mice. The present study indicates that guinea pig γ_2 antibodies are effective opsonins in promoting immune clearance of *E. coli* bacteria. Finally, it should be noted that reliance on a single indicator of antibody formation, in this instance the PCA reaction, would have led to failure to detect the immune response in guinea pigs immunized with *E. coli* bacteria.

Summary. Guinea pigs hyperimmunized with *E. coli* bacteria emulsified in complete Freund's adjuvant produced large amounts of $7S\gamma_2$ globulins. However, animals immunized by this method, as well as others initially immunized with bacteria administered intraperitoneally or intravenously, failed, with one exception, to produce $7S\gamma_1$ guinea pig skin sensitizing anti-*E. coli* antibodies.

We are indebted to Dr. A. M. Staub, Pasteur Institute, Paris, for a supply of purified *E. coli* 0 111:B4 polysaccharide prepared by alkali hydrolysis and to Dr. H. L. Spiegelberg, Department of Medicine, N. Y. U., for testing the opsonizing activity of guinea pig antibody fractions.

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Intracellular Site of Newcastle Disease Virus Nucleic Acid Synthesis.* (28584)

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Synthesis of Newcastle disease virus (NDV) protein has been demonstrated by immunofluorescence to take place in the cytoplasm of infected cells(1,2). Determination of the intracellular site of NDV nucleic acid synthesis, however, has been hindered by both failure to isolate infective NDV-RNA from cells and inability to differentiate synthesis of viral RNA from cellular RNA. An approach to the localization of NDV-RNA synthesis was suggested recently by the demonstration that several RNA-containing viruses, including NDV, can multiply to high titer in cells in which cellular DNA-dependent RNA synthesis has been inhibited by actinomycin D(3,4,5), and that NDV-RNA can be isotopically labeled in such cells(6).

In the experiments reported herein, actinomycin D† was used to differentiate synthesis

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† Actinomycin D was supplied by Dr. Karl Folkers, Merck Sharp and Dohme Research Laboratories.

of NDV-RNA from cellular RNA and the intracellular site of viral RNA synthesis determined by the autoradiographic technique.

Materials and methods. Many of the materials and experimental procedures used have been reported(7,8,9,10), and are in part described below, with modifications and additions.

Cell culture. In brief, the cell culture system employed in experiments designed to measure virus multiplication and incorporation of C^{14}-uridine into RNA consisted of HeLa cells grown in S-bottles with a flat culture area of 2000 mm^2. Complete monolayers were used in these bottles and each consisted of approximately 2×10^6 cells. In autoradiographic experiments HeLa cells were grown on 11×38 mm^2 coverslips in Leighton tubes. Each coverslip culture was composed of an incomplete monolayer of approximately 200,000 cells.

Virus. The allantoic fluid seed of NDV (Hickman strain) contained 3.2×10^9 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 200/1.

Virus assay. Infected cells were disrupted by freezing and thawing 2 ×, followed by sonic vibration for 2 minutes in a Raytheon 10 KC magnetostrictive oscillator at 9000 cycles per second. Infective NDV was assayed by the fluorescent cell-counting technique(7) and infective virus enumerated in terms of cell-infecting units (CIU).

Isotope methods. In autoradiographic experiments H^3-uridine with a specific activity of 3.28 c/mM was used. Following exposure to H^3-uridine, the cultures were fixed in acetic acid-alcohol in proportions of 1:3 for ½ hour and then washed in McIlvaine's buffer pH 3 for ½ hour and stripping film applied according to the method of Doniach and Pelc (11).

In scintillation counting experiments uridine-2-C^{14} with a specific activity of 5.7 μc/mg was used. Following exposure to the isotope the cultures were processed for counting according to methods described previously (10). In all isotope experiments the medium was supplemented with either unlabeled thymidine or 5-fluoro-2 deoxyuridine to inhibit incorporation of labeled uridine into DNA.

The effect of actinomycin D on NDV multiplication was studied by measuring production of infective virus in HeLa cells which had been treated with actinomycin D at a concentration sufficient to inhibit completely cellular RNA synthesis.

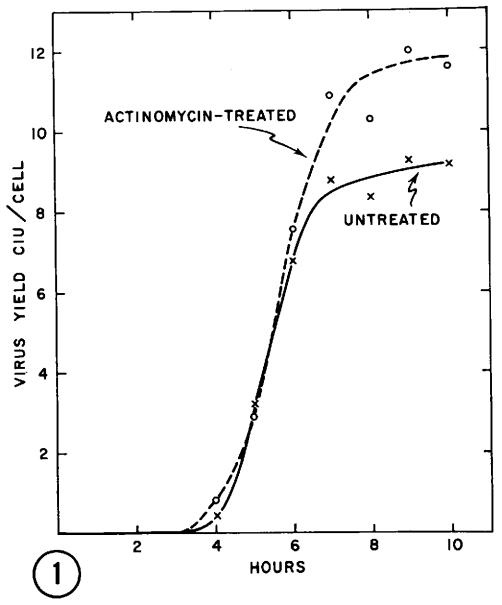
Bottle cultures containing complete monolayers of HeLa cells were treated with actinomycin D, 2 μg/ml, for one hour prior to virus inoculation. NDV at a virus/cell multiplicity of 200/1 was then inoculated together with actinomycin. After a 1-hour adsorption period, the cultures were washed 4 times with PBS, and growth medium which contained actinomycin was added. Two bottle cultures were removed at hourly intervals thereafter. Infected controls were treated similarly except that no actinomycin was used. The yield of infective virus was measured by the fluorescent-cell counting technique.

NDV production began in HeLa cells 4-5 hours after infection and maximum titers were reached at 6-7 hours (Fig. 1). The same time course of virus replication was observed in actinomycin-treated cells except that slightly higher titers of virus were produced.

Isotope labeling of NDV nucleic acid. The ability to label NDV-RNA with isotope was next studied by measuring the incorporation of C^{14}-uridine into RNA of actinomycin D-treated NDV-infected HeLa cells.

Bottle cultures which contained complete monolayers of HeLa cells were treated with actinomycin, 2 μg/ml, for 1 hour. NDV at a virus/cell multiplicity of 200/1 was then inoculated together with C^{14}-uridine diluted in growth medium to give .04 μc/ml. The medium was supplemented with actinomycin, 2 μg/ml, and thymidine, 0.2 mg/ml. At hourly intervals 4 bottles were collected and processed for scintillation counting of C^{14}-uridine incorporation into RNA. Controls were treated similarly except that no virus was used.

Actinomycin D at 2 μg/ml completely inhibited RNA synthesis in noninfected cells (Fig. 2). In NDV-infected cultures, however, incorporation of C^{14}-uridine into RNA



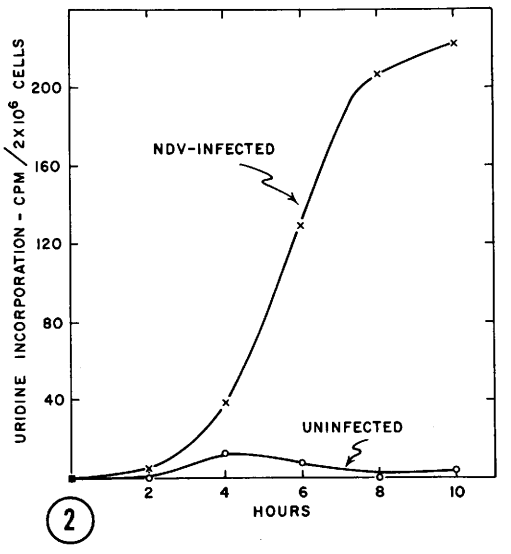


FIG. 1. Effect of actinomycin D 2 μg/ml on NDV multiplication in HeLa cells.

FIG. 2. Incorporation of C^{14}-uridine into RNA of actinomycin D-treated NDV-infected and uninfected HeLa cells.

began at 3-4 hours after infection and increased exponentially until the eighth hour. The time course of RNA synthesis in virus-infected cells is closely related to that of production of infective virus and is therefore evidence of the viral nature of this RNA synthesis although the possibility exists that a portion of the RNA synthesized is non-viral or viral but nonfunctional. These results furthermore suggest that NDV nucleic acid is synthesized just prior to its incorporation into infective virus, and in this respect is similar to poliovirus(12).

Localization of the intracellular site of NDV nucleic acid synthesis. The above demonstration that measurable amounts of RNA are synthesized and can be isotopically labeled in actinomycin D-treated NDV-infected HeLa cells provided a method to determine the intracellular site of NDV nucleic acid synthesis by autoradiography.

Coverslip cultures containing incomplete monolayers of HeLa cells were grown in Leighton tubes and treated for one hour with actinomycin, 2 μg/ml. NDV was then inoculated at a multiplicity of 200/1 together with H^3-uridine diluted in growth medium to give a specific activity of .04 μc/ml. The medium was supplemented with actinomycin, 2 μg/ml, and 5-fluoro-2 deoxyuridine at 10^{-6} M concentration. FUDR has been shown not to interfere with NDV multiplication(13). Noninfected controls were treated similarly except that no virus was used. At hourly intervals coverslips were removed and processed for autoradiography.

Incorporation of H^3-uridine into RNA in actinomycin D-treated NDV-infected cells began 2-3 hours after infection and the grains appeared predominantly over the cytoplasm (Fig. 3). Those few grains which are seen over the nucleus may be the result of either isotope scatter or the small amounts of cytoplasm which normally overlie the nucleus. At 4, 5, and 6 hours, progressively larger numbers of grains appeared diffusely distributed over the cytoplasm. All grains could be removed by treatment of the coverslip cultures with RNAase prior to application to stripping film. No grains appeared over actinomycin-treated noninfected cells.

Discussion. The autoradiograph experiments described above demonstrate that NDV-ribonucleic acid synthesis takes place in the cytoplasm of infected HeLa cells. It should be emphasized that not all grains observed in these autoradiographs necessarily emanate from viral RNA and some may reflect nonviral RNA synthesis. However, since

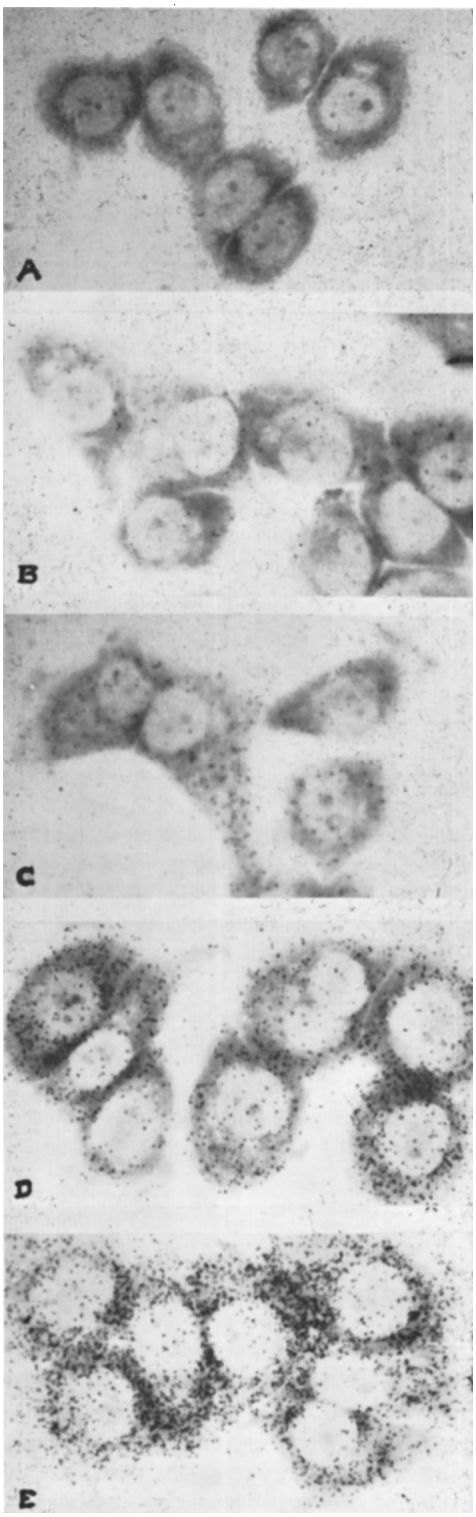


FIG. 3. Radioautographs of actinomycin D-treated NDV-infected HeLa cells in which H^3-uridine has been incorporated into RNA: A, 2 hr following infection; B, 3 hr following infection; C, 4 hr following infection; D, 5 hr following infection; E, 6 hr following infection.

all grains throughout the infectious cycle appear over the cytoplasm, that percentage of new RNA which is viral must therefore be synthesized in the cytoplasm. Synthesis of NDV protein also takes place in the cytoplasm as determined by the fluorescent antibody technique. The first appearance of new NDV protein in these cells, however, is at 2 hours after infection, at a time when no detectable amount of viral RNA has been synthesized. Since new viral RNA must direct synthesis of fluorescent-stainable virus protein, one must conclude that the methods described herein are not sufficiently sensitive to detect early synthesis of viral RNA.

It should be noted in the experiments described herein that although large amounts of H^3- and C^{14}-uridine are incorporated into NDV-RNA in actinomycin D-treated infected cells, only 8-12 infective virus particles per cell can be extracted and measured. This marked discrepancy between production of large amounts of virus RNA and small yields of infective virus exists also with NDV protein, where more virus protein is synthesized than is incorporated into infective virus particles(8). The possibility exists, however, that much of the viral RNA synthesized in HeLa cells may not be functional. In support of this hypothesis is the demonstration that protein synthesis in NDV-infected HeLa cells is completely inhibited by the sixth hour after infection(9). The NDV-RNA synthesized thereafter may be unstable and nonfunctional, as is the case in bacteria treated with chloramphenicol where all RNA synthesized after cessation of protein synthesis is defective and nonfunctional (14). The labeling of NDV-RNA with C^{14} now permits a qualitative comparison between viral RNA synthesized both before and after cessation of protein synthesis. On the other hand, it is entirely possible that some of the RNA synthesized in infected cells may be nonviral, and in this respect, NDV may be similar to poliovirus(4). The

final explanation for low yields of virus in comparison to large production of virus precursors may be that a major percentage of virus is inactivated or lost in the extraction procedures.

Of special interest in the experiments described above is the enhancement of NDV replication in HeLa cells treated with actinomycin D. Barry *et al.*(5) reported that NDV replicates to normal titers in actinomycin D-treated chorioallantoic membranes whereas Kingsbury(6) and Granoff (personal communication) observed an inhibition of NDV replication in treated chick fibroblasts. These results are in contrast with those reported here but this discrepancy may be accounted for on the basis of differences in either cell type or virus strain. The slight enhancement of NDV replication in actinomycin D-treated HeLa cells, however, indicates that the sequential events in the replication of this virus can take place independently of DNA synthesis. A reasonable explanation for this enhancement may be that cellular RNA normally competes with viral RNA for biosynthetic sites in the cytoplasm. Cessation of cellular RNA synthesis by actinomycin may remove this competition and permit viral RNA to replicate and direct synthesis of viral protein more efficiently.

Summary. Under conditions in which cellular RNA synthesis was inhibited by actinomycin D, incorporation of C^{14}-uridine took place into RNA of NDV-infected cells and was measured by scintillation counting. In autoradiograph experiments employing H^3-uridine, synthesis of NDV-RNA was demonstrated to take place in the cytoplasm of infected cells.

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Bromination of Phthalein Dyes by the Uterus of the Dogfish, *Squalus acanthias.* (28585)

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The natural occurrence of bromine compounds in marine animals such as the shellfish *Murex brandaris* and *M. tranculus*(1), and the coral fan *Gorgonia verrucosa*(2) indicates that enzymatic bromination may be of importance in their biogenesis. Previously we showed that the uterus of the pregnant spiny dogfish, *Squalus acanthias*, converted phenol red into bromphenol blue after intrauterine administration of the dye(3). Bromination of phenol red was also observed by an enzyme system isolated from the mold *Cal-*