's medium, 0.1 ml per culture. At intervals after inoculation, a coverslip was washed four times in PBS, and placed in 1 ml of tritiated nucleoside at a concentration of 1 μc per ml in protein-free Eagle's medium which also contained 10^{-7} M FUDR. Following ½ hour incubation at 37°C, the coverslip was washed six times in PBS, and incubated in 1 ml of 10^{-7} M FUDR in unlabeled protein-free Eagle's medium for an additional ½ hour at 37°C. The coverslip was then washed six times in PBS, and fixed in acetic alcohol and processed for autoradiographs. Control cultures were treated identically, except that no FUDR was used. Following fixation in acetic alcohol, the cultures which had been incubated with tritiated cytidine or deoxyuridine were treated with RNAase at 37°C for 1 hour. The concentration of the enzyme was 30 μg per ml of PBS. These cultures were fixed for a second time, and processed for autoradiographs together with other cultures.

It was found that HeLa cells treated with FUDR for 5 minutes failed to incorporate either deoxyuridine or cytidine into DNA. Thymidine was incorporated into DNA in FUDR-treated cells as effectively as in controls. Furthermore, the amount of tritiated thymidine incorporated into cells which had been pretreated with FUDR for 4 hours was approximately the same as that incorporated into untreated cells, and there was no difference in the number of cells containing grains.

Thus, FUDR blocks DNA synthesis almost instantly after addition to HeLa cells. The results obtained support the concept that FUDR acts by inhibiting the methylation of deoxyuridine monophosphate to thymidine monophosphate (5). The inability of cells treated with FUDR to incorporate cytidine

into DNA suggests that synthesis of DNA cannot proceed in the absence of thymidine, and that there is no appreciable thymidine pool in HeLa cells.

Effect of FUDR on Incidence of Mitosis.—Mitotic activity of HeLa cells treated with FUDR was determined. The concentration of FUDR necessary to inhibit mitosis, as well as the kinetics of inhibition was investigated.

Coverslip cultures with complete monolayers of HeLa cells were placed in flat-bottomed Petri dishes, and inoculated with 10-fold dilutions of FUDR in protein-free Eagle's medium,

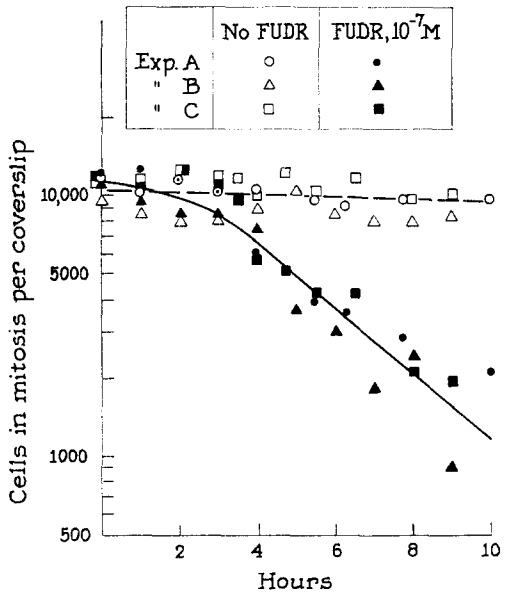

FIG. 1. Effect of FUDR on the incidence of mitosis in HeLa cell monolayers.

0.1 ml per coverslip. At the end of 1 hour at 37°C in an atmosphere of 5 per cent CO_2 in air, the cultures were placed in Leighton tubes which contained growth medium plus various dilutions of FUDR.

At intervals, the coverslip cultures were examined microscopically and the number of cells in mitosis were counted within a square formed by a microgrid inserted into the ocular of the microscope. Cells in metaphase, anaphase, and telophase were readily identified. Two coverslips from each dilution series were examined, and 2 squares per culture were selected at random and scored. The mean number of mitotic cells per square was calculated, and the result expressed as the number of mitotic cells per coverslip culture. The cultures originally contained approximately 400,000 cells. The incidence of mitosis in infected cultures was related to that in uninfected control cultures.

After 9 hours of incubation of cultures in the presence of FUDR at concentrations ranging from 10^{-3} to 10^{-7} M, approximately 80 per cent inhibition of mitosis was observed at each concentration. There was no inhibition of mitosis

at 10^{-8} M FUDR until 24 hours after inoculation, and no inhibition at all at higher dilutions. The kinetics of inhibition of mitosis was studied with 10^{-7} M FUDR.

Coverslip cultures containing complete monolayers of HeLa cells were inoculated as above with 10^{-7} M FUDR in protein-free Eagle's medium, and counts of mitotic cells made at hour intervals. Control cultures were treated similarly, but received no FUDR.

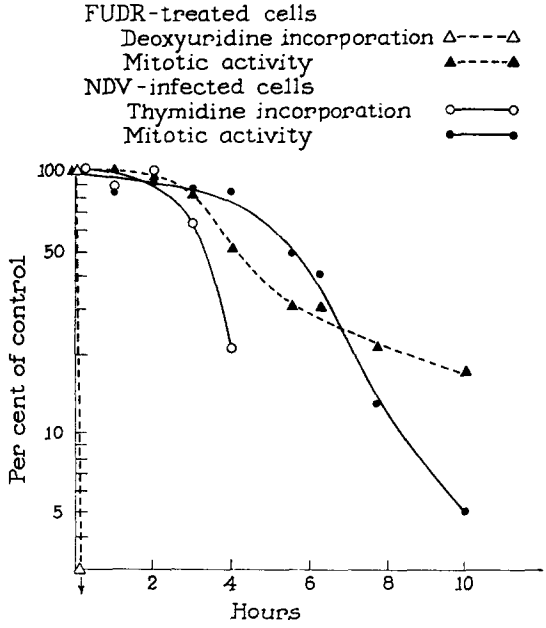

FIG. 2. Effect of FUDR and NDV infection on DNA synthesis and incidence of mitosis in HeLa cell monolayers.

As can be seen in Fig. 1, FUDR-treated cells showed a slight decrease in the incidence of mitosis at 4 to 5 hours, and an 80 per cent decrease at 8 hours. Control cultures showed no such decrease in mitotic activity.

Twenty-four hours after inoculation, inhibition of mitosis was in excess of 95 per cent in the FUDR-treated cells. The presence of a small percentage of cells in mitosis 24 hours after addition of FUDR suggests that some of the cells may be resistant to the effects of FUDR and can synthesize thymidine by an alternate pathway. Such resistant cell populations have been reported among bacteria (6).

Relation of Inhibition of DNA Synthesis to Inhibition of Mitosis in FUDR-treated, and in NDV-infected Cells.—Experiments were carried out in which parallel observations were made on inhibition of mitosis by FUDR and by NDV.

Coverslip cultures with complete monolayers of HeLa cells were inoculated with either 10^{-7} M FUDR or with NDV at a multiplicity of 500:1, and counts of mitotic cells made at hourly intervals. Control coverslips were treated similarly, but received no FUDR or NDV.

The time courses of inhibition of mitosis in FUDR-treated, and in NDV-infected cells in one such experiment are plotted in Fig. 2, together with curves describing inhibition of DNA synthesis in each group. The latter curves are based on results of experiments described in the preceding sections. As can be seen in Fig. 2, the interval between inhibition of DNA synthesis and inhibition of mitosis was shorter in NDV-infected than in FUDR-treated cells.

Since the interval between inhibition of DNA synthesis by FUDR and subsequent inhibition of mitosis is the shortest possible time in which inhibition of DNA synthesis can result in inhibition of mitosis, it is clear that inhibition of mitosis by NDV infection is not mediated through interference with DNA synthesis.

DISCUSSION

The results of the systematic study of the virus reproductive sequence and of the cellular consequences of infection presented in a previous communication (1) and above, lend themselves to an analysis of possible causal relationships between certain events which occur in NDV-infected HeLa cells. Fig. 3 summarizes most of the information secured in the study of cells infected at a virus/cell multiplicity of 500:1.

The sequence of biological events was as follows: both newly made virus antigen and the first infective virus particles appeared 2½ hours after infection of cultures. Infective virus reached a peak at 4 to 5 hours and then declined. Virus antigen reached a plateau after the decline in infective virus had begun. A decrease in mitotic activity occurred a short time before production of infective virus ceased, and it became marked at 6 to 8 hours as the virus antigen reached a plateau. Production of markedly damaged cells was a late event.

Studies on cellular synthesis of DNA, RNA, and proteins showed the following: inhibition of DNA and protein synthesis became evident at about the same time as newly made virus antigen and infective virus particles first appeared. Progressive decrease in synthesis of DNA and protein coincided with rapid increase in virus antigen and infective particles, and synthesis of DNA and protein stopped as the amounts of virus materials produced were reaching maximal levels. Inhibition of DNA and protein synthesis preceded inhibition of mitosis by 2 to 3 hours, and the development of marked cellular damage by several hours. Incorporation of labeled uridine into RNA was only slightly reduced at the time of cessation of both virus production and synthesis of DNA and protein; a considerable amount of incorporation of precursor was still taking place when cells undergoing marked degenerative changes began to appear in large numbers.

In what follows, the biochemical alterations are used as a basis for discussion of possible mechanisms involved in 4 separate processes: (*a*) cessation of production of infective virus, (*b*) inhibition of mitosis, (*c*) production of cell damage, and (*d*) viral interference.

Cessation of Virus Production.—The striking time relationship between inhibition of both DNA and protein synthesis, and cessation of production of infective virus suggests that the latter may be due to inhibition of either DNA or protein synthesis. That cessation of virus production was due to inhibition

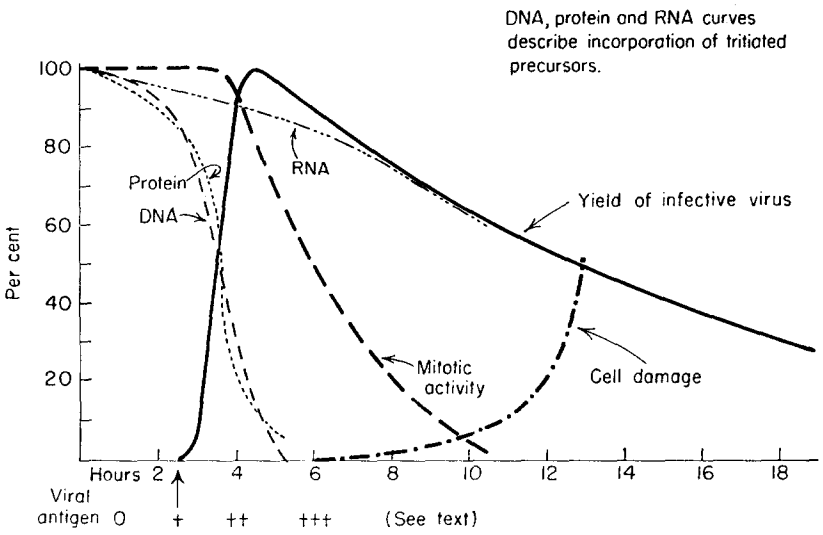

FIG. 3. Temporal relationships between aspects of virus multiplication and cellular response. NDV/HeLa cell multiplicity—500:1.

of DNA synthesis is unlikely, however, for the following reason: 5-fluoro-2-deoxyuridine, an antimetabolite which inhibits cellular DNA synthesis, does not interfere with the multiplication of NDV (7).

It should also be emphasized that since the DNA synthetic phase of the mitotic cycle is only 5 to 6 hours long (8), only about 30 per cent of cells are synthesizing DNA at any given time, and thus in the 4 hour interval following infection, less than 50 per cent of infected cells in the culture are affected by this biochemical alteration, whereas virus production apparently ceases in all cells.

As to a possible relationship between inhibition of protein synthesis and cessation of virus production, the following can be said. The continued presence after the 6th hour of large amounts of antigen in cells does not necessarily mean that the supply was sufficient to permit further virus production. Studies of

another virus-cell system (9) have suggested that surprisingly large quantities of virus precursors are required for production of infective particles. Furthermore, the antigen present at 6 hours may represent only one of the essential proteins which may be required for the manufacture of infective particles. Therefore, the available evidence leaves open the possibility that there is a causal relationship between inhibition of protein synthesis, and cessation of production of infective virus.

The possibility also exists, however, that cessation of virus production may be due to failure of synthesis of functional viral RNA. This possibility is suggested by the observation that the RNA produced in bacteria, in which protein synthesis has been inhibited by chloramphenicol, appears to be defective and non-functional (10).

Finally, the possibility should be considered that cessation of virus synthesis is due to production of interferon (11) which has been demonstrated in several different cell types incubated with a variety of infective or inactivated viruses (12). However, there is evidence that infective myxoviruses fail to induce the production of interferon in HeLa cells (13, 14). It is also unlikely that significant amounts of interferon are introduced in the virus-infected allantoic fluid used as the inoculum, since the seed virus was harvested before expected time of interferon production by the infected chorioallantoic membrane (12). Finally, it has been shown (12, 15) that interferon neither stimulates nor depresses cell division in tissue culture. Thus, it is highly unlikely that it has significant effects on cellular nucleic acid and protein synthesis. Early and marked inhibition of these processes in NDV-infected cells would therefore require another explanation.

Inhibition of Mitosis.—In a previous communication (1), the kinetics of inhibition of mitosis of NDV-infected HeLa cells was described and related to other biological aspects of virus-cell interaction. In brief it was found that inhibition was not due to marked degenerative cell changes, since these occurred much later. Furthermore, production of infective virus did not *per se* affect the ability of cells to undergo mitosis and to divide.

It should be noted in Fig. 3 that mitotic activity decreased at about the same time that production of infective virus ceased. This raises the possibility that the same biochemical alteration may be responsible for both cessation of virus production and inhibition of mitosis.

Experiments with FUDR indicated that the inhibition of mitosis observed in NDV-infected cells is probably not due to inhibition of DNA synthesis. Thus, the possibility exists that inhibition of mitosis results from inhibition of protein synthesis, which follows a parallel course to the inhibition of mitosis, but occurs 2 to 3 hours earlier. Little work has been done on proteins which are essential for mitosis and cell division. Mazia has isolated a protein component of the mitotic apparatus of sea urchin eggs, which comprises 12 per cent of cellular protein (16). It is known that a disulfide-rich protein is necessary for the con-

summation of mitosis and division in sea urchin eggs (17). Proteins of this kind may also be required in mammalian cells, and interference with their synthesis may preclude successful mitosis. However, the possibility cannot be ruled out that altered RNA synthesis in virus-infected cells may be, at least in part, responsible for inhibition of mitosis.

Quite different results have been obtained with herpes simplex virus which causes a considerable increase in the DNA content of infected HeLa cells (18), but at the same time inhibits cell division within 1 hour after infection when inoculated at a multiplicity of 1:1 (19). The exact nature of the DNA synthesized after infection has not been determined. It should be emphasized that in herpes virus–infected cells, fragmentation and margination of nuclear chromatin are the earliest cytopathological changes; they are later followed by rounding and lysis of cells (20).

In pseudorabies virus–infected rabbit kidney cells in culture an increase in DNA occurs, accompanied by proliferation of cell nuclei (21). Amitotic nuclear division is followed by cellular degeneration. It is of interest that x-ray-irradiated cells show no increase in DNA and no nuclear division despite active multiplication of the virus.

In KB cells infected with type 2 adenovirus the DNA and protein content increased continuously starting within 12 hours after infection, until 48 hours (9). RNA content increased until about the 24th hour, and then declined. However, cell division ceased 10 hours after infection. The production of infective virus reached a plateau at about 24 hours. The decline in RNA was attributed to partial cell lysis and loss of RNA from the cell.

Production of Cell Damage.—As can be seen in Fig. 3, and as has been emphasized above, the development of marked cell damage is a late phenomenon in virus-cell interaction, and is not directly related to production of infective virus. It is of interest in this connection that influenza virus, which fails to produce any infective progeny in HeLa cells, nevertheless causes degeneration and death of cells (22).

The striking relationship between the time at which DNA and protein synthesis becomes completely inhibited, and the onset of marked cell damage, strongly suggests that the degeneration and ultimate death of cells may well be due to the profound metabolic derangements which develop in virus-infected cells. It should be emphasized that specific biochemical alterations by themselves may not be the immediate cause, but may initiate series of cellular reactions culminating in structural disintegration. Thus, inhibition of protein synthesis may result in the inability of the cell to replace essential proteins which are necessary for structural and functional integrity. When the cellular supply of these components is exhausted, gross alterations may occur. Heterogeneity of the HeLa cell populations used may be responsible for the gradual development of these alterations.

Although the precise time relationships between biochemical alterations and

changes in cell structure have not been described in other virus-cell systems, it is of interest to compare the biochemical alterations observed in NDV-infected cells with those found in infections with other cytocidal viruses. Early stimulation of synthesis of macromolecules has been observed in several virus-cell systems (9, 18, 21, 23–25). Stimulation of biosynthetic activities during an early phase of infection has also been reported for poliovirus-infected HeLa cells incubated in a maintenance medium which did not permit growth of cells (26–28). In contrast, abrupt cessation of synthesis of macromolecules was observed in poliovirus-infected HeLa cells incubated in a rich medium permitting growth of cells (29, 30). No early changes in synthesis of DNA, RNA, or protein were observed in foot-and-mouth disease virus–infected bovine kidney cells (31).

The great variation in early cellular biochemical alterations produced by cytocidal viruses does not necessarily mean that each virus causes structural disintegration of the infected cell by a different mechanism. Indeed, it is not unreasonable to postulate that the final events which lead to cellular disintegration may be similar.

Viral Interference.—It was found in earlier studies on interference between NDV and the NWS strain of influenza A virus (1) that interference by NDV with NWS was quantitatively dependent on the length of the interval between inoculation of the two viruses, and also on the multiplicity of NDV; *i.e.*, on the amount of multiplication of NDV which has taken place prior to infection of HeLa cells by the challenge virus (NWS). The present demonstration that multiplication of NDV leads to progressive inhibition of cellular biosynthetic activities appears to provide an adequate explanation for the earlier finding of quantitative dependence of interference on the amount of NDV multiplication.

SUMMARY

The ability of NDV-infected HeLa cells to synthesize DNA, protein, and RNA was investigated by measuring the incorporation of tritiated precursors into these substances at intervals after infection of cells with a virus/cell multiplicity of 500:1. A significant decrease in incorporation of precursors into DNA and protein was first observed at 3¼ hours after infection. By 4¼ hours, an 80 to 90 per cent decrease had occurred, and by 5¼ hours, incorporation of precursors into DNA and protein was almost completely inhibited. Incorporation of precursor into RNA decreased gradually following infection; by the 10th hour, a 40 per cent decrease had occurred.

These results, integrated with earlier observations on biological aspects of infection, suggest the following causal relationships among events in NDV-infected cells: (*a*) The cessation of virus production is probably caused by inhibition of protein or RNA synthesis, and is not due to inhibition of DNA synthesis or to interferon. (*b*) The production of infective virus does not *per se* interfere

with the ability of an infected cell to divide, nor is inhibition of mitosis caused by either inhibition of DNA synthesis or development of marked degenerative changes in infected cells. Inhibition of mitosis may be the result of inhibition of protein or RNA synthesis. (*c*) Marked cell damage could have been caused by inhibition of protein, DNA, or RNA synthesis. (*d*) Interference by NDV with the multiplication of influenza virus was probably due to the inhibitory effects of NDV on cellular biosynthetic activities.

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