# *THE ROLE OF PROTEIN SYNTHESIS IN THE ECLIPSE PERIOD OF NEWCASTLE DISEASE VIRUS MULTIPLICATION IN HELA CELLS AS STUDIED WITH PUROMYCIN**

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The virus eclipse period is defined as the interval between infection and the first discernible presence of new virus or virus components in cells. Many events take place during the early part of the eclipse period which lead to the reorganization of cellular metabolic processes and synthesis of virus precursor material, infective nucleic acid, and infective virions.

In the T1, T3, T7, and 201S bacteriophage-*Escherichia coli* cell systems, it is likely that infection and replication of viral DNA can take place in the absence of protein synthesis.[1] This suggests, therefore, that these bacterial host cells normally contain all enzymes and metabolic processes necessary for synthesis of viral nucleic acid. The T2 phage DNA, however, contains 5-hydroxymethylcytosine, a base not found in its host cell, and so must synthesize special enzymes prior to viral nucleic acid synthesis.[2, 3] The initial part of the T2 eclipse period, therefore, requires cellular protein synthesis. There have been no comparable studies on the requirements for protein synthesis in the eclipse period of animal viruses.

In a previous communication on Newcastle disease virus (NDV)-HeLa cell interaction,[4] an early and marked inhibition of protein synthesis was reported to occur in infected cells prior to inhibition of mitosis and cell degeneration. In order to determine whether or not a causal relationship exists between NDV-induced inhibition of protein synthesis and subsequent cytopathic alterations, a metabolic inhibitor of protein synthesis, puromycin dihydrochloride,† was selected to study the cellular consequences of inhibition of protein synthesis. In the course of these experiments, it was found that puromycin could inhibit both protein synthesis and NDV multiplication in HeLa cells. Furthermore, on removal of the drug from the culture media, both protein synthesis and virus multiplication resumed.

These reversible inhibitory characteristics of puromycin permitted an investigation of the protein synthesis dependence of the NDV eclipse period. It was found that initiation of infection and completion of an early part of the virus eclipse period could take place in the presence of puromycin. These experiments will be described herein and two hypotheses presented to account for this phenomenon.

*Materials and Methods.*—Many of the materials and experimental procedures used in the performance of these studies were reported in previous communications[4-6] and are in part described below. Modifications and additions are also included.

*Cell culture:* In brief, the cell culture system employed in experiments designed to measure virus multiplication consisted of HeLa cells grown on 11 × 38 mm coverslips in Leighton tubes. Each culture consisted of an incomplete monolayer composed of approximately 200,000 cells. In isotope experiments, S-bottles[7] with a flat culture area of 1,000 $mm^2$ were employed. S-bottles were planted with $2.5 \times 10^5$ cells in 2 ml of growth medium. The bottles were gassed with 5 per cent $CO_2$ in air, stoppered, and incubated at 37°C for 48 hr. At this time, the monolayers were complete and each was composed of approximately 900,000 cells.

*Virus:* The allantoic fluid seed of NDV contained $3.2 \times 10^9$ CIU (cell-infecting units) per

ml.[5] 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 225:1.

*Isotope technique:* For isotope experiments, L-leucine-u-$C^{14}$ with a specific activity of 54.4 $\mu$c/mg was diluted in incomplete Eagle's medium, i.e., protein-free Eagle's medium[8] in which the unlabeled leucine concentration was reduced to 13 mg/liter.

The medium was removed from S-bottles which contained uninfected or NDV-infected monolayer cultures of HeLa cells and replaced with incomplete Eagle's medium containing $C^{14}$-L-leucine, 2 ml per bottle. The bottles were gassed with 5 per cent $CO_2$ in air, stoppered, and incubated at 37°C. After an appropriate interval, the monolayers were washed rapidly twice with phosphate buffered saline (PBS)[9] and treated with 0.5 *N* perchloric acid (PCA) at 4°C for 15 min. The bottles were then washed twice with cold 0.5 *N* PCA and drained. When the bottles were dry, 1.5 ml of 0.2 *N* NaOH was added to each. The bottles were then stoppered and incubated at 60°C for two hr. A 0.5 ml aliquot from each bottle was added to each of two screw cap glass bottles which contained 15 ml of scintillation liquid, and the counts per minute per bottle were measured in a Packard-Tri-carb scintillation counter. Four bottles per point were used in each experiment. The scintillation liquid consisted of naphthalene, 375 gm; 1,4-bis-2-(5-phenyloxazolyl-benzene), 2.2 gm; 2,5-diphenyloxazole, 22.5 gm; dioxane, 3 kg; and methanol, 200 ml.

*Protein determinations:* Protein determinations were performed by the method of Lowry *et al.*[10] The mean protein content of a complete monolayer of HeLa cells in 24 S-bottles was 422 $\gamma$ per bottle, and the standard deviation was 31.2 $\gamma$. Since the variation in protein among bottle cultures is small, incorporation of $C^{14}$-L-leucine into protein is expressed as "counts per minute," calculated as the mean of four bottles per point.

*Results.—Effect of puromycin on cellular protein synthesis:* The effect of various concentrations of puromycin on inhibition of protein synthesis in HeLa cells was determined by measuring the incorporation of $C^{14}$-L-leucine into protein of cells.

Monolayer cultures of HeLa cells in S-bottles were inoculated with half-log dilutions of molar puromycin in growth medium, 2 ml per bottle, and incubated for one-half hr at 37°C. The puromycin media was then replaced with incomplete Eagle's media containing 0.03 $\mu$c of $C^{14}$-L-leucine per ml and supplemented with the appropriate concentration of puromycin, 2 ml per bottle. Following one-half hr incubation at 37°C, the cultures were processed for scintillation counting.

As can be seen in Figure 1, $10^{-4}M$ (54.5 $\mu$g per ml) puromycin completely inhibited protein synthesis in HeLa cells within 45 min following treatment. An 80 per cent inhibition of protein synthesis was produced by $10^{-5}M$ puromycin and 20 per cent inhibition by $10^{-6}M$. No inhibition was produced by $10^{-7}M$.

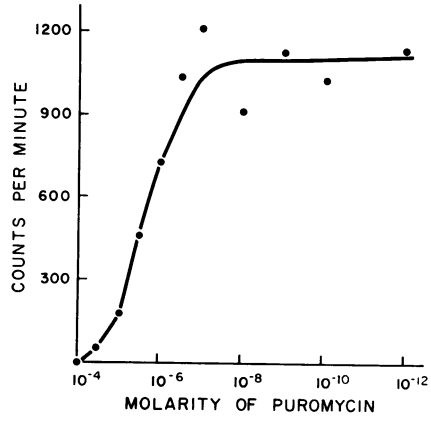


FIG. 1.—Effect of puromycin on protein synthesis in HeLa cells.

*Effect of puromycin on NDV multiplication.*—The inhibitory effect of puromycin on NDV multiplication in HeLa cells was determined by adding tenfold dilutions of molar puromycin to cultures at hourly intervals after infection. Further virus multiplication was measured by staining virus antigen with fluorescent antibody.

Leighton tubes which contained coverslip cultures of HeLa cells were inoculated with 1.5 ml of a $10^{-2}$ dilution of NDV seed in protein-free Eagle's medium. After

a one-hr adsorption period at 37°C, the coverslip cultures were removed, washed in PBS, and placed in tubes which contained growth medium for further incubation. At the time of virus inoculation or at hourly intervals thereafter, tenfold dilutions of molar puromycin were added to cultures. All coverslip cultures were collected 5 hr after infection and stained with fluorescent antibody.

As described in Table 1, $10^{-4}M$ (54.5 $\mu$g per ml) puromycin completely inhibited virus antigen synthesis. The amount of antigen observed at 5 hr was equal to that

TABLE 1

INHIBITION OF NDV MULTIPLICATION IN HeLa CELLS BY PUROMYCIN

| Molar concentration of puromycin | Virus antigen 5 hr after infection — Hr at which puromycin added | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 |
| $10^{-4}$ | 0 | 0 | + | 2+ | 3+ | 4+ |
| $10^{-5}$ | ± | + | 2+ | 3+ | 4+ | 4+ |
| $10^{-6}$ | + | 2+ | 3+ | 4+ | 4+ | 4+ |
| $10^{-7}$ | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ |

present in cells at the time of addition of puromycin as determined by nonpuromycin-treated infected control cultures. No increase in antigen, therefore, occurred in the presence of $10^{-4}M$. A $10^{-5}M$ concentration of puromycin produced a marked but incomplete inhibition of virus synthesis. A slight inhibition occurred in cells inoculated with $10^{-6}M$ puromycin, and no inhibition was seen with $10^{-7}M$.

These experiments demonstrate the dependence of virus multiplication on HeLa cell protein synthesis; complete inhibition of protein synthesis was necessary for cessation of virus multiplication. Furthermore, virus antigen synthesis could be arrested at any stage of the replication sequence by puromycin in sufficient concentration to inhibit cellular protein synthesis completely.

*Resumption of protein synthesis in HeLa cells on removal of puromycin:* A desirable characteristic of a metabolic inhibitor employed in studies of virus-cell interaction is reversal of effect. The ability of puromycin-treated HeLa cells to resume protein synthesis following removal of the drug from the culture medium was therefore studied.

S-bottles with complete monolayers of HeLa cells were inoculated with growth media which contained $10^{-4}M$ puromycin, 2 ml per bottle, and incubated at 37°C for two hr. Puromycin was then removed, the monolayer cultures washed four times with growth medium, and 2 ml of growth medium added to each bottle. Incubation at 37°C was continued. At the time of removal of puromycin or at various intervals thereafter, a 2 ml volume of incomplete Eagle's medium which contained 0.05 $\mu$c of $C^{14}$-L-leucine per ml was added to each bottle. Following 10 min incubation at 37°C, the cultures were processed for scintillation counting. The protein synthesis capacity of cells incubated in the presence of puromycin was measured by supplementing the isotope with $10^{-4}M$ puromycin.

As can be seen in Figure 2, within 15 min following removal of puromycin from the media, HeLa cells resumed protein synthesis at a rate 75 per cent of prepuromycin-treatment levels. At 30 min following removal of puromycin, 100 per cent restoration of protein synthesis was achieved.

It was next of interest to compare the resumption of puromycin-induced inhibition of protein synthesis in virus-infected cells with that in uninfected control cells.

S-bottles with complete monolayers of HeLa cells were inoculated with an

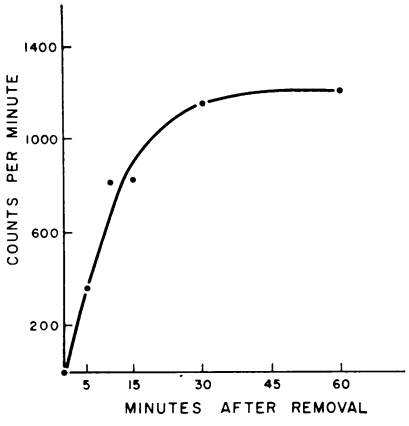


FIG. 2.—Resumption of protein synthesis in HeLa cells following removal of $10^{-4}M$ puromycin from culture media.

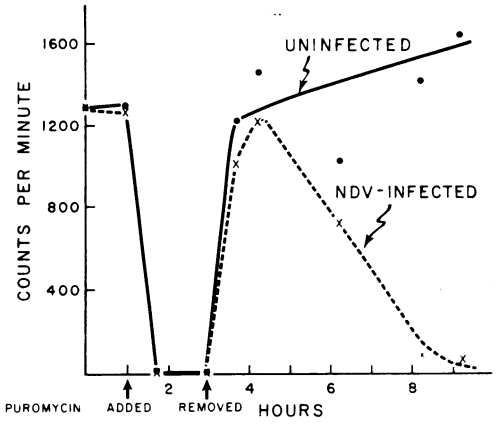


FIG. 3.—Effect of addition and removal of $10^{-4}M$ puromycin on protein synthesis in uninfected and NDV-infected HeLa cells.

NDV/cell multiplicity of 225:1. Following 1 hr incubation at 37°C, the mono layers were washed with PBS, growth medium with $10^{-4}M$ puromycin was added, 2 ml per bottle, and the cultures were reincubated for 2 hr. The cultures were then washed with growth medium, growth medium was added, 2 ml per bottle, and the bottles were incubated further at 37°C. The ability of HeLa cells to synthesize protein was determined at intervals throughout the experiment by measuring the incorporation of $C^{14}$-L-leucine into protein after a 30-min pulse. The isotope was diluted in incomplete Eagle's medium to give 0.03 $\mu$c per ml and supplemented with $10^{-4}$ $M$ puromycin when appropriate.

As illustrated in Figure 3, protein synthesis in both NDV-infected and uninfected control cultures was completely inhibited within 45 min after addition of puromycin. Within 45 min of removal of puromycin, cells of both groups had resumed pretreatment levels of protein synthesis. Thereafter, control cultures continued to synthesize protein at an increasing rate commensurate with a dividing population of cells, whereas virus-infected cells underwent a marked inhibition of protein synthesis. The rate of this final inhibition was similar to that found in NDV-infected cells in experiments without puromycin,[4] but the time course was delayed approximately 2 hr, a period equal to the duration of puromycin treatment. Thus, the inhibitory effects of puromycin on protein synthesis in both NDV-infected and uninfected control cultures were rapidly reversible on removal of the drug.

*Resumption of NDV multiplication in HeLa cells on removal of puromycin:* It was next of interest to determine whether the resumption of protein synthesis in NDV-infected cells which follows removal of puromycin from the culture would lead to resumption of virus synthesis.

Leighton tubes which contained coverslip cultures of HeLa cells were inoculated with an NDV/cell multiplicity of 225:1 as described above. Puromycin was added at 1 hr and removed at 3 hr after virus inoculation as in the preceding experiment, and at hourly intervals after virus inoculation coverslips were collected and stained with fluorescent antibody. Controls consisted of infected coverslip cultures incubated throughout the experiment in the absence of puromycin and of

infected cultures incubated in the presence of $10^{-4}$ $M$ puromycin after the first hr.

As described in Table 2, in infected cultures incubated without puromycin, new virus antigen was first detected two hr after infection, and the amount of antigen

TABLE 2

EFFECT OF ADDITION AND REMOVAL OF PUROMYCIN ON NDV MULTIPLICATION IN HELA CELLS

| | Virus antigen — Hr after infection | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| No puromycin | 0 | 0 | + | 2+ | 3+ | 4+ | 4+ | 4+ |
| Puromycin ($10^{-4}$ $M$) added at 1 hr | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Puromycin ($10^{-4}$ $M$) added at 1 hr, removed at 3 hr | 0 | 0 | 0 | 0 | + | 2+ | 3+ | 4+ |

increased until the fifth hour with little antigen produced after this time. Cultures which received puromycin one hr after infection and which were incubated in the presence of the drug thereafter never produced detectable amounts of virus antigen. When puromycin was removed three hr after infection from such cultures, however, virus antigen was detected one hr after removal, and the amount of antigen increased progressively until the seventh hour. Virus antigen synthesis appeared to resume at the point at which puromycin had originally been added, and the rate of synthesis, once begun, was similar to that in infected cells in experiments without puromycin. Thus, the resumption of protein synthesis in virus-infected cells which follows removal of puromycin from the culture medium is associated with a resumption of virus antigen synthesis.

It should be noted here that in these experiments the NDV eclipse period, defined as the interval between infection and first detection of new virus antigen, normally two hr in duration, was prolonged by puromycin treatment to four hr. A similar prolongation of the virus eclipse period, with the endpoint being production of infective nucleic acid, has been found in polio virus-infected HeLa cells;[11] the eclipse was extended by an interval equal to the length of puromycin treatment.

*Prolongation of the NDV eclipse period:* The prolongation of the NDV eclipse period by puromycin focused attention on this aspect of virus multiplication. It was of interest to determine the maximum interval by which the NDV eclipse could be prolonged by puromycin and still permit virus antigen synthesis on removal of the drug from the culture medium. The rapidity of resumption of virus antigen synthesis under these conditions was also studied. In the following experiments, HeLa cells were treated with puromycin for 1 hr before virus inoculation, thus inducing inhibition of cellular protein synthesis at the time of infection. A 10-min adsorption period for the virus inoculum was employed in order to relate the subsequent appearance of virus antigen more precisely to the time of infection.

Coverslip cultures of HeLa cells were placed in Leighton tubes which contained growth medium supplemented with $10^{-4}$ $M$ puromycin, 1.5 ml per tube. After a 1-hr incubation period at 37°C, the cultures were transferred to fresh tubes which contained a 1.5 ml volume of NDV diluted in protein-free Eagle's medium to give a virus/cell multiplicity of 225:1 and supplemented with $10^{-4}M$ puromycin. After a 10-min incubation period at 37°C, the coverslips were washed four times in PBS and once in $10^{-4}M$ puromycin diluted in PBS and returned to the original tubes which contained puromycin in growth medium. At intervals thereafter, groups of

coverslips were washed in PBS and placed in fresh tubes containing growth medium for further incubation. One coverslip in each group was harvested every 15 min after removal of puromycin, fixed, and stained with fluorescent antibody.

As described in Table 3, virus antigen first appeared two hr after infection in nonpuromycin-treated cells and the eclipse period could be extended to 12 hr by treatment with puromycin. Twelve hr was the longest interval studied, for at this time over 50 per cent of the cells had undergone marked degenerative changes due to puromycin. This effect of puromycin on cell structure will be described in detail elsewhere. Thus, HeLa cells retain their capacity to synthesize virus antigen over an extended period of absence of protein synthesis.

TABLE 3

EFFECT OF PUROMYCIN ON THE NDV ECLIPSE PERIOD IN HeLa CELLS

| Duration of puromycin* treatment (hr) | | Hours between virus inoculation and detection of new virus antigen (eclipse period) | Hours of protein synthesis between virus inoculation and detection of new virus antigen |
|---|---|---|---|
| Before virus inoculation | After virus inoculation | | |
| 0 | 0 | 2 | 2 |
| 1 | 1 | 2 | 1 |
| 1 | 2 | $2\frac{3}{4}$ | $\frac{3}{4}$ |
| 1 | 4 | $4\frac{1}{2}$ | $\frac{1}{2}$ |
| 1 | 7 | $7\frac{3}{4}$ | $\frac{3}{4}$ |
| 1 | 11 | 12 | 1 |

* $10^{-4}$ *M*.

Of special interest in these experiments was the demonstration that HeLa cells which had undergone 4 hr of treatment with puromycin following infection, with no protein synthesis during this interval, could, on resumption of protein synthesis, produce detectable amounts of virus antigen in one-half hr. That these puromycin-treated cells can synthesize as much virus antigen in the one-half hr of protein synthesis which follows removal of the drug as can nonpuromycin-treated cells in the 2 hr of protein synthesis which follows virus inoculation clearly indicates that some aspect of the virus eclipse period takes place in the presence of puromycin. Furthermore, the more rapid rate of virus antigen formation which follows resumption of protein synthesis in cells treated for four hr with puromycin as compared with cells treated for one hr after infection, suggests that virus precursors may accumulate in cells during extended periods of puromycin treatment. The slower rate of antigen synthesis in cells following treatment for more than four hr may be attributable to puromycin-induced cellular degenerative changes.

Finally, attention should be directed to the fact that the virus eclipse period in cells treated with puromycin for the first hour following virus inoculation is identical in duration to that in nonpuromycin-treated cells, i.e., two hr. This suggests that the virus synthetic processes which occur in the first hour of the eclipse period are independent of protein synthesis.

*Discussion.*—The inhibitory effects of puromycin on both protein synthesis and NDV multiplication in HeLa cells illustrate the dependence of virus multiplication on protein synthesis mechanisms. Virus antigen synthesis can be arrested at any stage of the replication sequence by inoculating concentrations of puromycin sufficient to inhibit cellular protein synthesis completely. Resumption of both cellular protein and virus antigen synthesis follows removal of the drug from the culture medium. This is of special interest, for amino acid incorporation into *Escherichia*

*coli* cell-free microsomes has been found to be irreversibly inhibited by puromycin.[12]

Puromycin has been shown to interrupt protein synthesis by preventing the transfer of activated amino acids from soluble RNA to ribosomes.[13] More recent studies suggest that puromycin splits off peptides from the ribosome RNA template by substituting itself for cellular aminoacyl-soluble RNA.[14] It is possible that puromycin inhibits NDV antigen synthesis in a similar manner by acting on the virus RNA template. If this is so, it constitutes a useful tool in the study of the mechanisms of virus multiplication.

Puromycin activity can be reversed by a number of compounds such as ribose, certain purines, and 4-amino-5-imidazolecarboxamide.[15] The removal of puromycin from the culture medium in the present experiments, combined with inactivation of intracellular puromycin by one of these agents, probably accounts for the rapid resumption of protein synthesis.

The prolongation of the NDV eclipse period by puromycin may be attributed to a temporary suppression of protein synthesis in the eclipse period. Virus genetic material and processes for virus synthesis are not irreversibly affected by puromycin, for virus multiplication promptly resumes on removal of the drug. It is remarkable that infected cells can resume virus antigen synthesis following 11 hr of puromycin treatment for at this time the cells are themselves undergoing puromycin-induced degenerative changes, probably attributable to the absence of protein synthesis during this interval. That resumption of protein synthesis following removal of the drug from these cultures leads to virus antigen formation rather than to exclusive synthesis of much needed cellular protein attests to the absolute control of virus over cellular protein synthetic mechanisms.

The demonstration that the NDV eclipse period in HeLa cells can be initiated and an early stage completed in the presence of puromycin suggests one of two hypotheses:

(1) Protein synthesis is not required for the initiation and completion of early processes in the virus eclipse period. If this hypothesis is true, the NDV eclipse period can be divided into protein synthesis-independent and dependent periods. The nature of the processes which occur during the first period is unknown. The following, however, can be said. Adsorption of virus to cell, penetration, and mobilization of virus ribonucleic acid must take place before biosynthesis of virus precursors begins. At high NDV/cell multiplicities, adsorption and penetration to an immune serum-resistant stage occur within one min following inoculation.[6] The time required for removal of the virus lipoprotein coat and release of nucleic acid is unknown, but this step could theoretically occupy most or all of the protein synthesis-independent period. Synthesis of virus RNA precursors and RNA would follow. Normal precursors of cellular RNA already present in cells could, of course, be diverted to synthesis of viral RNA. Newcastle disease virus nucleic acid synthesis could theoretically take place in the absence of prior or concomitant protein synthesis, for such a phenomenon is believed to occur in the T1, T3, T7, and 201S bacteriophage-*Escherichia coli* cell systems.[1] On the other hand, studies with poliovirus have shown that virus RNA is synthesized within minutes prior to virus protein synthesis.[16] If NDV is similar to poliovirus in this respect, virus RNA would be synthesized late rather than early in the eclipse period.

(2) The second hypothesis is that puromycin itself produces changes in HeLa cells

which are similar to those which occur in the early stages of the NDV eclipse period. The nature of these changes is unknown at present. Rabinovitz and Fisher,[17] however, have suggested that puromycin causes the continuous premature release of protein from the synthetic site of ribosomes. If NDV multiplies in association with ribosomes, the release of cellular peptides from these synthetic sites might logically constitute the first step in the utilization of cellular biosynthetic sites for virus replication. Thus, puromycin-treated cells may have, in effect, completed an early stage of the virus eclipse period. The precise characterization of the alterations produced in cells by puromycin may therefore increase our understanding of the early mechanisms of virus multiplication.

If the second hypothesis is correct, one could not unconditionally state that there is a protein synthesis-independent period in the NDV eclipse period, for, theoretically at least, virus multiplication could require protein synthesis for the accomplishment of this ribosome stripping effect.

*Summary.*—The role of protein synthesis in the multiplication of Newcastle disease virus (NDV) in HeLa cells was studied. Puromycin dihydrochloride at $10^{-4}$ *M* concentration was found to inhibit completely both cellular protein synthesis and NDV antigen synthesis. On removal of the drug from the culture media, both protein synthesis and virus antigen synthesis promptly resumed.

The NDV eclipse period, defined herein as the interval between infection and the first discernible presence of new virus antigen, normally 2 hr in length, could be prolonged to 12 hr by treatment with puromycin. HeLa cells which had been treated with puromycin for 4 hr following infection could synthesize as much virus antigen in the one-half hour of protein synthesis which followed removal of the drug as could nonpuromycin-treated cells in the 2 hr of protein synthesis which followed virus inoculation. Thus, it appears that some aspect of the virus eclipse period takes place in the presence of puromycin and that virus precursors accumulate in cells during extended periods of puromycin treatment.

Finally, the virus eclipse period in cells treated with puromycin for the first hour following virus inoculation was identical in duration to that in nonpuromycin-treated cells, i.e., 2 hr. This suggests either that the virus synthetic processes which occur in the first hour of the eclipse period are independent of protein synthesis or that puromycin itself produces changes in HeLa cells which are similar to those which occur in the early stages of the NDV eclipse period.

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† Puromycin dihydrochloride was supplied by B. L. Hutchings of Lederle Laboratories, N. Y.

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# *NUCLEAR RNA CHANGES OF NERVE CELLS DURING A LEARNING EXPERIMENT IN RATS*

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As the nerve cells are rich in ribonucleic acid (RNA), the suggestion has been made that the RNA of the neurons may be linked with the capacity of the central nervous system to store information.

The RNA and protein synthesis and the enzymic activities of neurons have been found to be linked with neural function (review, see Hydén[1]). From these results the senior present author has proposed a thought mechanism model for intraneuronal molecular storage of information[1,2] which has been based on the production of specific proteins in each neuron following changes of the RNA base composition after adequate stimulation. The altered RNA base composition, which may persist for a long time, might arise through electrical patterns associated with sensory and motor activity.

Results are presented on the altered base ratio composition of nuclear RNA and cytoplasmic RNA from neurons of rats, subjected to a "trial-and-error" type of learning experiment during which a pattern of sensory and motor abilities was established in the rats. Concomitantly, the adenine/uracil ratio of the nuclear RNA increased significantly, and an increase of the total amount of the nerve cell RNA occurred. Requisite control experiments were carried out which excluded the possibility that the chemical changes observed were due to demands on the neural function *per se*.

The present paper, the first in a series, presents the results obtained from vestibular Deiters' nerve cells. These cells were chosen because they were directly involved in this learning performance, and because we wanted to use neurons from phylogenetically old parts of the brain in these first experiments and to avoid complicated cortical areas.

*Material and Experimental Setup.*—White rats of the Sprague-Dawley strain weighing 150–200 gm were used. A wooden cage (95 × 85 × 45 cm) with one side wall of glass was used for the experiments. At a height of 75 cm on one of the end walls, a small platform was arranged with a feeding cup. A 90-cm long steel wire, 1.5 mm in diameter, was strung between the floor and the platform. The experimental rats, kept on a minimal amount of food but with free access to water, were individually placed in the cage for 45 minutes daily. The only way for the animals to satisfy their food hunger was to learn how to balance up to the platform, an exceedingly difficult