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Viral Inhibition of Mammalian Cell DNA Synthesis

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VIRAL INHIBITION OF MAMMALIAN CELL DNA SYNTHESIS

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by

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Preface

Eukaryotic cells replicate their DNA during a defined time period in the interphase between mitoses. Non-proliferating cells are generally found to be blocked in interphase prior to the DNA-synthetic period. It therefore appears that the control of cellular proliferation may be intimately related to regulation of DNA synthesis. Defining the mechanism for the regulation of DNA replication is a central problem in the biology of higher animal cells. The introductory section of this thesis attempts to bring together the currently available evidence into a coherent model for *in vivo* regulated DNA replication.

Animal viruses can be used as tools to study cellular DNA replication. Certain RNA viruses which multiply in the cytoplasm of host cells inhibit cellular DNA synthesis. The work described in this thesis represents an effort to locate the step in cellular DNA synthesis which is primarily affected in consequence of the multiplication of three cytoplasmic RNA viruses, mengovirus, Newcastle disease virus, and reovirus type 3. The general method of analysis used and the results obtained are discussed within the framework of the model presented in the Introduction.

SUMMARY

Previous studies have shown that the replication of DNA is a regulated process within the life cycle of the cell. DNA replication generally requires pre-existing DNA, the four deoxyribonucleotide triphosphate precursors, DNA polymerase, and polynucleotide ligase. Moreover, evidence from bacterial and mammalian systems suggests that *in vivo* DNA replication involves another factor which is necessary for the initiation of DNA synthesis. A model has been constructed which relates the replication sections of mammalian chromosomes to the bacterial chromosome. Using this model, a hypothesis is developed to explain the requirement for protein synthesis in the replication of DNA *in vivo* under conditions where all currently measurable factors involved in DNA synthesis are adequately represented. It is proposed that the initiation of DNA synthesis on individual replication sections may stoichiometrically require an initiator protein synthesized in the cytoplasm.

Animal virus infection can lead to an inhibition of cellular DNA synthesis. According to the replication scheme above, this inhibition could be effected in any of three ways: 1) degradation of cellular template DNA, 2) interference with the synthesis or polymerization of precursor deoxyribonucleotides, or 3) interference with the regulatory system, particularly initiation. Using this outline, an attempt has been made to locate the step in cellular DNA replication which is primarily affected during the multiplication of three cytoplasmic RNA viruses, mengovirus, Newcastle disease virus, and reovirus type 3. All studies were performed in the L line of mouse fibroblasts.

Mengovirus and Newcastle disease virus (NDV) infection leads to inhibition of L cell DNA synthesis concurrently with inhibition of cellular protein synthesis; both inhibitions become evident about 2 hours after infection. Reovirus inhibits cellular DNA synthesis in the absence of a detectable effect on protein synthesis.

Rate zonal sucrose density gradient analysis was performed on phenol-extracted double-stranded DNA and on alkali-denatured single-stranded DNA from cells infected with mengovirus, NDV, and reovirus. In no case was there detectable breakdown in the high molecular weight cellular DNA (100-200 million daltons) even late in infection when cellular DNA synthesis was 80% inhibited.

There is no apparent change in the enzymatic capability of infected cells to synthesize or polymerize deoxyribonucleotides as measured by *in vitro* enzyme assays. DNA polymerase, dCMP-deaminase, TdR-kinase, TMP-kinase, and TDP-kinase activities are unchanged by mengovirus, Newcastle disease virus, and reovirus infection.

Measurements were made of the growth of daughter DNA chains *in vivo* using rate zonal gradient analysis of pulse-labeled DNA chains after alkaline denaturation of total DNA into single strands. Pulse-labeled daughter chains were found to undergo a progressive 100-fold increase in size during a chase of several hours. This chain growth was shown to depend upon polymerization of precursor nucleotides. Thus it can be assumed that precursor depletion or polymerase inactivation during viral inhibition would be revealed in a decreased rate of chain elongation. However, a normal rate of elongation is maintained during the inhibition of DNA synthesis in mengovirus, Newcastle disease virus, and reovirus infections. This finding is in agreement with the results of measurements of the activities of selected enzymes essential to DNA synthesis.

The evidence suggests that virus-induced inhibition of DNA synthesis reflects a reduction in the number of active replication sections in infected cells as compared to uninfected control cells. This in turn makes it highly likely that the initiation process is primarily affected during each of the three virus infections herein investigated.

DNA synthesis and protein synthesis are inhibited concurrently during mengovirus and Newcastle disease virus infection. Comparative studies with chemical inhibitors of protein synthesis show that the maintenance of DNA synthesis requires continual protein synthesis. Gradient analysis of DNA chain growth after chemically-induced inhibition of protein synthesis demonstrates that daughter chain elongation *in vivo* does not require concurrent protein synthesis.

These findings, considered in conjunction with the model for *in vivo* regulation of DNA synthesis, suggest that the inhibition of cellular DNA synthesis in mengovirus and Newcastle disease virus infection may result from inhibition of initiator protein synthesis. Reovirus infection may lead to a selective inhibition of initiator protein synthesis without detectable effect on overall protein synthesis, or it may in some way interfere with the normal transport of initiator protein from cytoplasm to nucleus.

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Abbreviations

ATP - adenosine 5'-triphosphate

dAMP - deoxyadenosine 5'-monophosphate

dATP - deoxyadenosine 5'-triphosphate

dCMP - deoxycytidine 5'-monophosphate

dCTP - deoxycytidine 5'-triphosphate

dGMP - deoxyguanosine 5'-monophosphate

dGTP - deoxyguanosine 5'-triphosphate

DNA - deoxyribonucleic acid

DNAase - deoxyribonuclease

dUMP - deoxyuridine 5'-monophosphate

EDTA - ethylenediamine tetracetic acid, sodium salt (versene)

FPA - p-fluorophenylalanine

FUdR - 5-fluoro-2'-deoxyuridine

RNA - ribonucleic acid

RNAase - ribonuclease

TCA - trichloroacetic acid

TDP - thymidine 5'-diphosphate

TdR - thymidine (always the deoxyribonucleoside)

TTP - thymidine 5'-triphosphate

Tris - tris(hydroxymethyl)aminomethane

****

c - curie, 2.2×10^{12} radioactive disintegrations per minute

cpm - counts per minute

G1 - interphase gap between mitotic and DNA synthetic phases of the cell cycle

G2 - interphase gap between DNA synthetic and mitotic phases of the cell cycle

in vitro - usually refers to a system of fractions derived from living cells

in vivo - in living cells

M - mitotic phase of the cell cycle

PFU - plaque forming units

p.i. - post-infection

S - DNA synthetic phase of the cell cycle

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I. INTRODUCTION

A. The Enzymatic Mechanism of DNA Replication

Considerable experimental evidence bearing on the enzymatic mechanism of DNA replication has accumulated during the past decade. The enzymes of polymerization, the DNA polymerases, appear to function in essentially the same manner whether they originate from bacterial or animal sources (Mitra and Kornberg, 1966). Although our primary interest concerns DNA synthesis in animal cells, evidence obtained from bacterial systems will frequently be utilized in outlining the enzymatic mechanism of DNA replication.

DNA polymerase activity has been recognized in extracts from thymus (Smith and Keir, 1963; Bollum, 1959), kidney (Ledinko, 1968; Kit et al., 1969), liver (Keir et al., 1962), and other tissues (Smellie, 1961). Polymerase activity has also been demonstrated in extracts from mouse (Littlefield et al., 1963), hamster (Keir et al., 1966), and human (Jungwirth and Joklik, 1965; Hopper et al., 1966) cell lines grown in continuous culture. DNA polymerase has been purified 15-fold from a continuous line of mouse cells (Gold and Helleiner, 1964), 100-fold from calf thymus (Bollum, 1966), and 75-fold from rat liver (Meyer and Simpson, 1968).

The DNA polymerase level in a cell-free extract tends to reflect the proliferative activity of the source material. Specific activities per mg. protein are 7- to 10-fold higher in crude extracts from regenerating rat liver than in extracts from normal rat liver (Bollum and Potter, 1959; Smellie, 1961). When stationary phase cells in the rabbit kidney cortex are excised and cultured in growth medium, DNA polymerase activity increases 4- to 6-fold coincident with onset of DNA synthesis (Lieberman et al., 1963).

In vitro studies have attempted to define the enzymatic mechanism whereby DNA polymerase replicates DNA. Studies of bacterial (Lehman et al., 1958; Richardson et al., 1963; Mitra and Kornberg, 1966; Kornberg, 1969) and mammalian (Bollum, 1960, 1963) DNA polymerase show that the enzyme catalyzes polymerization in the 5' to 3' direction. The 3'-(deoxyribose) hydroxyl on the growing end of a

template-bound, daughter DNA strand makes a nucleophilic attack on the α-nucleotidyl phosphorus of an incoming deoxyribonucleoside 5'-triphosphate, and inorganic pyrophosphate is eliminated. There is an absolute requirement for the parental template strand of DNA; monomer nucleotides are incorporated into the daughter strand as determined by Watson-Crick base pairing. The replication fidelity of this biosynthetic mechanism has been demonstrated by *in vitro* production of new, infectious DNA using single-stranded ϕX174 phage DNA and a defined DNA polymerase system from bacterial cells (Goulian and Kornberg, 1967; Goulian *et al.*, 1967).

Since DNA polymerase catalyzes chain growth in a 5' to 3' direction (Mitra and Kornberg, 1966), simultaneous replication of the antiparallel parental strands of native DNA should involve growth of the two daughter chains in opposing directions. However, autoradiographic studies of the *in vivo* duplication of *E. coli* chromosomal DNA (Cairns, 1963) and Chinese hamster cell chromosomal DNA (Huberman and Riggs, 1968) indicate growth of both daughter chains in the same direction. Genetic evidence, to be discussed later, supports the autoradiographic analysis of *E. coli* chromosomal replication. Both daughter strands, in bacterial and mammalian DNA, appear to be synthesized simultaneously, one strand growing in the 5' to 3' direction and the other seemingly in the 3' to 5' direction. Hence, there seems to be a contradiction between the enzymatic capabilities of DNA polymerase and *in vivo* DNA synthesis. This dilemma can be resolved in a manner suggested by Okazaki and coworkers (Okazaki *et al.*, 1968). The autoradiographic, macroscopic picture (Figure 1, left side) implies a 5' to 3' growth of one daughter chain at the growing fork, and a 3' to 5' growth of the other. The Okazaki model (Figure 1, right side) suggests that DNA polymerase replicates DNA in a 5' to 3' direction, producing short chains of "nascent" daughter DNA in the region of the growing fork. These newly synthesized short chains are then rapidly joined to the two longer daughter chains by a different enzyme, a ligase. Attachment of short chains to longer daughter chains would not be discernible by autoradiography, as it occurs below the level of autoradiographic resolution.

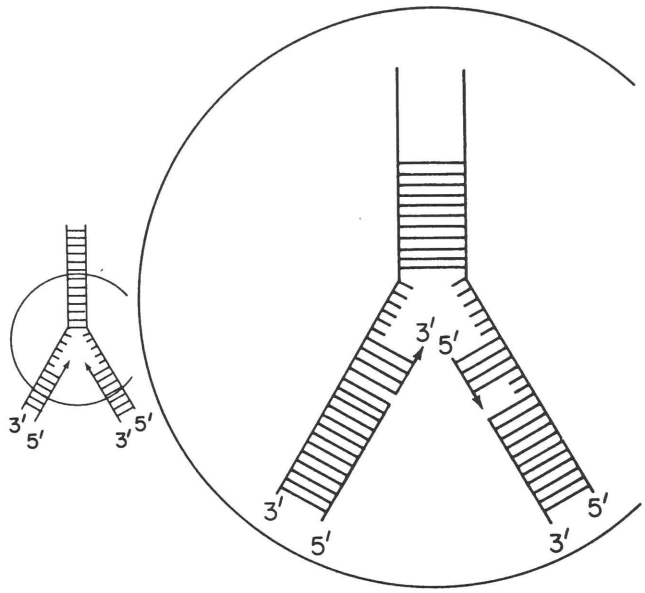


Figure 1. Scheme for discontinuous synthesis of high molecular weight DNA.

The Okazaki model predicts that it should be possible to isolate small pieces of newly synthesized DNA after denaturation of total DNA to single strands. Radioactively labeled precursor nucleotides should be first incorporated into nascent short segments. These segments should then be rapidly integrated into the longer daughter chains. Examination of E. coli DNA in pulse-chase experiments revealed daughter DNA chains possessing these properties (Okazaki et al., 1968). Further studies using T4 phage mutants have provided additional support for the Okazaki model (Newman and Hanawalt, 1968; Hosoda and Mathews, 1968; Masamune and Richardson, 1968; Sugimoto et al., 1968).

Recent reports indicate that short, nascent DNA segments are intermediates in DNA synthesis in higher cells also. With Chinese hamster cells a brief pulse of radioactive thymidine labels pieces of DNA sedimenting at less than 10 S (Taylor et al., 1968; Schandl and Taylor, 1969). These short pieces are integrated into much larger 70 S pieces during a chase of several hours with unlabeled thymidine. Similar results have been obtained with Hela cells (Painter, 1968) and rat liver cells (Tsukada et al., 1968).

The joining of smaller daughter chains into larger chains may be effected by a polynucleotide ligase. Such ligase enzymes have been purified from bacterial (Zimmerman et al., 1967; Weiss and Richardson, 1967; Weiss et al., 1968) and mammalian (Lindahl and Edelman, 1968) sources. Purified polynucleotide ligases catalyze the formation of a phosphodiester bond between adjacent (nascent-daughter) DNA chains when such chains are bound to a complementary (parental) strand of DNA. Some polynucleotide ligases use the energy of ATP and others the energy of nicotinamide adenine dinucleotide to form the phosphodiester linkage which joins the adjacent DNA chains.

To summarize, current evidence supports the Okazaki model. Short pieces of newly synthesized DNA are rapidly attached to larger daughter chains. This joining appears to be effected by polynucleotide ligases. Thus, normal in vivo DNA replication may require the presence, in close proximity, of both DNA polymerase and polynucleotide ligase.

Finally, it should be pointed out that *in vivo* DNA replication is a regulated event occurring under cellular control. In the next section we will discuss what is known about controlled DNA replication, especially as applicable to nucleated, eukaryotic cells.

B. Cellular Control of DNA Synthesis

In higher, eukaryotic cells, chromosomal DNA is duplicated in interphase before cell division (Howard and Pelc, 1953). DNA replication is temporally constrained to a period of synthesis, S, located in interphase. Cellular proliferation thus involves DNA synthesis followed by division, DNA synthesis, division, etc. When conjoined with the intermediate time gaps, this pattern describes a cyclic sequence known as the cell cycle (Fig. 2). Cell cycle studies can be readily carried out with continuous cell lines (Puck, 1964; Puck and Steffen, 1963; Puck *et al.*, 1964; Clever, 1967). G1, S, G2 and M phases vary in duration from cell type to cell type, but are constant for any one cell type under specified growth conditions (Clever, 1967). Figure 2 depicts the phase durations for the L cell (Stanners and Till, 1960; Clever, 1967).

Finer levels of control are apparent within the synthetic period, S. Autoradiographic analysis reveals multiple foci of independent syntheses on each chromosome (Taylor, 1960). Activation of these sites occurs in a defined temporal sequence within S phase (Hsu, 1964). Temporal control over activation operates on a subchromosomal level. Translocation of a segment of an early replicating, autosomal chromosome to a late replicating, sex chromosome does not change the early replication pattern of the translocated segment (Stubblefield, 1966). Finally, it has been demonstrated biochemically that DNA replicated early (or late) in one S phase is replicated early (or late) in the next S phase (Mueller and Kajiwara, 1966).

By what mechanism is the enzymatic machinery of DNA replication so precisely regulated? Available evidence suggests that the cytoplasm plays a role. Nuclear transplantation and cell fusion studies, as well as *in vitro* experiments with cell fractions, demonstrate the presence of cytoplasmic regulatory factors.

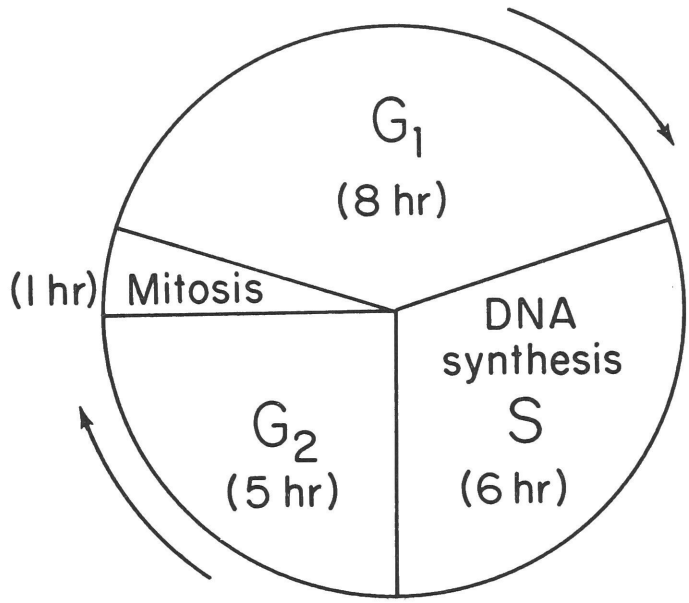


Figure 2. The cell cycle.

1. Evidence for Cytoplasmic Control

Prescott and Goldstein (1967) have examined the regulation of DNA synthesis using nuclear transplantations among amoebae. DNA synthesis can be induced in a G2-phase nucleus by transplanting it into an S-phase cell. Conversely, DNA synthesis in an S-phase nucleus ceases upon transplantation into a G2-phase cell. Control experiments show that transplantation of S-phase nuclei into S-phase cells does not inhibit nuclear DNA synthesis; and, transplantation of G2-phase nuclei into G2-phase cells does not initiate nuclear DNA synthesis.

Transplantation experiments utilizing the large ciliate, Stentor coeruleus, have yielded similar findings (de Terra, 1967). Transplantation of G1-phase nuclei into S-phase cells initiates DNA production in the transplanted nuclei. Synthesis ceases in S-phase nuclei upon transplantation into G1-phase cells. Control experiments show that transplantation of G1-phase nuclei into G1-phase cells does not initiate synthesis, and, that transplantation of S-phase nuclei into S-phase cells does not inhibit ongoing synthesis.

In summary, transplantation experiments suggest that DNA synthesis is controlled by the presence of factors residing in the cytoplasm of cells. DNA synthesis could be positively controlled by a cytoplasmic stimulatory factor present in S-phase, but not in G1- or G2-phase cells. Alternatively, DNA synthesis could be negatively controlled by the presence of a cytoplasmic inhibitory factor present in G1- and G2-, but not in S-phase cells. Additional findings tend to support the positive control hypothesis and rule out inhibitory cytoplasmic substances. When S-phase cells of stentor are grafted to G1-phase cells of equal size, DNA synthesis is initiated in the nucleus of the G1-phase cell, not inhibited in the nucleus of the S-phase cell (de Terra, 1967). Similarly, fusion of animal cells active in DNA synthesis with inactive cells can stimulate the inactive cells to undergo synthesis (Harris, 1965). Hen erythrocytes, rat lymphocytes, and rabbit macrophages do not replicate DNA when cultured separately or when fused with themselves. When these cells are fused

with HeLa cells, the presence of HeLa cell cytoplasm can initiate DNA synthesis in their inactive nuclei. Since DNA synthesis in HeLa cell nuclei is not inhibited in heterokaryons, the presence of an inhibitor in the cytoplasm of inactive cells is unlikely. Thus it appears that nuclear DNA synthesis can be initiated in the nuclei of normally inactive cells by admixture with the cytoplasm of proliferating cells.

When supplied with deoxyribonucleotide triphosphates, isolated nuclei can produce small amounts of DNA without the addition of exogenous, denatured, template DNA. Under these conditions, hen erythrocyte nuclei do not make much DNA (Thompson and McCarthy, 1968). Addition of cytoplasm from rapidly proliferating mouse L cells causes a 25-fold increase in synthetic rate. Cytoplasm from normal, stationary phase mouse liver does not stimulate. The cytoplasmic factor causing this increase is not DNA polymerase.

In conclusion, several lines of evidence point to a positive control over DNA synthesis. The cytoplasm seems to contain factors which are necessary, and perhaps sufficient, to cause initiation of nuclear DNA synthesis at appropriate times in the cell cycle. It is appropriate then to consider in more detail a regulatory mechanism which uses positive effectors or initiators.

2. Control in Bacteria

In 1963, Jacob and coworkers proposed a model for the control of DNA synthesis in bacterial systems. They concluded that the evidence then available indicated the necessity for definite units of DNA replication; units over which positive control was exerted by the presence or absence of a diffusible initiator substance. In their model the unit of replication, the replicon, carries two specific determinants. One determinant is a structural gene controlling the synthesis of a specific initiator. A second determinant is a specific DNA site, a replicator, with which the initiator interacts to instigate replication of DNA.

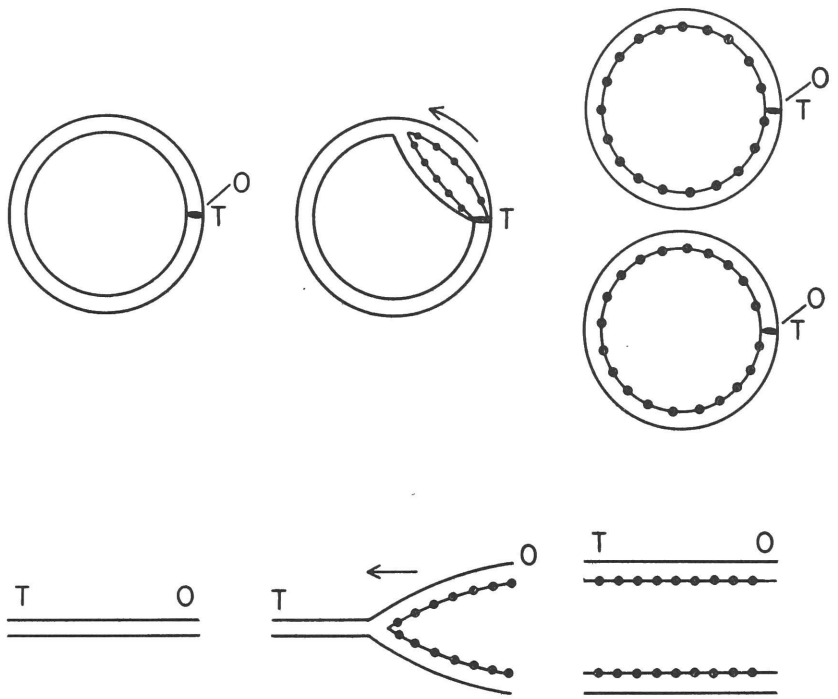
Autoradiographic and genetic evidence indicates that the bacterial chromosome replicates as a single unit. The elegant

autoradiographic studies of Cairns (1963) demonstrate that the E. coli chromosome consists of a single, circular piece of double-stranded DNA. Chromosome replication involves the progressive, sequential duplication of the circular DNA molecule at a single growing fork as depicted in Figure 3(a). Genetic analysis of the bacterial chromosome indicates that replication occurs sequentially from a definite point of origin to a point of termination. The duplication of an integrated E. coli prophage takes place abruptly at a definite time in the replication cycle of cell DNA (Nagata, 1963). This time relates directly to where the prophage is integrated into the circular genetic map of the bacterium.

Another genetic approach using B. subtilis confirms this result (Yoshikawa and Sueoka, 1963). Frequencies of various genetic markers were compared in the exponential and in the stationary phases of growth using a transformation assay. The frequency of each marker in exponential growth was directly correlated with its position on the chromosomal genetic map. Markers nearer the point of origin of chromosome replication were present at higher frequencies than markers located toward the point of termination of replication.

There is evidence that replication of the bacterial chromosome requires the presence of an initiator, a special protein. Maaløe and Hanawalt conducted experiments utilizing a mutant of E. coli that required thymine, arginine, and uracil (Maaløe and Hanawalt, 1961; Hanawalt et al., 1961). They concluded that the need for protein and/or RNA synthesis was only expressed at a particular stage in chromosome replication. Protein and/or RNA synthesis was necessary to initiate, but not to sustain, ongoing chromosomal replication. More recently, it has been shown that amino acid deprivation, in particular, causes replication to stop at a particular point on the bacterial chromosome (Lark et al., 1963).

Treatment of E. coli with phenethyl alcohol can selectively stop DNA synthesis without affecting overall RNA and protein syntheses (Lark and Lark, 1966). Chromosomal duplication continues until a particular region of the chromosome is reached. Replication ceases



(O) origin (site of initiation)

(T) terminus

Figure 3a. Replication of the E. coli chromosome in the normal circular form and in a hypothetical linear form.

at the same region at which it ceases when protein synthesis is inhibited. The location of this region is an inherited property and can be considered as the initiation point (origin) for chromosomal duplication. Results of phenethyl alcohol studies indicate that two kinds of proteins are involved in the initiation process. The synthesis of one kind is blocked by chloramphenicol, but not by phenethyl alcohol. This protein accumulates in cells treated with phenethyl alcohol and supplied with amino acids to support protein synthesis. After removal of phenethyl alcohol, this protein manifests itself by allowing several cycles of replication to occur in the presence of otherwise inhibitory concentrations of chloramphenicol. The second kind of protein involved in initiation does not accumulate in the presence of phenethyl alcohol since amino acids must be present for DNA synthesis to begin after removal of phenethyl alcohol. The synthesis of this second protein appears to be sensitive to phenethyl alcohol, but resistant to chloramphenicol treatment.

We can draw the following conclusions concerning the replication of the bacterial chromosome:

a. The bacterial chromosome replicates as a unit.

b. Replication is sequential, starting from a particular region of the chromosome, the origin.

c. Initiation of replication requires the synthesis and presence of initiator protein.

3. Model for Control in Mammalian Cells

Autoradiographic studies on animal cell chromosomes demonstrate that DNA duplication involves the regulated replication of units much smaller than complete chromosomes. What are these control units like, and what properties do they have in common with the bacterial chromosome?

Studies of structural units of mammalian DNA replication can be performed on pulse-labeled DNA isolated for autoradiography. The chromosomal DNA of Chinese hamster cells is found to be replicated in many tandemly-joined sections (Huberman and Riggs, 1968). Within each

active "replication section", two growing forks diverge from a single median point of origin and move toward the two termini of the section. The independently replicated sections have average lengths of 30 μ (60 x 10^6 daltons), but lengths may range up to 100 μ. A recent study on the phenol extractability of units of newly synthesized DNA tends to confirm this size estimate based on autoradiography (Friedman and Mueller, 1969). An average mammalian cell nucleus contains 3 x 10^6 μ of DNA (DuPraw, 1968). Therefore, the chromosomal DNA complement of a single cell would be equivalent to ca. 100,000 replication sections.

HeLa cell DNA is also replicated in sections on long, continuous DNA strands (Cairns, 1966). A consideration of the time required for in vivo chromosome replication with the linear rate of DNA replication leads to this implication: the mammalian chromosome, of necessity, averages about 100 simultaneously active replication sections throughout S phase. More indirect, double-label, density gradient studies confirm the autoradiographic findings (Painter et al., 1966; Taylor, 1968a; Taylor and Miner, 1968). From these indirect approaches one can conclude that, on the average, 25-250 simultaneously active replication sections must exist per chromosome during S phase.

Figure 3(c) diagrammatically shows the progressive activation of a small number of replication sections during the replication of a region of chromosomal DNA. As shown in Figure 3(b), a replication section can be visualized to be the result of an origin to origin fusion of two small "bacterial-like" chromosomes. The product of such a fusion would be replicated by two growing forks diverging from a common origin and would present an autoradiographic picture similar to that of a single replication section.

Enzymatic digestion studies and patterns of chromosome breakage and segregation are most easily explained if the backbone of the chromatin fiber, and in turn of the chromosome, is taken to be a very long piece of DNA (MacGregor and Callan, 1962; Sasaki and Norman, 1966; DuPraw, 1965; Taylor, 1963; Huberman and Riggs, 1966; Terasima and Tsuboi, 1969; Taylor et al., 1957). If such is the case, then

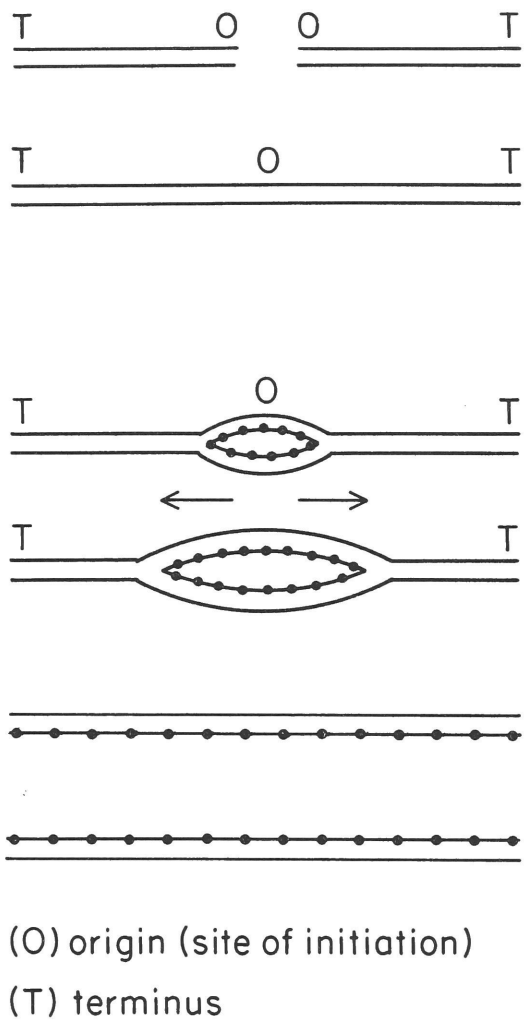


Figure 3b. Model for the formation of a replication section from two linear chromosomes fused origin to origin.

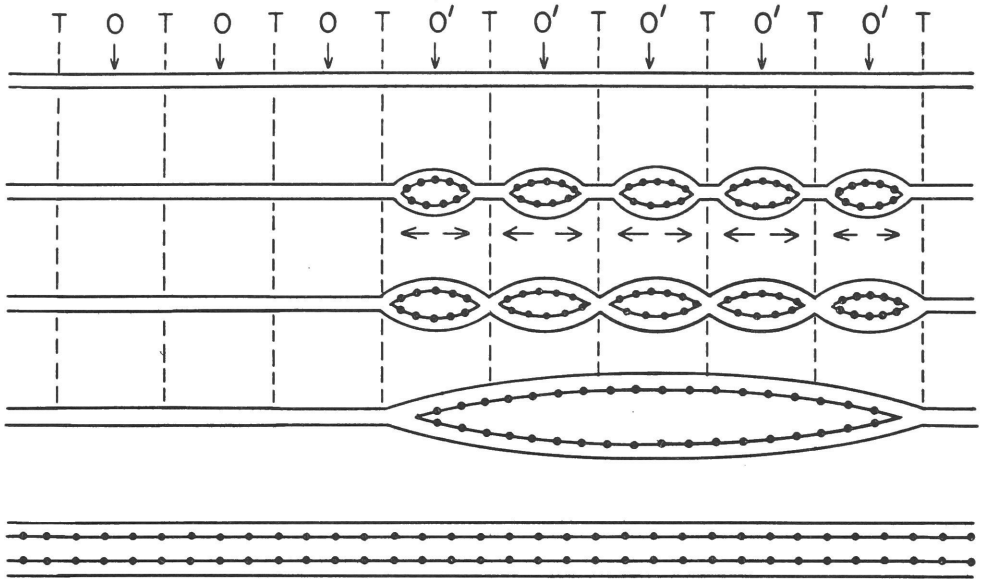


Figure 3c. Replication of a region of mammalian chromosomal DNA through activation of replication sections.

the multi-focal initiation of DNA synthesis on an intact chromosome indicates multiple regions of simultaneous DNA replication on a long piece of chromosomal DNA. Each region of synthesis could represent the replication of a group of coordinately controlled replication sections. In fact, the autoradiographic evidence obtained from studies of isolated DNA indicates that groups of adjacent replication sections do undergo simultaneous replication (Huberman and Riggs, 1968). Perhaps a group of adjacent replication sections, such as the group of three with origins O′ in Figure 3(c), makes up a larger regulatory unit.

The asynchronous pattern of replication of these groups of replication sections (chromosomal regions) can be explained in several ways. One initiation factor may be responsible for all initiations. A declining series of site-determined affinities for this factor could result in the progressive, ordered replication of all of the chromosomal DNA. On the other hand, different initiation factors might be sequentially produced during S phase. These would then initiate synthesis at specific receptor sites. Both possibilities would demand identifiable differences among groups of coordinated replication sections representing chromosomal regions of DNA synthesis. Such differences might reflect regional differences in the macromolecular configuration of chromatin or in the specific base sequences defining the origins of replication sections.

As mentioned above, the initiation factors involved in bacterial DNA replication appear to be proteins (Maaløe and Hanawalt, 1961; Lark and Lark, 1966). Furthermore, the bacterial initiator proteins appear to be sufficiently unstable so that concurrent protein synthesis is generally required to initiate replication at the chromosome origin (Fig. 3a). If the mammalian chromosome is functionally related to the bacterial chromosome, as indicated by previous discussion, then initiation at the origins of replication sections (Fig. 3c) may require the presence of initiator proteins. Furthermore, continual protein synthesis may be required if these initiator proteins are in any way unstable.

Puromycin and cycloheximide are potent inhibitors of protein synthesis characterized by defined modes of interaction with the translational machinery (Williamson and Schweet, 1965; Columbo et al., 1966; Felicetti et al., 1966). Treatment of mammalian cells with these drugs does not affect the cellular levels of deoxyribonucleotide precursor enzymes or of DNA polymerase for several hours (Bennett et al., 1964; Powell, 1962a,b; Littlefield and Jacobs, 1965; Kit et al., 1969). Yet cellular DNA synthesis is rapidly inhibited upon addition of these inhibitors of protein synthesis. In the first 15 minutes after cycloheximide addition to mammalian cells, only 10-20% of the normally synthesized amount of DNA is made.

The protein essential to DNA synthesis may be a structural protein whose synthesis is normally coupled to DNA synthesis; for example, a lysine-rich histone (Robbins and Borun, 1967). However, in analogy to bacterial DNA replication, it is possible that initiation of DNA synthesis on replication sections may require the presence of initiator protein. If only initiation were blocked, DNA synthesis would continue on previously initiated sections until termini were reached. Even if initiator protein levels are immediately reduced, completion of synthesis on those sections already initiated could take a half hour or more (Cairns, 1966; Huberman and Riggs, 1968). This would account for the small amount of residual DNA synthesis which occurs after complete inhibition of protein synthesis.

These conclusions are in agreement with earlier studies of Mueller and coworkers (1962) on DNA synthesis in synchronized HeLa cells treated with puromycin. They suggested that each chromosomal locus or site of DNA synthesis becomes activated through a process requiring protein synthesis. As we have pointed out, each of these chromosomal sites may represent a group (or groups) of coordinately controlled replication sections. Thus, the need for protein synthesis in initiation of DNA synthesis at a chromosomal site reflects the need for initiator protein at the level of each replication section.

Although current experimental evidence is limited, several characteristics of initiator proteins in higher animal cells can be postulated. The rapidity of the effects of inhibitors of protein synthesis would indicate that initiator proteins required in DNA synthesis are unstable. This instability can be rationalized if they are stoichiometrically utilized in the initiation process. Poxvirus DNA replication in mammalian cells is a regulated process (Joklik, 1968). We can speculate that control is effected through initiator proteins. In support of this is the fact that puromycin inhibits ongoing viral DNA synthesis in HeLa cells (Kates and McAuslan, 1967). A protein essential for DNA synthesis accumulates when viral DNA synthesis is blocked by FUdR while protein synthesis is allowed. After removal of FUdR, the accumulated protein is sufficient to support DNA synthesis for several hours under otherwise inhibitory concentrations of puromycin. This situation is somewhat analogous to the build-up of initiator protein in E. coli when DNA synthesis is blocked by phenethyl alcohol (Lark and Lark, 1966) or thymine starvation (Lark, 1966). Initiator protein would seem to accumulate only in certain situations where it is not stoichiometrically utilized in the initiation process.

Perhaps under appropriate conditions a considerable amount of initiator protein can be stored up. The DNA synthetic phases during the early, synchronous cleavages of the sea urchin egg are very interesting in this regard (Black et al., 1967). Puromycin is ineffective in blocking DNA synthesis during the first synthetic phase, S1. However, it does effectively block DNA synthesis in all subsequent S phases. This finding can be readily explained on the basis of the current model. In this unique situation initiator protein is accumulated to a degree sufficient to carry out all initiations during the first S phase. This supply of protein is stoichiometrically used up during the first S phase. Thus new initiator protein must be synthesized for all subsequent synthetic phases.

Control over DNA synthesis might ultimately reside at the level of transcription of messenger RNA for initiator protein. Such

messenger RNA would have to be quite unstable to couple the transcription process and DNA synthesis effectively. Treatment of mammalian cells with actinomycin D inhibits DNA synthesis, but with a 30 minute time lag compared with the more immediate effect of puromycin (Taylor, 1965). Similarly, in poxvirus DNA replication, the messenger for the stoichiometrically utilized (initiator) protein is much more labile than messengers for the viral-induced thymidine kinase and DNA polymerase (Kates and McAuslan, 1967). Therefore it appears that the messenger RNA's for initiator proteins may be sufficiently labile to couple DNA replication tightly to the transcription process.

Finally, nuclear transplantation and cell fusion experiments suggest that initiator proteins are found and perhaps synthesized in the cytoplasm. Such proteins would then have to move from the cytoplasm into the nucleus to initiate nuclear DNA synthesis. Migratory proteins which move from cytoplasm to nucleus, and which may possess some affinity for the nucleus, have been reported in amoeba (Byers et al., 1963a,b) and in L cells (Zetterberg, 1966a,b).

In summary, the following properties can be proposed for DNA replication in the chromosomes of higher animals:

a. Duplication of chromosomal DNA involves the replication of thousands of tandemly-linked replication sections which functionally divide a long backbone molecule of DNA (Fig. 3c).

b. Each replication section can be visualized to consist of two small "bacterial-like" chromosomes linked origin to origin (Fig. 3b). The replication sections, in addition to being much smaller (by a factor of ca. 30), are replicated at a slower linear rate of DNA duplication than the bacterial chromosome, i.e. 1-2 μ/min versus 30 μ/min (Huberman and Riggs, 1968; Cairns, 1963, 1966).

c. Initiation of DNA replication occurs at the origin of the replication section just as it occurs at the origin of the bacterial chromosome. The initiation process requires

the presence of initiator protein which is stoichiometrically used up in the process.

d. Regulation of DNA replication is effected at the level of initiation of groups of coordinately controlled replication sections. Initiator protein synthesis is in turn controlled through the regulated transcription and translation of unstable messenger RNA. Different initiator proteins may be sequentially produced throughout S phase and thus activate specific chromosomal sites at different times. On the other hand, there could be a sequential initiation at sites possessing decreasing affinities for attachment of initiator protein. In either case the orderly, but asynchronous, replication of chromosomal DNA ultimately implies identifiable site differences in chromatin configuration and/or base sequences.

e. Synthesis of initiator protein is cytoplasmic with subsequent transport to the nucleus. Hence, interference with transcription, translation, or transport processes can inhibit initiation.

f. Throughout the DNA synthetic phase, S, new groups of replication sections are undergoing initiation as other groups complete their replication. Inhibition of initiation would not affect the currently active replication sections. Since each replication section is relatively small, the rate of cellular DNA synthesis will rapidly decline if active sections complete replication under conditions where no new sections can be initiated.

In summary, a model of positive regulation for the in vivo control of cellular DNA replication can be constructed and partially supported by experimental evidence. It will be shown in the next section that a virus infection can severely disrupt the biosynthesis

of cellular DNA in animal cells. The viral-induced inhibition of cellular DNA synthesis will be examined in the light of the enzymatic and regulatory mechanisms outlined above.

C. Cellular DNA Synthesis in Animal Virus Infections

Viral infection commonly influences the replication of cellular DNA. In the present studies our interest has been directed towards virus-induced inhibition of cellular DNA replication. Animal viruses with widely differing properties are capable of inhibiting cellular DNA synthesis (Table I). It should be pointed out, however, that a number of viruses, such as parainfluenza virus, SV5, may multiply extensively in certain cell types without affecting cellular DNA synthesis (Holmes and Choppin, 1966). Also, papovavirus infection can induce cellular DNA synthesis in confluent monolayer cultures of cells in which DNA synthesis has become repressed (Dulbecco et al., 1965; Kit et al., 1967).

The data in Table I indicate that the onset of inhibition of cellular DNA synthesis usually coincides with the onset of viral nucleic acid synthesis. This temporal relationship does not necessarily imply a direct causal relationship. Both phenomena probably depend on viral gene function. After infection, time is required for the synthesis of virus-specific enzymes necessary for replication of viral nucleic acid. During this preliminary period products may also be synthesized which are capable of affecting a function essential for cellular DNA replication. The subsequent build-up in viral genetic material would be expected to lead both to an increase in the rate of viral nucleic acid replication and to augmented synthesis of products which have effects on cellular metabolism.

The polymerization of cellular DNA is an enzymatic process occurring within the cell nucleus. It could be argued that the production of viral DNA and virus particles within nuclei of infected cells might well interfere with the enzymatic process of cellular DNA synthesis, while the production of viral RNA and virus particles within infected cell cytoplasm might not directly interfere with the

TABLE I

Viruses that Inhibit Cellular DNA Synthesis

Virus group	Virus	Length of growth cycle (Hrs.)	Onset of replication of viral nucleic acid (Hrs. p.i.)	Onset of inhibition of cellular DNA synthesis (Hrs. p.i.)
DNA Viruses				
Papova	Polyoma	36-50	12	12
"	SV 40	50-80	12-16	12
Adeno	Adeno 5	24	6	6
"	Adeno 2	24	12	12
"	Adeno 12	24	18	18
Herpes	Pseudorabies	13	2	2
"	Equine abortion	18	8	8
Pox	Vaccinia	12	1	1
RNA Viruses				
Picorna	Polio	6	3	3
"	Maus-Elberfeld	8	-	4
"	Mengo	10	3	2
Myxo	Newcastle Disease	5	2	2.5
Reo	Reo 3	15	7	10

References:

Polyoma: Branton & Sheinin, 1968; Dulbecco et al., 1965; Gershon et al., 1965; Sheinin, 1967, 1966.
SV40: Kit, 1967; Kit et al., 1967; Sauer et al., 1966.
Adeno 5: Ginsberg et al., 1967; Ginsberg, 1967.
Adeno 2 & 12: Doerfler, 1969.
Pseudorabies: Kaplan and Ben-Porat, 1963.
Equine Abortion: O'Callaghan et al., 1968.
Vaccinia: Joklik & Becker, 1964.
Polio: Holland & Peterson, 1964.
Maus-Elberfeld: Holoubek & Rueckert, 1964.
Mengo: Baltimore & Franklin, 1962.
Newcastle Disease: Wheelock & Tamm, 1961; Wheelock, 1963.
Reo 3: Gomatos & Tamm, 1963; Kudo & Graham, 1965.
General Reference: Horsfall & Tamm, 1965.

process of synthesis of cellular DNA. However, as Table I indicates, nuclear DNA-containing viruses (papova-, adeno-, and herpesviruses) and cytoplasmic RNA-containing viruses (picorna-, myxo-, and reoviruses) can inhibit cellular DNA synthesis with equal facility. Thus the ability of viruses to inhibit cellular DNA synthesis does not depend on the site of viral nucleic acid biosynthesis per se, or whether the viral genome is DNA or RNA. Moreover, it should be pointed out that at least some of the proteins of nuclear DNA-containing viruses (adeno- and herpesviruses) are synthesized in the cytoplasm and subsequently transported to the nucleus where they combine with viral DNA to form virus particles (Velicer and Ginsberg, 1968; Roizman *et al.*, 1967). In these instances, virus-specific proteins, if they be inhibitory to cellular functions, could act either on cytoplasmic or nuclear processes involved in cellular DNA synthesis.

Autoradiographic studies of cells infected with pseudorabies (Kaplan and Ben-Porat, 1963), polio- (Ackermann *et al.*, 1966), Newcastle disease (Wheelock and Tamm, 1961b), or reovirus (Gomatos and Tamm, 1963) have shown that viral inhibition of cellular DNA synthesis is not associated with a reduction in the proportion of S phase cells in infected cultures. Declining grain counts indicate a declining rate of DNA synthesis in individual cells in S phase. Hence it would appear that inhibition of cellular DNA synthesis by these viruses takes place at the level of ongoing synthesis in individual S phase cells.

In our approach, we have endeavored to separate *in vivo* DNA synthesis into its component parts and then determine which step is affected during virus-induced inhibition. From previous discussion, we may propose that the inhibition of cellular DNA replication could take place at any of three levels. Inhibition could result from 1) degradation of cellular template DNA, 2) interference with the enzymatic processes directly related to deoxyribonucleotide production or polymerization, or 3) interference with normal *in vivo* regulation--particularly initiation of DNA synthesis. In subsequent sections we will consider these possibilities in detail utilizing three cytoplasmic RNA viruses belonging to the picorna-, myxo-, and reovirus groups.

D. RNA Viruses that Inhibit Cellular RNA Replication

The inhibition of cellular DNA synthesis during infection with cytoplasmic RNA-viruses is of particular interest. These viruses utilize RNA precursors and thus would appear less likely to interfere with cellular DNA metabolism through precursor competition. The viral synthetic processes appear to be totally cytoplasmic, yet a nuclear process, cellular DNA replication, is adversely affected.

Cellular DNA synthesis is inhibited during infection with certain members of three major groups of RNA-viruses, the picornaviruses, myxoviruses, and reoviruses. Among these, mengovirus, Newcastle disease virus, and reovirus 3 have growth and inhibitory characteristics which have been most thoroughly investigated. These characteristics are summarized in Table II.

1. Mengovirus

Mengovirus is a member of the group of small, lipid-free, RNA-containing viruses known as picornaviruses. There is a natural reservoir for mengovirus in rodent populations, but a wide variety of primates are susceptible to infection (Andrewes and Pereira, 1967). Table II summarizes the properties of the virion and the growth characteristics of mengovirus in L cells. After a latent period of 4 hours there is a period of exponential multiplication lasting through 7 hours p.i. and producing a yield of up to 1000 PFU/cell (Franklin and Baltimore, 1962; Amako and Dales, 1967). Infected cells often display some aggregation of chromatin by 2 hours p.i. Cells infected with either the wild type mengovirus or the small plaque variant do not lyse until the 10th hour of infection (Franklin and Baltimore, 1962; Amako and Dales, 1967). Cells infected with the unstable large plaque variant start to lyse during the 6th hour of infection (Amako and Dales, 1967).

Infection of L cells with mengovirus results in rapid inhibition of cellular RNA and protein synthesis evident by 0.5 to 1.0 hour p.i. (Franklin and Baltimore, 1962). Virus-directed RNA and protein syntheses become detectable 2.5 to 3.0 hour p.i. in the cytoplasm of infected cells and rapidly increase thereafter (Krug and Franklin, 1964;

TABLE II

Properties and Growth of Three RNA Viruses

Virus and group	Virion size (mμ)	M.W. and stranded-ness of nucleic acid	Site of synthesis	Latent period	Growth cycle	Virus yield (PFU/cell)	Effects on host biosynthesis (time for 20% inhibition) RNA	DNA	Protein
Mengovirus (picorna)	27	$2x10^6$ S.S.	Cyt.	4 hr	11 hr	200 -1000	Inhibition (0.5-1.0 hr)	Inhibition (1.5-2 hr)	Inhibition (0.5-1.0 hr)
Newcastle Disease Virus (myxo)	100 -200	$7.5x10^6$ S.S.	Cyt.	3 hr	5 hr	0.1 -1.0	None detected	Inhibition (2.5 hr)	Inhibition (2.5 hr)
Reovirus 3 (reo)	75	$17x10^6$ D.S.	Cyt.	6 hr	16 hr	200 -1000	None detected	Inhibition (10 hr)	None detected

Baltimore et al., 1963; Plagemann and Swim, 1966; McCormick and Penman, 1967). The rates of viral RNA and protein syntheses may reach levels equivalent to 30-50% of the rates of cellular RNA and protein syntheses in uninfected cultures.

Infection of L cells causes early inhibition of cellular DNA synthesis, detectable by 1.5 to 2.0 hours p.i. (Franklin and Baltimore, 1962). It has been suggested that the inhibition of cellular DNA synthesis is secondary to virus-induced inhibition of protein synthesis because of the temporal relationship between these inhibitions (Franklin and Baltimore, 1962). DNA isolated by phenol extraction as late as 8 hours after infection has a sedimentation coefficient similar to that of DNA extracted from uninfected cells, and there is no loss of acid-precipitable radioactivity from prelabeled DNA. Other experiments have shown that there is no detectable release of lysosomal enzymes through 9 hours p.i. (Amako and Dales, 1967).

2. Newcastle Disease Virus

Newcastle disease virus (NDV) is primarily a pathogen of the respiratory tract of domestic and wild fowl (Andrewes and Pereira, 1967). It is a member of the parainfluenza subgroup of the myxovirus family. Virus particles have a diameter of 100-200 mμ and consist of an inner coiled helix of ribonucleoprotein surrounded by an outer lipoprotein envelope derived from the plasma membrane. Each infected cell of a chick allantoic membrane produces an average of 1000 infective particles. The virus yield from the HeLa and L cell lines is only 0.1-1.0 PFU/cell (Wheelock and Tamm, 196la; Wilcox, 1959). In these cell lines viral antigenic proteins are produced without production of corresponding amounts of infectious virus.

The most complete studies of the biology of NDV-infected cells have been carried out in HeLa cells (Wheelock and Tamm, 1959, 196la,b). In these cells the latent period of viral growth is approximately 3 hours. Exponential multiplication then begins and continues until 5 hours p.i. Cellular protein and DNA syntheses are detectably

inhibited by 2.5 to 3.0 hours p.i. There is nearly complete inhibition of these cellular synthetic functions by 5 hours p.i. On the other hand, the overall rate of RNA synthesis has declined only 10% by 5 hours p.i. and 20% by 8 hours p.i. Thus there appears to be marked inhibition of protein and DNA syntheses without comparable inhibition of RNA synthesis. The possibility has not been investigated that the RNA synthesized at later times in infection might be largely virus-specific RNA. Wheelock and Tamm (1961b) have suggested that the inhibition of cellular DNA synthesis might be due to virus-induced inhibition of cellular protein synthesis.

3. Reovirus 3

Reoviruses occur in man and other animals and cause a variety of respiratory-enteric disease pictures (Andrewes and Pereira, 1967). Reovirus type 3, used in the present investigation, is structurally an icosahedral particle 76 mμ in diameter, which contains 17-19 million daltons of double-stranded, base-paired RNA (Gomatos et al., 1962; Gomatos and Tamm, 1963; Bellamy et al., 1967).

Reovirus 3 grows to high titers in mouse fibroblast L cells with a yield of 200-1000 PFU per cell (Gomatos et al., 1962). The latent period in L cells is commonly 8 hours, but it can be shortened to 5 to 6 hours when cells are infected with virus at high input multiplicities (Gomatos et al., 1962; Kudo and Graham, 1965; Silverstein and Dales, 1968). Virus growth reaches a plateau by 13 to 16 hours p.i.

Virus-directed RNA synthesis is detectable by 5 hours p.i. in the presence of 0.5 μg/ml of actinomycin D, a concentration at which normal amounts of new infective virus are produced (Shatkin, 1965). Viral RNA synthesis occurs at a rate less than 5% of the rate of cellular RNA synthesis in uninfected cells. This contrasts with mengovirus infection of L cells, where, late in infection, the rate of mengovirus RNA synthesis reaches a level equivalent to 50% of the rate of cellular RNA synthesis in uninfected cells (Franklin and Baltimore, 1962). There is, in fact, no detectable difference between

the rates of RNA synthesis in reovirus-infected cells and uninfected cells if actinomycin is not used to depress cellular RNA synthesis (Gomatos and Tamm, 1963; Kudo and Graham, 1965). Autoradiographic analysis has not demonstrated any differences in nuclear (i.e. cellular) RNA synthesis between uninfected and infected cells.

Autoradiographic analysis of monolayer cultures infected with reovirus 3 reveals no inhibition of overall protein synthesis even at 16 hours p.i. (Gomatos and Tamm, 1963). However, in infected suspension cultures overall protein synthesis seems to decline after 9 hours p.i. with 40% inhibition by 14 hours p.i. (Kudo and Graham, 1965). In neither study was there an attempt made to distinguish cellular from viral protein synthesis.

Cellular DNA synthesis, however, is markedly inhibited in both monolayer and suspension cultures of L cells infected with reovirus (Gomatos and Tamm, 1963; Kudo and Graham, 1965). Inhibition is detected by 10 hours p.i. in monolayer, and by 12 hours p.i. in suspension cultures. By 16 hours p.i., cellular DNA synthesis is 50-70% inhibited. Autoradiographic evidence indicates that infection inhibits DNA synthesis within each cell in S phase (Gomatos and Tamm, 1963). In monolayer cultures, the inhibition of DNA synthesis during infection with reovirus 3 does not appear to be associated with inhibition of protein synthesis. However, the finding of inhibition of protein synthesis in reovirus-infected suspension cultures leaves open the possibility of a relationship between inhibition of cellular DNA and protein synthesis.

4. Résumé

Infection with reovirus type 3 inhibits cellular DNA synthesis without necessarily inhibiting RNA and protein synthesis. Infection with Newcastle disease virus inhibits protein and DNA synthesis without a marked effect on RNA synthesis. Infection with mengovirus inhibits cellular RNA, protein and DNA synthesis. The characteristics of these three viruses have been discussed and are summarized in Table II.

We have previously analyzed cellular DNA replication with respect to the enzymatic mechanism of nucleotide polymerization, and *in vivo* regulation of DNA synthesis. Below we shall present results of a comparative study of the inhibitory effects caused by these RNA viruses in one cell type, the L cell.

II. MATERIALS AND METHODS

A. Growth Media for Cells

Eagle's minimum essential medium (MEM) supplemented with 5% fetal calf serum (MEMFS) was used to grow uninfected L cells in monolayer cultures (Eagle, 1959).

Infected monolayer cultures of L cells were grown in reinforced Eagle's medium (REM) supplemented with 5% fetal calf serum (REMFS). Reinforced Eagle's medium contained 2x the amino acid concentration and 4x the vitamin concentration of Eagle's MEM (Bablanian et al., 1965). The concentration of sodium bicarbonate was 3.7 g/l and that of glucose was 4.5 g/l. In addition, reinforced Eagle's medium contained ferric nitrate, 0.1 mg/l, and sodium pyruvate, 0.11 g/l.

Suspension cultures of L cells were grown in Eagle's spinner medium (SpMEM) supplemented with 7% fetal calf serum (SpMEMFS) (Eagle, 1959). This medium differs from MEM in that calcium chloride has been omitted; the concentration of NaH_2PO_4 has been increased 10-fold; and "non-essential" amino acids, alanine, asparagine, aspartic acid, glycine, glutamic acid, proline, and serine, have been included at 0.1 mM each.

Fetal calf serum was purchased from Grand Island Biological Co., Grand Island, N. Y.

All media contained penicillin (500 units/ml), streptomycin (0.1 mg/ml) and mycostatin (25 units/ml). All growth media containing fetal calf serum also contained tylosin tartrate (50 μgm/ml) (Friend et al., 1966).

B. Diluents

Phosphate buffered saline (PBS) contained 0.0081 M KH_2PO_4, 0.0015 M Na_2HPO_4, 0.137 M NaCl, 0.0027 M KCl, 0.0009 M $CaCl_2$, and 0.0005 M $MgCl_2$ (Dulbecco and Vogt, 1954).

PBS-def refers to PBS without Ca^{++} and Mg^{++}.

C. Cell Cultures

The L(929) mouse fibroblast line (Sanford et al., 1948) used in these studies came from Dr. P. J. Gomatos (Gomatos et al., 1962).

Monolayer cultures were grown to confluency in 32 oz prescription bottles at 37°. Cells were dispersed from the monolayer with 1 ml of 0.25% trypsin-0.05% versene in PBS-def(T-V). The suspended cells were diluted to 10 ml with growth medium (MEMFBS), and 0.5 ml of diluted cells was added to 40-50 ml of growth medium in a new prescription bottle. Bottles were gassed with 5% CO_2-air and incubated at 37°.

Suspension cultures of L cells were maintained in spinner growth medium (SpMEMFBS) in spinner vessels (Bellco Glass Co.) at 37°. Suspension cultures were initiated from monolayer cultures by dispersal with T-V. The dispersed cells were diluted with SpMEM to a concentration of 1×10^5 cells/ml. Suspension cultures were periodically diluted to maintain the cell density at less than 4×10^5 cells/ml. The generation time was similar to that in monolayer, i.e. 20 hr.

Monolayer cultures in 60-mm plastic petri dishes (Falcon Plastics Co.) were initiated either by plating of trypsinized cells from monolayers grown in bottles or by direct plating of cells from suspension culture. Cells transferred to plastic dishes from suspension culture attached within 30 min.

D. Growth and Assay of Viruses

1. Reovirus

Reovirus type 3, Dearing strain, was obtained from Dr. P. J. Gomatos (Gomatos et al., 1962) and further passaged 2x in L cells.

Seed virus was prepared by inoculating confluent 60-mm monolayer plates of L cells with 30-50 PFU/cell in 0.3 ml of inoculum. Adsorption was carried out at 37° in a humidified atmosphere of 5% CO_2-air. The inoculum was redistributed by tilting of plates every 30 min for 2 hr, and then 3 ml of REMFS was added. Twenty-six hr later, the cells were scraped into the medium and the total suspension was frozen and thawed 3 x. Cellular debris was removed by centrifugation, and the supernatant was stored at -55°. Virus seeds contained on the average 8×10^8 PFU/ml.

Plaque assays were performed as follows: ten-fold dilutions of virus were prepared in Eagle's MEM, and confluent monolayer cultures of L cells in 60-mm petri dishes were inoculated with diluted virus, 0.3 ml/plate. Four plates were used for each dilution. The cultures were incubated at 37° in 5% CO_2-humidified air. The inocula were redistributed by tilting the plates every half hour. After a 2-hr adsorption period, each plate received 10 ml of an overlay medium made up of equal volumes of 1.8% Bacto-Difco agar and 2x concentrated REMFS. After incubation for 5 days, 4 ml of a second overlay of 0.9% Bacto-Difco agar-0.02% neutral red was added to each culture. Plaques were read 24 hr later.

Reovirus growth curves in monolayer cultures were conducted as follows: L cells in 60-mm petri dishes were washed once with MEM. Infection was initiated (time zero) by inoculation at a multiplicity of 100 PFU/cell. The virus inoculum in 0.3 ml MEM was redistributed at 15 min. After adsorption for a total of 30 min, the residual inoculum was removed and the plates were washed 2x with MEM. The plates were incubated for 20 min with anti-reovirus immune serum and then washed 3x more with MEM. Finally, 5 ml of REMFS medium was added to each plate. At appropriate intervals the cells and media from three plates were harvested and total infective virus determined by plaque assay.

Reovirus growth curves in cells in suspension culture were determined as follows: cells were concentrated to 1 x 10^6 cells/ml and transferred into SpMEM medium containing reovirus at a concentration equivalent to 100 PFU/cell. Cells were incubated in a 50 ml centrifuge tube rotated at 50 rpm around its long axis on a spinner wheel. After 30 min at 37°, the cells were pelleted by centrifugation. Cells were resuspended in an equal volume of fresh SpMEM and recentrifuged. The pelleted cells were resuspended to a concentration of 2 x 10^6 cells/ml in fresh SpMEM containing anti-reovirus immune serum. After treatment with immune serum for 20 min, the cells were washed 2x with fresh medium. The infected cells were finally resuspended in SpMEMFS to a concentration of 4 x 10^5 cells/ml. Duplicate 5 ml

aliquots were removed at intervals and infectivity assays were performed to measure total virus present.

The ability of infected cells to exclude trypan blue was determined by the addition of trypan blue (0.5% in PBS) to a final concentration of 0.05%. A hemocytometer was used to enumerate affected suspension cells whereas a microscope with an eyepiece grid was used in determinations of affected cells in monolayer culture.

Anti-reovirus immune serum was prepared by two intravenous injections, two weeks apart, of purified reovirus (1×10^9 PFU in 1 ml/injection). At the time of the second intravenous injection, one ml of a suspension of purified virus was injected subcutaneously. Serum was collected 26 days after the first injection.

Reovirus purification was carried out as follows (Gomatos, 1966): six liters of L cells at 4×10^5 cells/ml were collected by centrifugation and resuspended in 360 ml of SpMEM containing reovirus at a concentration equivalent to 20 PFU/cell. The cell suspension was incubated with stirring at 37° for 2 hr and then diluted to 3 l. with SpMEMFBS. After 26 hr, cells were collected by centrifugation at 1200 x G for 20 min at 4°. The medium was decanted and the pelleted cells were resuspended in 5 ml of PBS. The cells were frozen and thawed 2x, and then sonicated for 60 sec at lowest resonance on setting 1 on a Branson LS-75 sonifier (regular probe). Debris was sedimented at 1200 x G for 5 min, and the supernatant was decanted and saved. The pellet was resuspended in 4 ml PBS, resonicated, and recentrifuged. The combined supernatants were treated with 2 μg/ml RNAase and 20 μg/ml DNAase for 30 min at 37°. Then chymotrypsin was added to 30 μg/ml and incubation was continued for 45 min at 37°. The virus suspension was cooled to 4° and extracted 2x with 5 ml of genesolv D., using a vortex mixer and centrifugation to separate the phases. The aqueous phase was collected and saved. The interface was reextracted with 2 ml of PBS. The combined aqueous phases were sonicated 30 sec at lowest resonance. CsCl was added to an average density of 1.37

and the solution was centrifuged to equilibrium in a SW39 rotor at 37,500 rpm for 40 hr. The major band of virus was in the center of the tube. The virus band was diluted with new CsCl solution and rebanded. The final band was passed through a Sephadex G-25 column to desalt the virus and transfer it into PBS. The final yield of purified virus was 200 PFU/cell. Purified virus had a 260/280 O.D. ratio of 1.37.

2. Mengovirus

The small plaque variant of Mengovirus was obtained from Dr. S. Dales (Amako and Dales, 1967), and plaque purified 3x.

Stock virus was prepared by inoculating confluent monolayer cultures of L cells in 60-mm petri dishes at a multiplicity of 50 PFU/cell in 0.3 ml of MEM. After a 1-hr adsorption period, with redistribution every 15 min, 3 ml of REMFS was added to each plate. After 13 hr, plates were transferred to a freezer (-15^{o}) and stored. Upon thawing, cells were scraped and the cell suspension was frozen and thawed an additional 2x. The suspension was clarified by centrifugation at 1200 x G for 30 min and the resulting supernatant was stored at -15^{o}. Virus stocks prepared in this manner contained 1×10^{9} PFU/ml.

Infectivity titrations were carried out on confluent monolayers of L cells in petri dishes. Each plate received 0.3 ml of appropriately diluted virus. The virus inocula were redistributed every 15 min during adsorption of virus to cells at 37^{o} under 5% CO_2-humidified air. After 1 hr the residual inoculum was removed from each plate, which then received 10 ml of overlay made up of equal volumes of 2% agarose (Bausch and Lomb) and 2x concentrated REMFS. Plates were incubated at 37^{o} for 36 hr and then 4 ml of 0.02% neutral red in 0.9% Bacto-Difco agar was added to each plate. Plaques were counted 36 hr later.

Mengovirus growth curves were determined in suspension cultures. L cells were concentrated to 8×10^{5} cells/ml and transferred into SpMEM containing mengovirus at 100 PFU/cell (time zero). Cells were

incubated in a horizontally rotated tube at 50 rpm for 30 min and then centrifuged. The pellet was resuspended in SpMEM to 4 x 10^5 cells/ml and the cells washed by two cycles of centrifugation in fresh medium. After washing, the cells were suspended in SpMEMFS to 1.5 x 10^5 cells/ml. At appropriate times, duplicate 5 ml aliquots of infected cells were removed and frozen. Plaque assays were carried out on virus harvested as previously described.

3. Newcastle Disease Virus

The Hickman strain of NDV was obtained from Dr. E. F. Wheelock (Wheelock and Tamm, 1961a,b). After plaque purification 3x on chick embryo cells, a seed virus pool was prepared by allantoic inoculation of 10-day-old chick embryos with 2 x 10^5 PFU of NDV per embryo. After incubation for 28 hr at 35°, the infected eggs were chilled overnight at 4°. The allantoic fluids were collected, clarified by centrifugation, immediately frozen and held at -55°. The virus stock used in all experiments contained 2.3 x 10^9 PFU/ml.

Monolayer cultures of chick embryo cells in petri dishes were used for the plaque assay of NDV. Confluent plates were inoculated with 0.5 ml of the appropriate ten-fold dilution in MEM (time zero). There was a 2-hr adsorption period with redistribution every 15 min. Adsorption was at 37° under 5% CO_2-humidified air. After the adsorption period, the inoculum was removed and 8 ml of a 1:1 mixture of 1.8% Bacto-Difco agar and 2x concentrated REM (with 0.2% yeast extract and 0.2% bovine serum albumin) was added for an overlay. Plates were incubated at 37° for 36 hr and then overlaid with 5 ml of 0.01% neutral red in 0.9% Bacto-Difco agar. Plaques were read after 16 additional hr.

The growth curve of NDV was determined in a manner similar to that used for reovirus in suspension. The input multiplicity was 100 PFU/cell. Anti-NDV immune serum was used after adsorption to lower the level of residual infective virus from the inoculum.

Anti-NDV immune rabbit serum was prepared by two 10-ml intravenous injections, two weeks apart, of infected allantoic fluid (2.3×10^{10} PFU/injection). Serum was collected 28 days after the first injection.

E. Chemicals

All deoxyribonucleosides and deoxyribonucleotides were purchased from P-L Biochemicals, Milwaukee, Wisconsin. 5-Fluoro-2'-deoxyuridine was made available by Dr. W. E. Scott of Hoffman-La Roche Inc., Nutley, N. J. Cycloheximide (actidione), Pentex bovine serum albumin and pronase B were purchased from Calbiochem Co., Los Angeles, Calif. Puromycin dihydrochloride was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. Ribonuclease, deoxyribonuclease, and chymotrypsin were obtained from Worthington Biochemical Corp., Freehold, N.J. Tris(hydroxymethyl)aminomethane (Tris) was obtained from Sigma Chemical Co., St. Louis, Missouri. Cesium chloride was purchased from Harshaw Chemical Co., Hastings-on-Hudson, N. Y. Sucrose was obtained from Schwarz Bioresearch, Inc., Orangeburg, N. Y.

All chemicals were of the highest purity available.

F. Cell Synchronization

A culture of cells can be synchronized with respect to DNA synthesis through the use of reversible inhibitors of DNA synthesis. Treated cells move through G2, M and G1 phases and accumulate at the beginning of S phase. Either thymidine at millimolar concentrations or 5-fluoro-2'-deoxyuridine (FUdR) at 10^{-6} M concentration can be used as a reversible inhibitor of DNA synthesis (Xeros, 1962; Galavazi *et al.*, 1966; Puck, 1964; Hsu *et al.*, 1964; Rueckert and Mueller, 1960).

1. TdR-FUdR-TdR Technique

L cells at 1×10^5 cells/ml in suspension were first blocked with 2 mM thymidine (added as 50x concentrated solution) for 14 hr. The cells were sedimented in a warm centrifuge, resuspended, and recentrifuged in warm, fresh SpMEM without thymidine. The pelleted cells were resuspended in fresh SpMEMFS at 1×10^5 cells/ml. After

8 hr, FUdR was added to a final concentration of 2×10^{-6} M (added as 100x concentrated solution). The cells were released from this second block 12-14 hr later by the addition of thymidine to 2×10^{-5} M (added as 100x concentrated solution). Cytidine-5-H^3 (0.5 μc/ml, 10^{-9} M) incorporation was used to measure DNA synthesis (with an alkaline digestion to remove label incorporated into RNA).

2. TdR Double-Block

The first block with excess TdR was similar to that described above. After 14 hr in 2 mM TdR, the cells were sedimented, washed and resuspended in SpMEMFS without TdR to 1×10^5 cells/ml. After 8 hr, TdR was added again to 2 mM. After an additional 12-14 hr, the second thymidine block was removed by centrifugation, resuspension, and recentrifugation in fresh SpMEM. The pelleted cells were then transferred to fresh growth medium. Although labeled thymidine can be used to measure DNA synthesis with this technique, cumulative incorporation of labeled cytidine tends to be more linear throughout the period of synthesis, S. Linear cumulative incorporation throughout the 6-hr synthetic period, S, requires either H^3-cytidine (0.5 μc/ml, 10^{-9} M), or H^3-thymidine (2 μc/ml, 2×10^{-6} M).

In general, either technique results in a nearly linear precursor incorporation after release from the second block. The period of linear DNA synthesis ranges from 5 to 7 hr, with a mean of 6 hr. Usually 80% of the cells in the synchronized culture divide within 14 hr after release from the second block.

G. Pulse-Labeling with Radioisotopes

Thymidine-6-H^3 was obtained from the Amersham/Searle Co. at specific activities of 25-30 c/mM. For pulse-labeling for periods over 20 min, unlabeled thymidine was added to make the final thymidine concentration 2×10^{-7} M and the isotope concentration 1-2 μc/ml. Isotope was added to cells as a 100x concentrated solution.

L-leucine-4,5-H^3 was obtained from New England Nuclear Corp. at a specific activity of 55.5 c/mM. Cells were transferred to medium containing one-half the normal complement of leucine 20-24 hr prior

to pulse-labeling. The final H^3-leucine concentration was 4-5 μc/ml. This isotope was also added to cells as a 100x concentrate.

Cytidine-5-H^3 was used to measure DNA synthesis in synchronized cells where FUdR was present. Cytidine-5-H^3 was obtained from Amersham/Searle at 23-25 c/mM and was used at 0.5 μc/ml.

Deoxycytidine-5-H^3 was used to label DNA for alkaline sucrose gradient analysis where FUdR treatment was given. Deoxycytidine-5-H^3 was obtained from Amersham/Searle at 14.7 c/mM and was used at 10 μc/ml final concentration.

Suspension cells growing in Bellco spinner vessels were given the treatments described in the various experiments. For pulsing, three 5 ml aliquots were removed from the spinner vessel and placed into 16 x 125 mm tissue culture tubes with screw caps (Falcon Plastics). A 50 μl aliquot of 100x concentrated isotope solution was added to each tube. The cells were kept in suspension for the duration of the pulse, usually 30 min, by gentle, horizontal, mechanical shaking. When longer periods of incorporation were used, cells were placed in tissue culture tubes and rotated horizontally at 50 rpm. Cell concentrations in pulse-labeling experiments were 1-2 x 10^5 per ml. To stop incorporation, cells were pelleted at 1000 x G for 5 min at 4^o. The supernatants were decanted, and the pellets quick-frozen in a dry ice-alcohol bath. Pellets were stored at -15^o until further analysis.

Cells in monolayer cultures were pulsed by the addition of 50 μl of 100x concentrated isotope solution to 5 ml of the overlay medium. The plates were swirled to distribute the isotope throughout the medium. At the end of the pulse, the medium was aspirated and the plates were washed with cold PBS. One ml of cold water was added and the plates were stored at -15^o. For further analysis, plates were thawed, the cells scraped from the dish and transferred to plastic tubes with additional water. Cells were then pelleted in the cold and the pellets analyzed as described below.

Pulse-labeling was conducted in a constant temperature room at 37^o. Treatments performed in the cold were conducted either on ice or at 4^o.

The cell pellets labeled with H^3-thymidine or H^3-cytidine were digested for 12-14 hr at 37° with 1 ml of 0.3 N KOH. The samples were then cooled to 4° and 0.5 ml of 50% trichloracetic acid (TCA)-5% $Na_4P_2O_7 \cdot 10\ H_2O$ was added. The precipitates were collected on Whatman glass fiber filters (GF/C) and washed 2x with two ml of 5% TCA-0.5% $Na_4P_2O_7 \cdot 10\ H_2O$ each time. This alkaline digestion removed all label incorporated into RNA and less than 3% of the label incorporated into DNA.

The cell pellets labeled with H^3-leucine were resuspended in 1 ml of cold water with the aid of a vortex mixer. One ml of 10% TCA was added and the cell suspension was held at 37° for 2 hr. The samples were cooled to 4°, collected on Whatman glass fiber filters (GF/C) and washed 2x with two ml aliquots of 5% TCA.

Glass fiber filters were dried in glass scintillation vials at 80°-90° for 2 to 4 hr. Then 3 ml of a toluene-based scintillation mixture (Liquifluor, Pilot Chemicals) was added to each vial. Samples were counted in a Packard Scintillation Spectrometer with a machine efficiency of 45-55%.

H. Phenol Extraction of Double-Stranded DNA

One million "labeled" cells were pelleted in a 12 ml centrifuge tube with a rubber-stoppered bottom (Kontes Glass Co.). The cell pellet was suspended in 1 ml cold water using a 1 ml plastic pipette (Falcon Plastic Co.). Then 3 ml of a solution containing 0.5% sodium lauryl sulfate, 0.05 M Tris (pH 7.5), and 0.01 M EDTA was added. The tube was stoppered with a silicon rubber stopper and gently inverted 50x by hand during the next 15 min. Then 2.5 mg of pronase B (previously heat-treated for 3 hr at 37°) in 0.5 ml water was added and the tube again inverted 50x by hand. The tube was incubated at 37° for one hr with 25 gentle inversions every 15 min. Then 4 ml of phenol (redistilled and saturated with 0.01 M Tris, pH 7.2 , 0.001 M EDTA) was added. The stoppered tube was gently inverted 100x by hand and placed overnight at room temperature. Afterwards the tube was again gently inverted 50x more. The bottom phenol phase was aspirated through a

23-gauge needle inserted into the rubber stopper in the tube bottom. The aqueous phase was extracted 2x more with 4 ml fresh phenol each time. The DNA-containing aqueous phase was transferred to a regular 12 ml glass centrifuge tube and extracted 3x with 5 ml ethyl ether each time. The residual ether was removed by blowing nitrogen over the surface of the solution. This solution of native DNA was stored at 4^{o} and analyzed on sucrose gradients within 4 days.

I. Neutral Sucrose Gradients

A neutral gradient was prepared as a 36 ml, linear 5-20% sucrose gradient made up in 0.01 Tris (pH 7.2), 1.0 M NaCl, and 0.001 M EDTA. The solution of native DNA (0.6 ml) was layered on top of this gradient. Gradients were centrifuged at 88,000 x G in the SW-27 rotor on a Spinco model L2 centrifuge for 5 hr at 4^{o}. C^{14}-labeled adenovirus 2-DNA was obtained from Dr. Walter Doerfler and used as the marker in all gradients. This marker DNA sediments at 32S under neutral conditions (Doerfler, 1969; Green et al., 1967). Fractions were collected from the bottom of the tube through a 16-gauge needle and pumped through a peristaltic pump to a fraction collector. Thirty fractions were collected from each gradient using timed collection. To each fraction was added 500 µg bovine serum albumin in 0.3 ml. Then 0.5 ml of 50% TCA-5% $Na_4P_3O_2 \cdot 10\ H_2O$ was added, and the precipitates were collected on glass fiber filters, washed, dried, and counted as previously described.

J. Alkaline Sucrose Gradients

Alkaline gradients were prepared as 36 ml, linear 5-20% sucrose gradients made up in 0.3 N NaOH, 0.5 M NaCl, and 0.01 M EDTA. Before loading with cells, an overlay of 0.8 ml of 0.5 N NaOH-0.1 M EDTA was placed on top of the sucrose solution. Aliquots (1-5 x 10^5 cells) of radioactively labeled cells were pelleted in PBS-def in 12 ml centrifuge tubes. The pelleted cells were resuspended in 0.4 ml cold water using a plastic 1 ml pipette and layered onto the gradient held at 4^{o}. After gently loading the cell suspension and adding adenovirus-2 C^{14} marker DNA*, 0.6 ml more of 0.5 N NaOH-0.1 M EDTA was layered over

* Adenovirus DNA has a sedimentation coefficient of 34S under alkaline conditions (Doerfler, 1969; Green et al., 1967).

the cell suspension. Loaded gradients were held at 4^{o} for 12 to 16 hr until they were centrifuged. Gradients were centrifuged in the SW-27 rotor on a Spinco model L2 centrifuge at 88,000 x G for 4 hr at 4^{o}. Alkaline gradients were collected and analyzed as previously described for neutral gradients.

When deoxycytidine-H^3 was used as a DNA label; the alkaline sucrose fractions were held at 37^{o} for 12 to 14 hr before addition of carrier BSA and precipitation with TCA.

General Comment. In theory, sucrose gradients prepared in this manner would not be expected to be isokinetic, i.e. field and solvent effects should not exactly cancel out to give a constant rate of sedimentation throughout the gradient. However, S values determined on DNA in these gradients were nearly identical to those obtained on the same DNA under the isokinetic conditions of Burgi and Hershey (1963). Molecular weights of DNA were estimated using the formulas of Studier (1965).

K. Assays for TdR- and TMP-Kinases

A pellet containing 6 x 10^7 washed cells was resuspended in 8 ml of PBS containing 1 mM TMP and 1/6000 (v/v) mercaptoethanol. Samples were frozen and held in dry ice until all other samples were accumulated. Samples were thawed in cold water and sonicated for 60 sec at setting 2 on a Branson LS-75 sonifier. Sonicated samples were centrifuged 1 hr in a Spinco S40 rotor at 104,000 x G at 4^{o}. The resulting supernatants were assayed as follows:

To measure the production of TMP from TdR, i.e. thymidine kinase, the supernatants were assayed at 1/10 dilution. At this dilution, thymidine remained at a saturating concentration throughout the incubation period.

The supernatants were assayed undiluted for the measurement of the production of TTP. In this case all of the labeled TdR-H^3 was converted to TMP-H^3 within 1 to 2 min. Since the supernatant solution contained unlabeled TMP at 1 mM, this represented an internal generation of a saturating concentration of "labeled" TMP.

One-half ml of cell supernatant was added to one-half ml of the following substrate solution: 0.2 M Tris, pH 7.9, 0.01 M ATP; 0.01 M $MgCl_2$, 0.01 M sodium phosphoglycerate; and 100 μc/ml of 6-H^3-TdR (10-25 c/mM; 3-7 x 10^{-6} M) (Kit et al., 1963).

Incubation was carried out at 37° for 20 min and then stopped by the addition of 0.1 ml of 6 N perchloric acid after the samples had been transferred to an ice bath. The precipitated protein was sedimented at 4° for 10 min at 600 x G. The supernatants were carefully decanted and neutralized with 0.1 ml of cold 4 N KOH. After 20 min at 4°, the precipitated $KClO_4$ was removed by a 20 min centrifugation at 700 x G at 4°. Once again the supernatant was quantitatively decanted. The deoxynucleotides in this supernatant were separated by the thin layer chromatographic (TLC) procedure to be described. Each sample spot on the TLC plate received 1 μg each of carrier TdR, TMP, TDP, and TTP in 1 μl.

L. Assay for dCMP-Deaminase

A sample of 1.5 x 10^7 cells was centrifuged and the cell pellet resuspended in 1 ml of buffer (1 x DBS) of the following constitution: 0.033 M Na_2HPO_4, pH 7.3; 0.03 M NaF; 0.0002 M dCTP (Hartwell et al., 1965).

Samples were frozen and thawed 4x and then assayed in duplicate. One-tenth ml aliquots of samples were mixed with 0.05 ml of a 3x concentrated DBS buffer containing 80 μc/ml H^3-dCMP (3.7 c/mM, Schwarz Bioresearch Co.). Assay mixtures were incubated for 60 min at 37°. The reaction was stopped by insertion of the tubes into boiling water for 3 min. The precipitated protein was sedimented by centrifugation for 5 min at 700 x G at 4°. Duplicate aliquots of the supernatant were assayed on thin layer chromatography by the standard procedure to be described. Each sample spot on the TLC plate received 1 μg of carrier dUMP and 1 μg of dCMP in 1 μl.

Enzyme activity was proportional to protein concentration in the extract and time of incubation within the ranges utilized.

M. Assay for DNA Polymerase

Aliquots containing 1.2-1.6 x 10^7 cells were swollen in 2 ml Na^+ RSB (a hypotonic buffer with 0.01 M NaCl, 0.01 M Tris, pH 7.4, 0.0015 M $MgCl_2$). After 5 min of swelling, the cells were homogenized in a Dounce homogenizer (25 strokes, tight pestle) (Jungwirth and Joklik, 1965). The nuclei were spun down at 800 x G for 10 min. Triplicate portions of the cytoplasmic supernatant were assayed as follows: One-half ml of the supernatant was mixed with one-half ml of substrate solution and incubated at 37° for 40 min.

The substrate solution contained 100 mM Tris (pH 8.1); 8 mM $MgCl_2$; 4 mM 2-mercaptoethanol; 320 μM dATP, dGTP, dCTP; H^3-TTP (4 μc/ml, 280 μM); 1.6 mM ATP; and 400 μg/ml heat-denatured salmon sperm DNA (Hopper et al., 1966). Salmon sperm DNA at 2 mg/ml in distilled water was denatured at 100° for 10 min and then quenched in a dry ice-alcohol bath.

Incorporation was stopped by the addition of 0.2 ml of 4 N KOH. A standard double digestion with KOH and precipitation with TCA was carried out as will be described for the nuclear DNA-synthesizing preparations.

Enzyme activity was proportional to protein concentration in extract and time of incubation within the ranges used. The cytoplasmic supernatant represented 80% of the DNA polymerase activity found in total cellular homogenates. No activity was detectable with native, double-stranded DNA as template.

N. Nuclear DNA-Synthesizing System

A cell pellet containing 1.2 x 10^8 cells was resuspended in a solution of 0.25 M sucrose - 0.003 M $CaCl_2$ by gentle pipetting. The cell suspension was homogenized in a Dounce homogenizer (50 strokes with a tight-fitting pestle). The homogenate was diluted to 6 ml with additional sucrose-$CaCl_2$ solution.

Nuclei were separated from cytoplasm by centrifuging for 8 min at 800 x G. The cytoplasmic supernatant was completely decanted and saved. The nuclei were resuspended in various solutions as indicated.

One-half ml samples (extracts from 1 x 10^7 cells) were incubated for 20 min at 37° with 0.5 ml of the following solution: 0.2 M sucrose, 0.02 glucose, 0.002 M $MgCl_2$, 0.02 M NaCl, 0.001 M ATP, 0.001 M phosphoenolpyruvate, 0.01 M K_2HPO_4 (pH 7.5), and a labeled deoxyribonucleoside or deoxyribonucleotide (usually H^3-TTP, 15 c/mM, New England Nuclear Corp.) (Friedman and Mueller, 1968).

Incorporation was stopped by pelleting the nuclei at 1200 x G for 5 min. One ml of 0.3 N KOH was immediately added to the pelleted nuclei and digestion was performed at 37°. After 12 hr, the solution was cooled to 4° and 0.3 ml of 50% TCA-5% $Na_4P_2O_7 \cdot 10\ H_2O$ was added.

The precipitated DNA was rapidly swirled on a vortex mixer and fragmented into smaller, more manageable pieces. These pieces were pelleted at 1200 x G for 30 min at 4°. The supernatant was discarded and the DNA-containing pellet was redigested in 1.0 ml of 0.3 N KOH for 2 hr at 37°. The solution was cooled and the DNA was precipitated with TCA as before. The precipitated, radioactive DNA was collected on glass fiber filters and counted as previously described.

O. Protein Synthesis in Cell Compartments

At appropriate timepoints 1 x 10^7 cells were pulsed with tritiated leucine for one hr as previously described. The cells were swollen in Na^+ RSB (see DNA polymerase assay) for 5 min and then homogenized in a Dounce homogenizer (25 strokes, tight pestle). Nuclei were spun down at 800 x G for 10 min. The cytoplasmic supernatant was saved as a single fraction. The nuclear pellet was extracted with 0.2 N HCl for 6 hr at 4° and then the soluble "histone" fraction was separated from the insoluble "residual" protein by centrifugation at 1200 x G for 30 min. The residual protein was dissolved in 0.3 N KOH. Aliquots of the various protein fractions were precipitated by the addition of an equal volume of 50% TCA, collected on glass fiber filters, and assayed for radioactivity as previously described. Other aliquots were assayed for protein content by the method of Lowry et al. (1951).

P. Thin Layer Chromatography of Nucleosides and Nucleotides

MN Polygram Cel 300 PEI (cellulose polyethylenimine) thin layer sheets were obtained from Brinkmann Instruments Co. The sheets were given a preliminary wash in the following manner: a 10% solution of sodium chloride was allowed to rise to a height of 5 cm in the layer, and then, without intermediate drying, the layer was developed in distilled water to the upper edge. The plate was dried in air, subjected to complete ascending development with distilled water, and redried (Randerath, 1966).

Carrier compounds were added as mixtures in 1 μl of distilled water. Samples were applied in 5 μl or 10 μl volumes. Continuous, gradient development of the plate was carried out: 1.0 M acetic acid to 4 cm, then 0.2 M LiCl for 2 min, 1.0 M LiCl for 6 min, and finally 1.6 M LiCl to 13 cm.

Nucleotides were located by UV fluorescence-quenching after the plate had been dried with a hair dryer. Areas containing nucleotides were cut out. These small pieces of cellulose polygram plastic sheet were placed in glass scintillation vials and dried at 80-90^{o} for several hr. The samples were counted in a Packard Tricarb Scintillation Spectrometer in 10 ml of scintillation fluid. Counting efficiencies were comparable to those obtained with glass fiber filters.

Q. Protein Determinations

Protein determinations were performed using the Lowry modification of the Folin-phenol reaction (Lowry *et al.*, 1951). Pentex brand bovine serum albumin was used for a standard. Concentrated standard solutions (1-2 mg) were stable for over three months if stored at 4^{o}.

R. DNA Determinations

DNA determinations were performed using the Burton modification of the diphenylamine reaction (Burton, 1956). Salmon sperm DNA was used for standard solutions.

III. RESULTS

A. Mengovirus Infection of L Cells--Viral Replication and Inhibition of Cellular DNA and Protein Synthesis

The growth kinetics of the small plaque variant of mengovirus are depicted in Figure 4. After a latent period of 4 hours, virus production increases exponentially, and by 7 hours the yield approaches 100 PFU/cell. New virus continues to be produced until the cells begin to lyse at 9 to 10 hours p.i. These growth kinetics were previously described by Amako and Dales (1967).

Rates of protein and DNA synthesis in cells were determined with appropriate radioactive precursors at various times after infection (Fig. 4). Half-hour pulses were used. Inhibition of cellular DNA and protein synthesis develops between one and two hours after infection. DNA synthesis subsequently declines to low levels, but the overall rate of protein synthesis levels off after the 3rd hour, and shows a slight increase about 5 hours p.i. The inhibition of cellular DNA synthesis follows kinetics similar to those previously reported (Franklin and Baltimore, 1962). However, the onset of inhibition of protein synthesis in our experiments is one hour later than has been previously reported (Franklin and Baltimore, 1962; McCormick and Penman, 1967). This may reflect differences in the virus strains or in the conditions used for infection of cells.

Results of cell fractionation studies (Table III) show that synthesis of nuclear proteins in infected cells continues to decrease after 3.5 hours, whereas cytoplasmic protein synthesis levels off between 3.5 hours and 4.5 hours p.i. The decline in the synthesis of the two major nuclear protein fractions is evidence that cell-directed protein synthesis becomes progressively inhibited after infection. It has been shown previously that most of the protein synthesis taking place after the 3rd hour p.i. is of viral origin (Krug and Franklin, 1964; McCormick and Penman, 1968). The synthesis of virus-directed proteins in the cytoplasm interferes with precise determination of the synthesis of cytoplasmic proteins of the cell, but our results suggest that the synthesis of both nuclear and cytoplasmic proteins of the cell continues to decline even after 3.5 hours p.i.

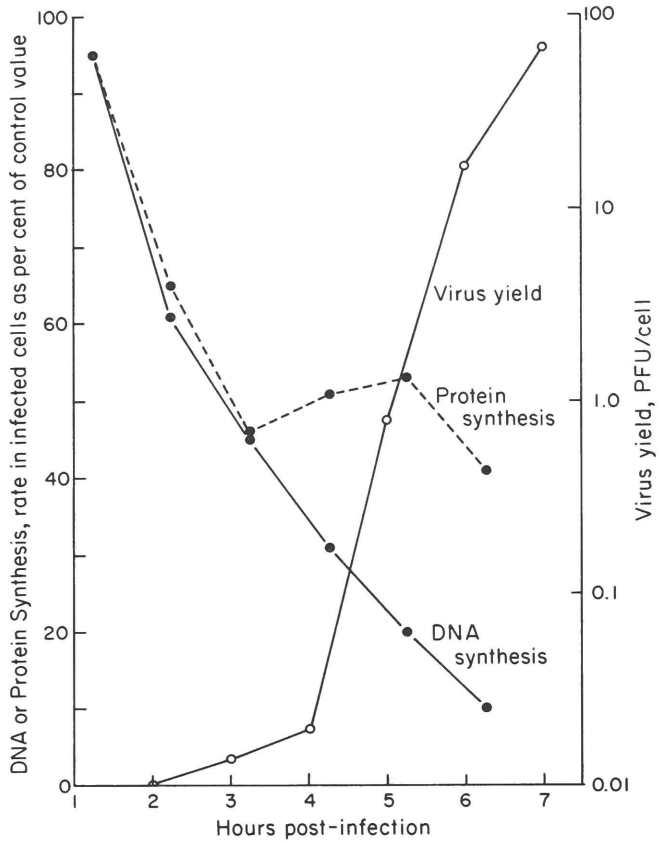


Figure 4. Mengovirus multiplication and the overall rates of protein and DNA synthesis in infected L cells. (Input multiplicity of 200 PFU/cell)

TABLE III

Localization and Rate of Protein Synthesis
in L Cells Infected with Mengovirus

Protein synthesis	Protein synthesis in uninfected cells, % of total	H^3-Leucine incorporated, specific activities of proteins, infected cells / uninfected cells			
		1.0 hr. p.i.[a]	2.5 hrs. p.i.	3.5 hrs. p.i.	4.5 hrs. p.i.
Cytoplasmic proteins	70	0.92	0.80	0.32	0.31
Nuclear histones	20	0.82	0.64	0.43	0.15
Nuclear residual proteins	10	1.04	0.78	0.54	0.28

[a] Post-infection; midpoint of one-hour pulse.

B. Newcastle Disease Virus Infection of L Cells--Viral Replication and Inhibition of Cellular DNA and Protein Synthesis

The kinetics of NDV growth and of inhibition of synthesis of DNA and proteins in infected L cells are shown in Figure 5. The results obtained in L cells are similar to those previously obtained in HeLa cells (Wheelock and Tamm, 1959, 1961a,b). The infective process is essentially non-productive in that less than one PFU per cell is produced in L cells infected at an input multiplicity of 100 PFU/cell. The main increase in virus takes place between 3 and 4 hours p.i. In electron microscopic studies we have seen large cytoplasmic aggregates of viral nucleoprotein by 6 hours p.i. Infected cells begin to swell and lyse after the 10th hour of infection.

The rates of protein and DNA synthesis were determined using half-hour pulses of appropriate radioactive precursors (Fig. 5). The onset of inhibition of cellular DNA synthesis is detectable by 2 hours p.i. Synthesis is inhibited 40% by 3 hours, and 90% by 5 to 6 hours p.i. Protein synthesis also becomes inhibited by 2 hours, and by 3 hours there is 30% inhibition. The rate of protein synthesis continues to decline, falling to 15% of the normal level in uninfected cells at 6 hours p.i.

Résumé

The inhibitions of cellular protein and DNA synthesis take place concurrently in NDV and mengovirus infections. In view of this temporal correlation it appeared desirable to investigate the possibility of a causal relationship between inhibition of cellular protein synthesis and inhibition of DNA synthesis. The effects of three chemical inhibitors of protein synthesis on cellular DNA synthesis were therefore determined.

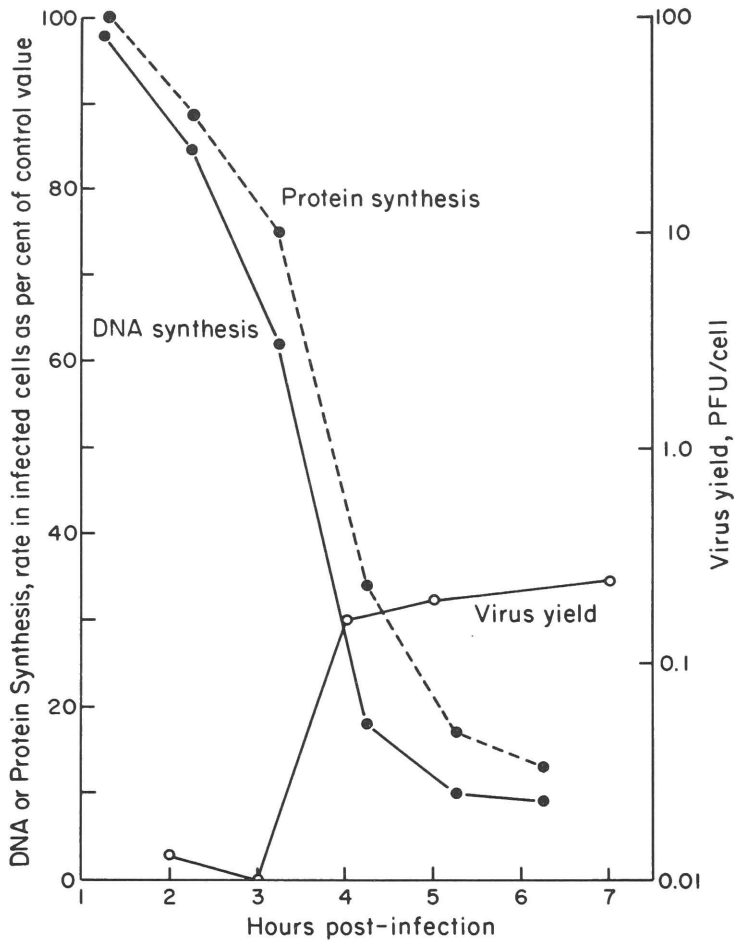


Figure 5. Newcastle disease virus multiplication and the overall rates of protein and DNA synthesis in infected L cells. (Input multiplicity of 200 PFU/cell)

C. Effects of Chemical Inhibitors of Protein Synthesis upon L Cell DNA Synthesis

Puromycin is an analog of aminoacyl(tyrosyl)-transfer-RNA and its incorporation into nascent peptide chains causes premature chain termination and release (Yarmolinsky and de la Haba, 1959; Williamson and Schweet, 1965). Treatment of L cells with puromycin at a concentration of 200 μg/ml causes 90% inhibition of protein synthesis within 30 minutes (Fig. 6, upper-left). DNA synthesis is inhibited by 75% in this interval (Fig. 6, lower-left). Comparison with the effects of puromycin at 20 μg/ml suggests that a correlation exists between the extent of protein synthesis remaining in treated cells and the residual level of DNA synthesis. However, while the rate of protein synthesis remains stable at a reduced level established within 30 minutes from addition of puromycin, the rate of DNA synthesis continues to fall.

Cycloheximide rapidly inhibits peptide chain elongation without premature chain termination or polysome breakdown (Felicetti *et al.*, 1966; Columbo *et al.*, 1966). Cycloheximide is a more potent inhibitor of protein synthesis than puromycin. Protein synthesis is inhibited more than 90% within 30 minutes from addition of cycloheximide to a concentration of 5 μg/ml (Fig. 6, upper-right). DNA synthesis is inhibited 75% in this interval (Fig. 6, lower-right). Similar effects are obtained with cycloheximide at a final concentration of 50 μg/ml. Other experiments have shown that DNA synthesis becomes 75% inhibited already within 5 minutes after addition of cycloheximide. The residual DNA synthesis declines gradually over the course of 2 to 3 hours.

p-Fluorophenylalanine (FPA) is incorporated into proteins in place of phenylalanine (Munier and Cohen, 1959). Treatment of L cells with FPA at 200 μg/ml inhibits cellular protein and DNA synthesis within 30 minutes (Table IV). The inhibition is not as marked as that obtained with puromycin or cycloheximide. In FPA-treated cells protein synthesis levels off at 50% of the control value, whereas DNA synthesis continues to decline even after the first 30 minutes of treatment (Table IV). Similar relationships are depicted in Figure 7.

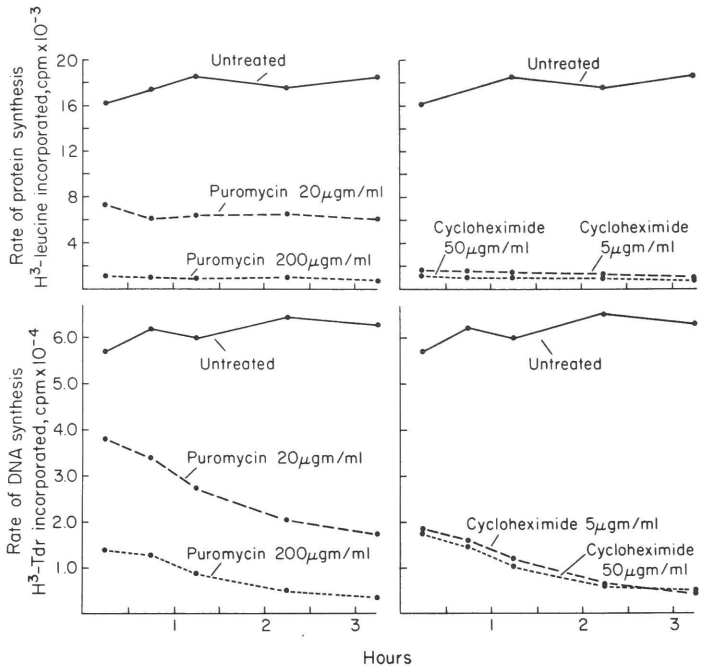


Figure 6. Effect of puromycin or cycloheximide treatment upon L cell protein and DNA synthesis. (Values are plotted at the mid-points of the half-hour intervals over which pulses were given. Drug treatment was initiated at time zero.)

TABLE IV

Inhibition of L Cell DNA and Protein Synthesis
by p-Fluorophenylalanine

Period of Precursor Incorporation	Untreated		FPA, 200 µgm/ml	
	H^3-Thymidine incorporated, cpm	H^3-Leucine incorporated, cpm	H^3-Thymidine incorporated, cpm	H^3-Leucine incorporated, cpm
30-0 min. Pre-addition	282,000	24,000	279,000	23,000
0-30 min. Post-addition	301,000	22,000	200,000	11,000
1-1.5 Hrs. Post-addition	293,000	24,000	111,000	11,000

All cells in Eagle's spinner medium without phenylalanine but with 7% undialyzed fetal bovine serum.

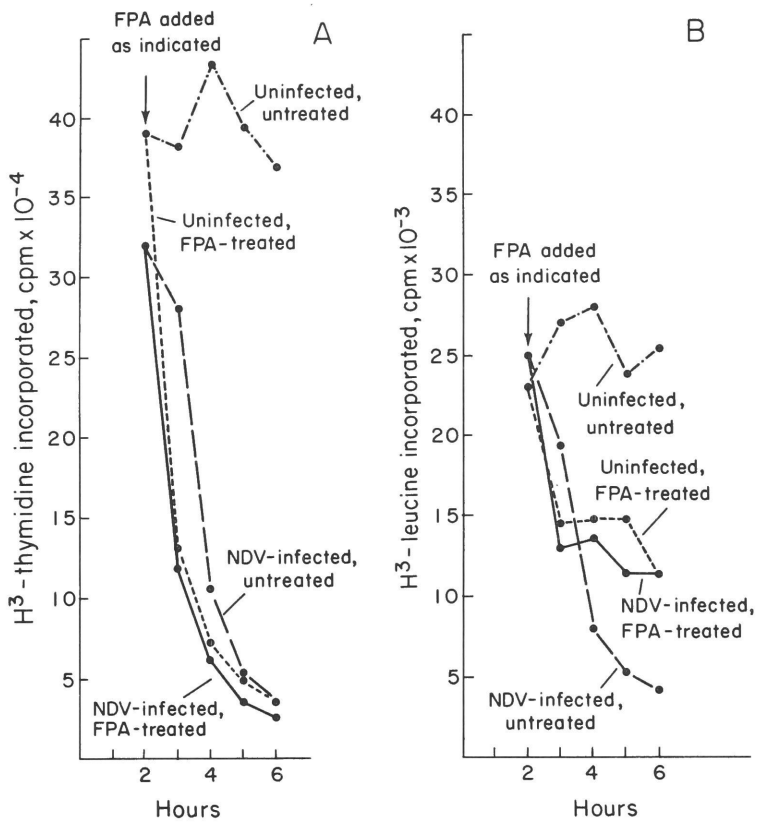


Figure 7. Effect of p-fluorophenylalanine treatment upon DNA synthesis and protein synthesis in uninfected and Newcastle disease virus-infected L cells. FPA was added to a final concentration of 200 μg/ml. Cells were grown in Eagle's spinner medium without phenylalanine but with 7% undialyzed fetal bovine serum. (Input multiplicity of 200 PFU/cell)

In addition, Figure 7 shows that FPA can block the viral inhibition of cellular protein synthesis to some extent. However, possible blocking of viral inhibition of cellular DNA synthesis by FPA could not be determined, as treatment with FPA inhibits cellular DNA synthesis to the same extent as does viral infection.

Résumé

Puromycin and cycloheximide inhibit protein synthesis through well-defined effects on the translational machinery (Williamson and Schweet, 1965; Felicetti et al., 1966; Columbo et al., 1966). Treatment of cells with these inhibitors of protein synthesis causes nearly immediate and marked inhibition of cellular DNA synthesis. On continued treatment, the residual DNA synthesis declines further. Thus it would seem that a major part of ongoing DNA synthesis requires concurrent protein synthesis. This need for concurrent protein synthesis could reflect a continual need for certain structural proteins in chromatin. However, we prefer to view the kinetics of DNA inhibition in terms of the model of in vivo regulation considered in the Introduction. It may be postulated that DNA synthesis continues after protein synthesis is inhibited only until accumulated initiator proteins are consumed, which means only until synthesis within active replication sections has been completed.

The fact that FPA treatment inhibits DNA synthesis indicates that the (initiator) protein necessary for DNA synthesis has highly specific structural constraints. Certain enzymes are unaffected by FPA substitution (Westhead and Boyer, 1961); but, on the other hand, the translation-inhibitory antiviral protein produced in response to interferon treatment of cells is inactivated by FPA substitution (Dianzani et al., 1969). This indicates that protein synthesis by itself is not sufficient to support ongoing DNA synthesis if the proteins produced contain FPA instead of phenylalanine, and are faulty.

The results of these studies with chemical inhibitors of protein synthesis underscore the possibility that the inhibition of DNA synthesis in NDV and mengovirus infections may be secondary to the inhibition of protein synthesis. Direct viral effects on the

biosynthetic machinery of DNA synthesis are also possible, of course. However, primary inhibition of cellular protein synthesis would be sufficient to cause the observed inhibition of cellular DNA synthesis in NDV- or mengovirus-infected cells.

D. Reovirus Infection of L Cells--Viral Replication and Inhibition of Cellular DNA Synthesis

The kinetics of reovirus multiplication were determined in monolayer (Fig. 8) and suspension (Fig. 9) cultures of L cells. In both experimental systems the latent period of viral growth is 6 hours, after which virus increases nearly exponentially for 6-8 hours in monolayer and 4 hours in suspension cultures. Cytoplasmic paracrystalline aggregates of virus particles form a perinuclear ring encompassing infected cell nuclei which is detectable by 10 hours p.i. in the phase-contrast or electron microscope.

Reovirus 3 reportedly has different effects on host L cell protein synthesis depending upon whether the infected cells are grown in suspension or monolayer. A previous autoradiographic study has shown that there is no demonstrable inhibition of protein synthesis during reovirus infection of L cells in monolayer (Gomatos and Tamm, 1963). However, the results of a subsequent study on cells in suspension culture suggested that reovirus infection causes some inhibition of protein synthesis (Kudo and Graham, 1965). In both systems, cellular DNA synthesis became inhibited during infection.

We were interested in determining why inhibition of protein synthesis was seen only in reovirus-infected cells grown in suspension cultures. To insure uniformity of cellular and viral growth conditions in all aspects except for monolayer or suspension maintenance of cells, experiments were carried out as follows: cells in suspension were inoculated with purified reovirus at an input multiplicity of 100 PFU/cell. Two hours after inoculation, half of the cells in suspension culture were plated onto dishes in which they attached and formed monolayers within 30 minutes. At appropriate times thereafter cells were pulsed for 30 minutes to measure the rates of DNA and

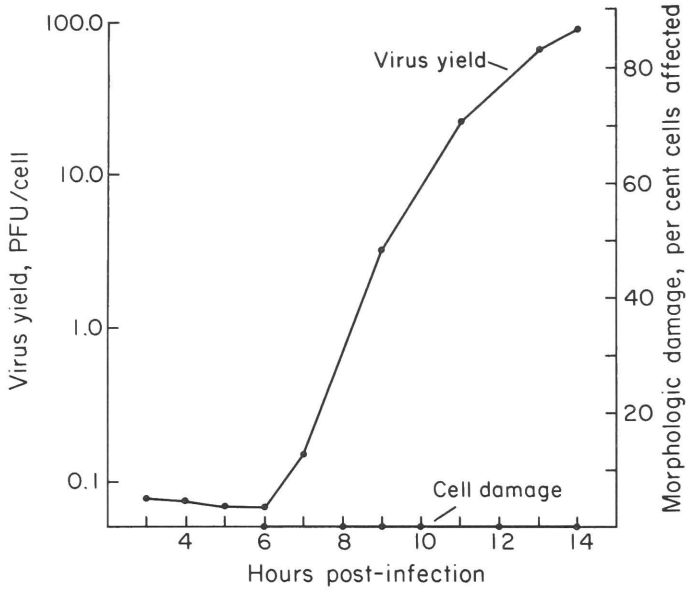


Figure 8. Growth and cytopathic effects of reovirus type 3 in monolayer cultures of L cells. (Input multiplicity of 100 PFU/cell)

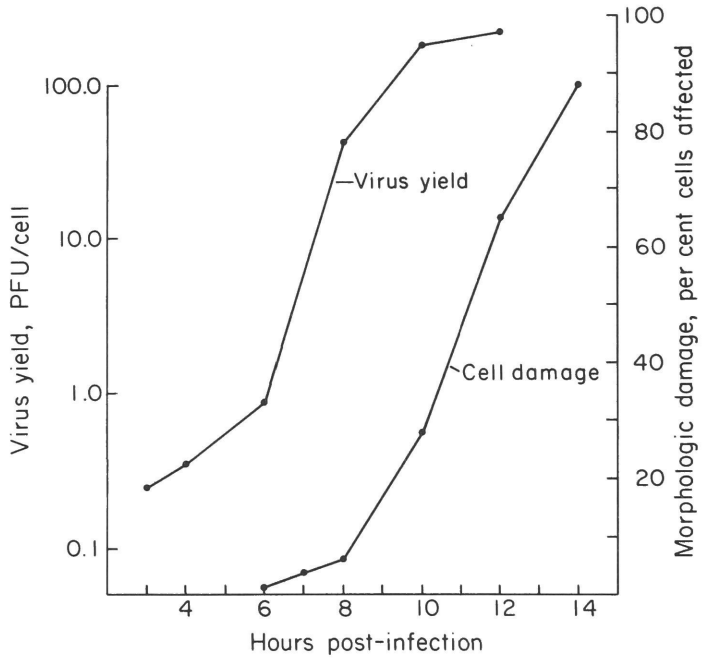


Figure 9. Growth and cytopathic effects of reovirus type 3 in suspension cultures of L cells. (Input multiplicity of 100 PFU/cell)

protein synthesis in suspension and monolayer. Figure 10 shows that in infected suspension cells, the rates of DNA and protein synthesis begin to decrease noticeably after the 8th hour of infection. By 12 hours p.i. DNA synthesis is inhibited 80%, while protein synthesis is inhibited only 40%. Figure 11 displays the results obtained from sister cells maintained in monolayer from 2 hours p.i. on. The inhibition of DNA synthesis is quite similar to that found in suspension cells except that it becomes detectable somewhat earlier, i.e. at 7 hours p.i. On the other hand, no inhibition of protein synthesis is observed in the infected monolayer culture even at 12 hours p.i. This contrasts with the results in suspension culture where protein synthesis is 40% inhibited at 12 hours p.i.

Microscopic examination indicated that infected cells in suspension culture show cytopathic changes earlier than cells in monolayer. Determinations were therefore made of the number of damaged cells by use of trypan blue exclusion and hemacytometer cell counts. It was found that the proportion of cells in the infected suspension culture unable to exclude dye rapidly increased after the 8th hour of infection. By 12 hours p.i. more than 60% of the cells in the infected suspension culture are damaged, i.e. either lysed or cannot exclude trypan blue (Fig. 9). Half of the damaged cells at 12 hours p.i. are completely lysed, a figure which corresponds to the decreased rate of protein synthesis depicted in Figure 10. Similar examination of infected monolayer cultures did not reveal any demonstrable cell damage through 14 hours p.i. (Fig. 8).

It has been demonstrated in mengovirus-infected L cells that overall protein synthesis levels off or displays a late increase when viral protein synthesis replaces cell-directed synthesis which has become inhibited (Fig. 4). Cell fractionation studies similar to those performed on mengovirus-infected cells were also performed on reovirus-infected L cells grown in monolayer. The results (Table V) do not reveal any appreciable decrease in the synthesis of proteins in any fraction through 12.5 hours p.i. Cell-directed synthesis, as reflected in the nuclear fractions, appears to be unaffected by reovirus infection.

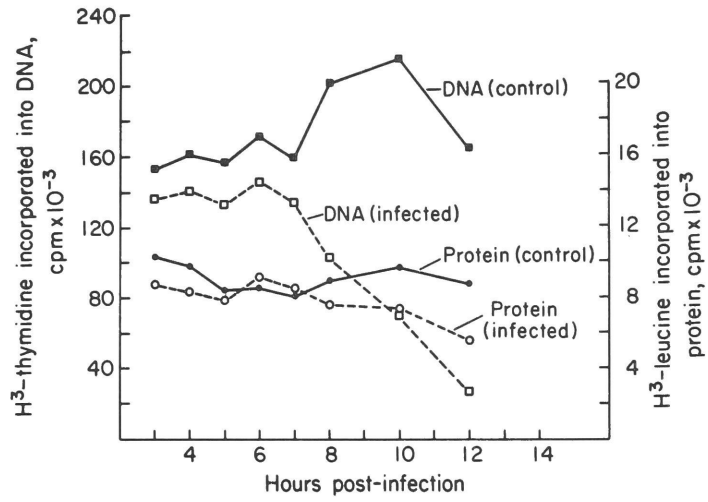


Figure 10. Effect of reovirus infection upon the overall rates of protein and DNA synthesis in suspension cultures of L cells. (Input multiplicity of 100 PFU/cell)

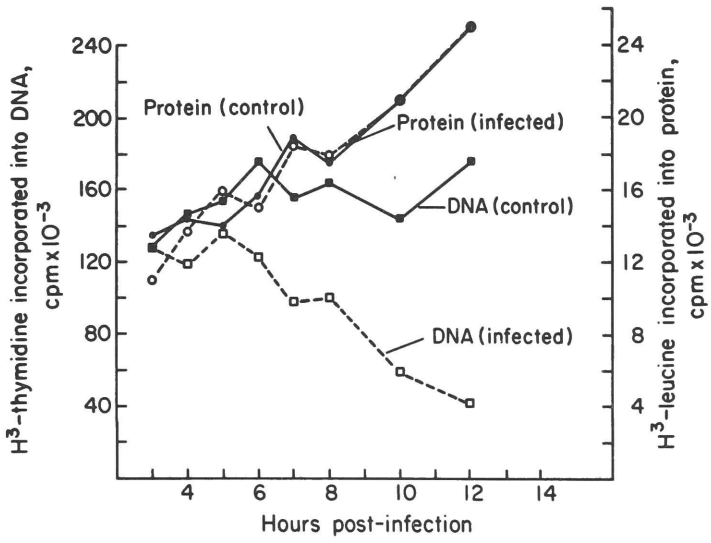


Figure 11. Effect of reovirus infection upon the overall rates of protein and DNA synthesis in monolayer cultures of L cells. (Input multiplicity of 100 PFU/cell)

TABLE V

Localization and Rate of Protein Synthesis
in L Cells Infected with Reovirus

Protein synthesis	H^3-Leucine incorporated, specific activities (cpm/mg protein), infected cells / uninfected cells			
	4.5 hrs. p.i.[a]	8.5 hrs. p.i.	10.5 hrs. p.i.	12.5 hrs. p.i.
Cytoplasmic proteins	1.04	1.08	0.94	0.93
Nuclear histones	0.99	1.06	0.98	0.93
Nuclear residual proteins	1.02	0.99	0.97	0.91

[a] Post-infection; midpoint of one-hour pulse.

Résumé

Reovirus 3 grows equally well in monolayer and suspension cultures of L cells. In infected monolayer cultures there is no detectable loss of cell integrity through 14 hours p.i. In contrast, infected suspension cells begin to swell and lyse after 8 hours of infection. These findings suggest that the inhibition of protein synthesis in infected suspension cells, reported by Kudo and Graham (1965), can be accounted for by cell loss. There is no inhibition of protein synthesis in infected cultures of monolayer cells in which cellular integrity is maintained. Fractionation studies on such cells indicate that there is no evident inhibition of cellular protein synthesis as might be revealed through effects on the synthesis of nuclear proteins of the cell. It appears that the inhibition of DNA synthesis during reovirus infection is specific and does not result from inhibition of cellular protein synthesis. This distinguishes reovirus inhibition of DNA synthesis from the inhibition during NDV and mengovirus infection where there is concurrent inhibition of protein synthesis.

E. Viral-Induced Inhibition of Cellular DNA Synthesis in Synchronized Cell Cultures

We have previously pointed out in the Introduction that cellular DNA synthesis is confined to one period, S, in the cell life cycle. Cells are distributed throughout all phases of the cell cycle in exponentially growing, asynchronous cultures. Asynchronous cultures may be unknowingly partially synchronized through technical manipulations and this may produce spurious results in metabolic-kinetic studies. Cell cultures can be synchronized, using reversible blockage of DNA synthesis with FUdR or with excess thymidine, so that all cells undergo the DNA replication phase simultaneously (Materials and Methods). The kinetics of viral inhibition of DNA synthesis can be studied by following DNA synthesis after reversal of these chemical blocks. Such experiments have been done to determine more precisely the kinetics of inhibition of cellular DNA synthesis in virus-infected cells. In all of the experiments described below, cumulative

incorporation of a DNA precursor was measured. Viral inhibition of DNA synthesis is represented by a divergence of the curves for infected cells from the more sharply rising curves for uninfected-control cells.

Suspension cells were synchronized so that essentially all cells were held at the G1-S interface with FUdR. Virus stock solutions were added directly to the cell suspensions to produce a final multiplicity of 200 PFU/cell. The FUdR block was then circumvented by the addition of exogenous thymidine and the cumulative incorporation of H^3-cytidine into DNA measured. The incorporation curves for synchronized cells infected with NDV or mengovirus are presented in Figure 12. Inhibition is evident in infected cells by 2 hours and becomes increasingly more complete so that there is very little additional incorporation of labeled cytidine into DNA in infected cells after 4 hours of infection. These results are in agreement with the kinetic data obtained using unsynchronized, pulse-labeled cells. Furthermore, a colorimetric measurement (diphenylamine reaction) of the amount of DNA synthesized in synchronized, infected cells (Table VI) confirms the radioactive incorporation findings, i.e. both techniques show that there is approximately a 50% reduction in the amount of DNA made in infected cells as compared to uninfected cells under these conditions.

More extensive studies were performed on synchronized cells infected with reovirus. Purified, concentrated reovirus was added to cell cultures either before or at the time of release from a DNA synthetic block. Figure 13 presents the results of synchronization with FUdR and Figure 14 the results utilizing a block of excess thymidine. In both instances, the inhibition of cellular DNA synthesis becomes clearly evident between 4 and 5 hours after infection and release from the block. It is apparent that infection 3 hours before release results in a more marked and rapid inhibition after release as compared to the effect of infection at the time of release. It should be noted that, in either case, synthesis of DNA in infected cells ceases at about the same time as in uninfected cells, i.e. by 5 to 6 hours after release. The lowered rate of DNA synthesis in

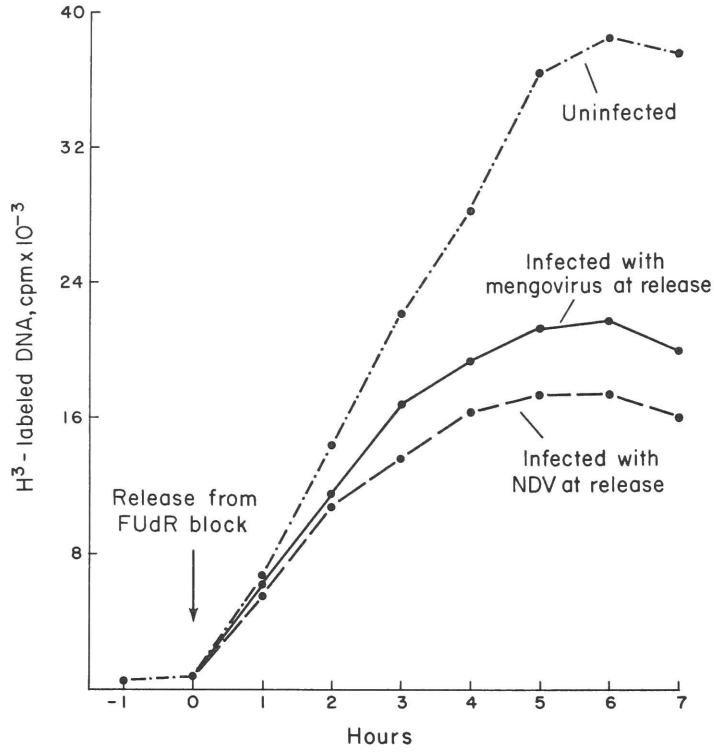


Figure 12. DNA synthesis in synchronized cultures of L cells during mengovirus and Newcastle disease virus infection. (Input multiplicity of 200 PFU/cell)

TABLE VI

Viral Inhibition of DNA Production as Measured by the Diphenylamine Reaction

Synchronized L cells[a]	Amount of DNA, µg/5x10^6 cells	DNA increase during 6 hr after release, µg/5x10^6 cells	DNA increase in infected cells, % of uninfected control
Suspension cultures			
Uninfected:			
Not released	110		
Released	185	75	
Newcastle disease virus[b]	145	35	47
Mengovirus[b]	140	30	40
Monolayer cultures			
Uninfected:			
Not released	130		
Released	235	105	
Reovirus[c]	130	0	0

[a] Synchronized with FUdR, released from FUdR block with thymidine.
[b] Infected at time of release.
[c] Infected at 9 hours pre-release.

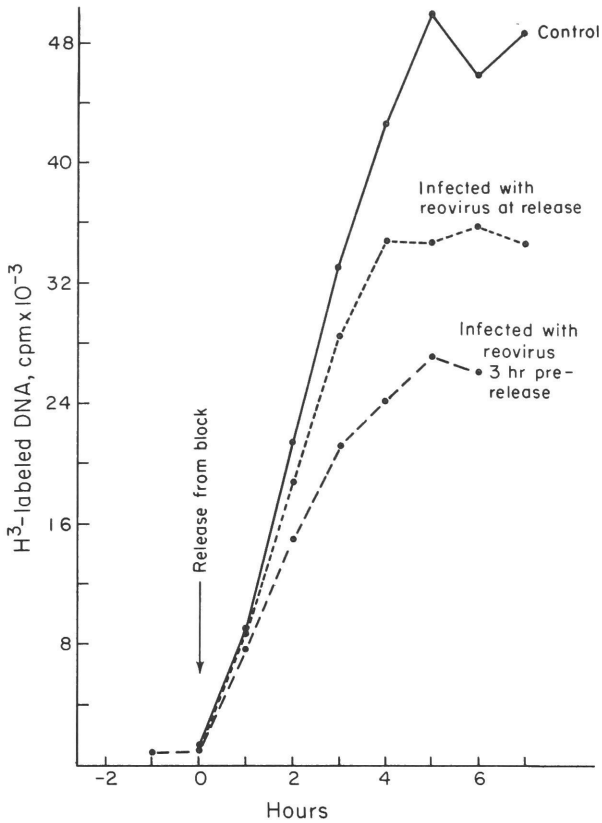


Figure 13. DNA synthesis in synchronized cultures of L cells during reovirus infection (a). (Input multiplicity of 100 PFU/cell)

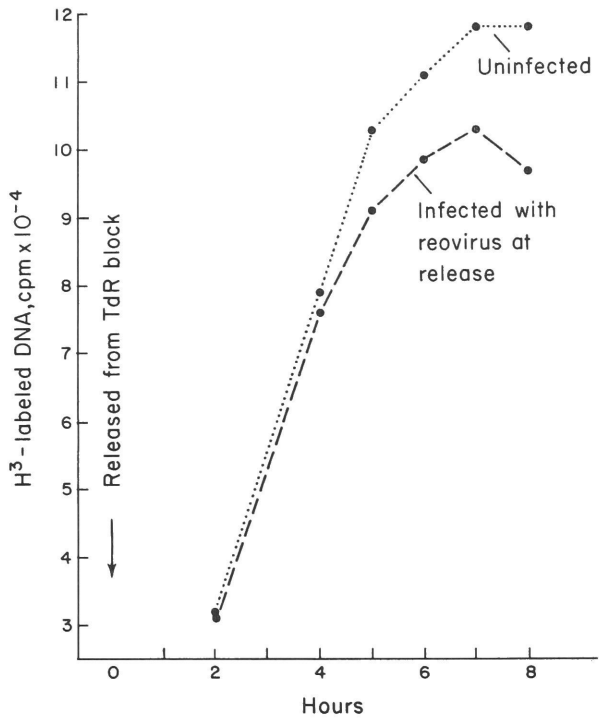


Figure 14. DNA synthesis in synchronized cultures of L cells during reovirus infection (b). (Input multiplicity of 100 PFU/cell)

infected cells apparently does not extend the time interval of S phase. Even though viral processes themselves may not completely inhibit DNA synthesis in 5 to 6 hours, complete inhibition is detected in cells infected at time of release and may reflect the cooperation of a cellular control mechanism involved in delimiting the S phase. When cells are infected 6 hours before release from an FUdR block (Fig. 15), they still can synthesize a significant amount of DNA during the first hour after release, but not later. Cells infected 4 hours before release make close to normal amounts of DNA for the first two hours after release, but incorporation declines rapidly thereafter (Fig. 15). However, a colorimetric measurement (diphenylamine reaction) of the amount of DNA synthesized in synchronized, infected cells demonstrates that essentially no new DNA is produced in cells infected 9 hours prior to release (Table VI).

Résumé

The overall kinetics of inhibition of DNA synthesis in NDV- or mengovirus-infected synchronized cultures of L cells are similar to the kinetics in infected asynchronous cultures. However, the time course of inhibition in synchronized reovirus-infected cells reveals novel features. DNA synthesis in asynchronous cells was previously shown to be inhibited only at 7 to 8 hours p.i. Inhibition can be detected as early as 4 hours p.i. in synchronized cells infected at time of release from a block in DNA synthesis. In addition, synthesis in cells infected 6 hours before release can exceed that predicted from the kinetics of inhibition in cells infected at time of release. The ramifications of these findings will be discussed later.

Finally, it should be pointed out that the results of a colorimetric assay for DNA confirm the results of radioactivity measurements. Lack of precursor incorporation thus directly reflects inhibition of DNA synthesis in all three virus infections.

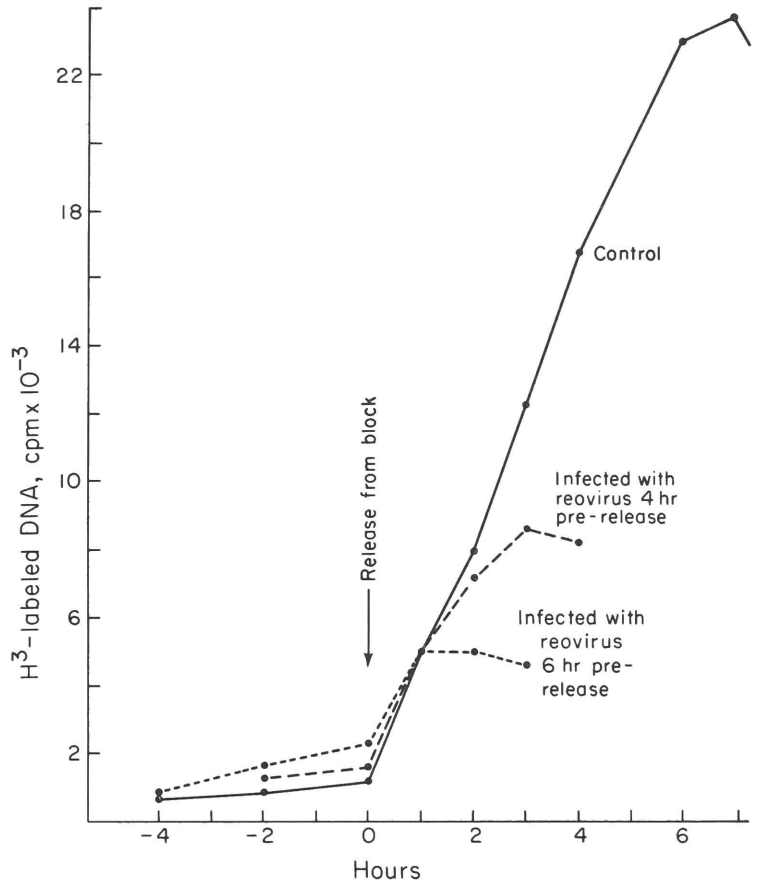


Figure 15. DNA synthesis in synchronized cultures of L cells during reovirus infection (c). (Input multiplicity of 100 PFU/cell)

F. Activities of Selected Enzymes Involved in DNA Synthesis

The *in vitro* (extract) activities of DNA polymerase and of most of the enzymes intimately related to thymidine triphosphate production (Table VII) correlate closely with cell proliferation (Bollum and Potter, 1959; Smellie, 1961; Lieberman *et al.*, 1963; Kara and Weil, 1967; Maley and Maley, 1960). Adequate levels of these enzymatic activities are apparently a requirement for DNA synthesis. The possibility was investigated that virus infection might in some way drastically reduce these essential enzyme activities and thereby inhibit DNA synthesis.

1. TdR-, TMP-, and TDP-Kinase Activities

Suspension cells were directly inoculated with stock solutions of NDV, mengovirus, or reovirus 3 at a multiplicity of 200 PFU/cell. At various times after infection, aliquots of cells were removed and cell-free extracts were assayed for their abilities to produce TMP from TdR (Table VIII) and TTP from TMP (Table IX). There is no decline in these enzyme activities in NDV or mengovirus infection at 3.5 hours p.i., by which time cellular DNA synthesis has been already inhibited 50%. At 5.5 hours, DNA synthesis has declined 90% in NDV- or mengovirus-infected cells, yet these enzyme activities have decreased only 15-20%. This small decrease in activity could reflect the normal decay of the enzymes as a result of the earlier virus-induced inhibition of cell protein synthesis.

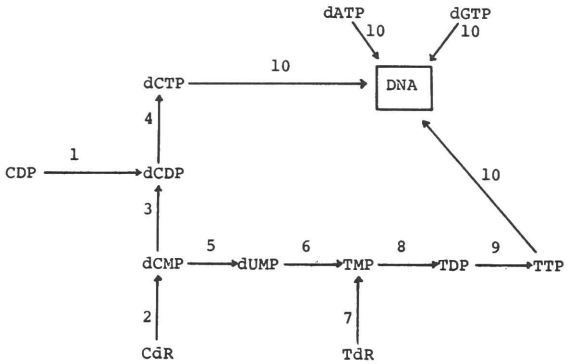
In reovirus-infected cells there is no detectable change in these enzyme activities at 8 hours p.i., by which time DNA synthesis has been inhibited 30%.

2. dCMP-Deaminase Activities

Suspension cells were infected with NDV or mengovirus, and monolayer cells were infected with reovirus. dCMP-deaminase activity was determined in extracts of infected cells at various times after infection, and compared to that in uninfected control cells (Table X). It can be seen that viral infection does not lead to significant changes in this enzyme activity even when cellular DNA synthesis has

TABLE VII

Scheme for the Biosynthesis of Pyrimidine Deoxynucleotides and of DNA



1. CDP - reductase
2. CdR - kinase
3. dCMP - kinase
4. dCDP - kinase
5. dCMP - deaminase*
6. TMP - synthetase
7. TdR - kinase*
8. TMP - kinase*
9. TDP - kinase*
10. DNA polymerase*

*Enzymes whose activities were measured in infected L cells.

TABLE VIII

Thymidine Kinase Activity in Extracts of Virus-Infected L Cells

Cell extract	Thymidine monophosphate produced, mμmoles/min./mg protein			
	1.0 hr. p.i.[a]	3.5 hrs. p.i.	5.5 hrs. p.i.	8.0 hrs. p.i.
Uninfected	1.80	1.75	1.62	
Newcastle Disease Virus	1.75	1.58	1.17	
Mengovirus	1.79	1.58	1.50	
Uninfected		1.80	1.75	1.62
Reovirus		2.10	1.45	1.86

[a] Post-infection.

TABLE IX

Production of Thymidine Triphosphate from Thymidine Monophosphate in Extracts of Virus-Infected L Cells

Cell extract	Thymidine triphosphate produced, mμmoles/min./mg protein			
	1.0 hr. p.i.[a]	3.5 hrs. p.i.	5.5 hrs. p.i.	8.0 hrs. p.i.
Uninfected	7.8	7.8	8.6	
Newcastle Disease Virus	7.8	8.1	6.8	
Mengovirus	8.5	8.3	7.3	
Uninfected		7.8	7.7	8.5
Reovirus		8.0	6.1	7.2

[a] Post-infection.

TABLE X

Deoxycytidine Monophosphate Deaminase Activity in Extracts of Virus-infected L Cells

Cell extract	Deoxyuridine monophosphate produced, μμmoles/min./mg protein					
	1.0 hr. p.i.(a)	3.5 hrs. p.i.	5.5 hrs. p.i.	5.0 hrs. p.i.	10.0 hrs. p.i.	12.0 hrs. p.i.
Uninfected	25.7	23.3	22.4			
Newcastle Disease Virus	27.2	22.2	22.2			
Mengovirus	25.3	24.3	26.2			
Uninfected				25.5	27.3	21.8
Reovirus				31.6	21.5	18.7

(a) Post-infection.

decreased 80%, i.e. at 5.5 hours p.i. with NDV and mengovirus, and 12.5 hours p.i. with reovirus.

3. DNA Polymerase Activities

Suspension cells were infected with NDV or mengovirus, and monolayer cells were infected with reovirus 3. DNA polymerase activity was determined in extracts of infected and control cells at various times after infection (Table XI). This activity did not significantly decline even when cellular DNA synthesis had been inhibited 80%, i.e. at 5.5 hours p.i. with NDV and mengovirus, and 12.5 hours p.i. with reovirus.

Résumé

The results obtained in studies of enzymes in the DNA-synthetic pathway make it unlikely that virus-induced inhibition of cellular DNA synthesis is brought about through effects either on DNA polymerase or on enzymes in thymidine metabolism. Extracts of infected cells contain normal dCMP-deaminase activity, TdR-, TMP-, and TDP-kinase activities, and DNA polymerase activity even at times when DNA synthesis has been inhibited *in vivo*.

G. The Stability of Native, Double-Stranded Cellular DNA During Infection

Experiments were undertaken to determine the stability of cellular DNA during the period of virus-induced inhibition of DNA synthesis. Cells were infected with NDV, mengovirus, or reovirus and given H^3-thymidine to prelabel the cellular DNA before onset of inhibition. Native, double-stranded DNA was phenol-extracted from these cells late in infection when DNA synthesis was inhibited by 80-90%. This phenol-extracted DNA was analyzed on neutral sucrose gradients (Figs. 16 and 17). The DNA from NDV-, mengovirus-, and reovirus-infected cells and from uninfected cells sediments as a fairly sharp band between 70 S and 80 S. These results suggest that there is no detectable breakdown of native cellular DNA during infection with any of these viruses. However, the presence of virus-induced single-strand breaks in cellular DNA cannot be ruled out by this technique.

TABLE XI

DNA Polymerase Activity in Extracts of Virus-Infected L Cells

Cell extract	Thymidine triphosphate incorporated, μμmoles/min./mg protein					
	1.0 hr. p.i.[a]	3.5 hrs. p.i.	5.5 hrs. p.i.	5.0 hrs. p.i.	10.0 hrs. p.i.	12.0 hrs. p.i.
Uninfected	25.1	26.4	26.2			
Newcastle Disease Virus	25.0	26.4	25.0			
Mengovirus	24.6	24.0	24.2			
Uninfected				25.0	24.0	27.0
Reovirus				24.0	23.0	24.0

[a] Post-infection.

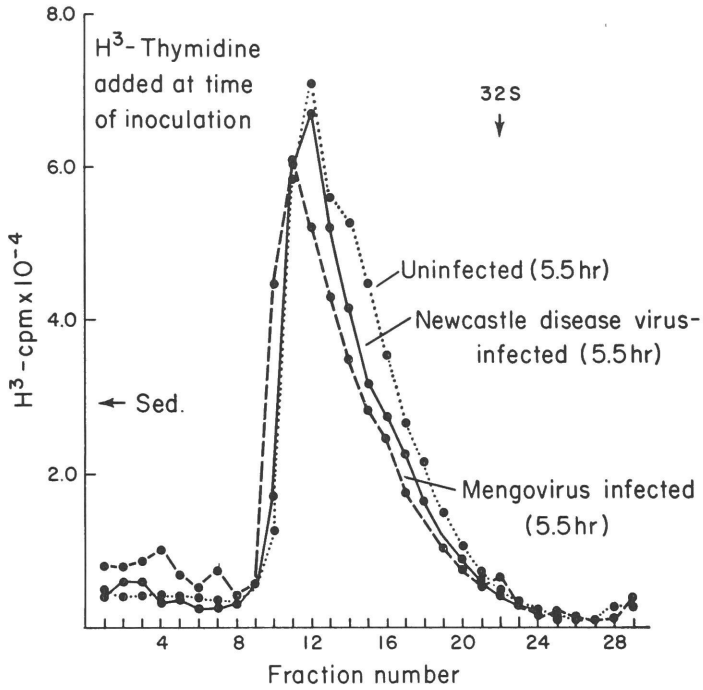


Figure 16. Double-stranded cellular DNA from mengovirus- and Newcastle disease virus-infected L cells. Labeled DNA was phenol-extracted from infected cells at 5.5 hr p.i. and subjected to rate zonal analysis on neutral sucrose gradients. (Input multiplicity of 200 PFU/cell)

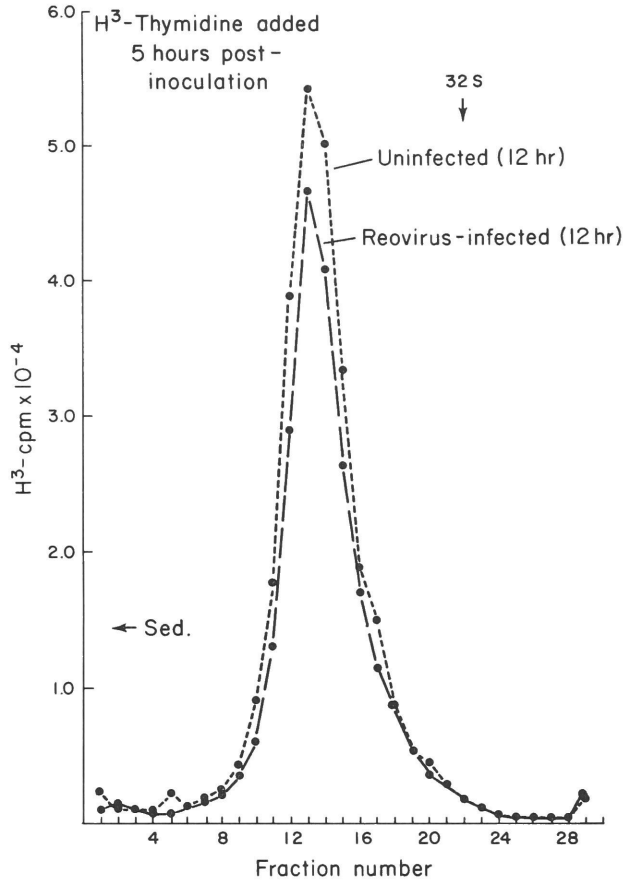


Figure 17. Double-stranded cellular DNA from reovirus-infected L cells. Labeled DNA was phenol-extracted from infected cells at 12 hr p.i. and subjected to rate zonal analysis on neutral sucrose gradients. (Input multiplicity of 100 PFU/cell)

H. DNA Chain Growth as Revealed in Pulse-Chase Experiments Followed by Alkaline Sucrose Gradient Analysis

1. Dynamics in Normal L Cells

Gentle lysis of cells on top of alkaline sucrose gradients removes protein from cell DNA and denatures it into single strands (Doerfler, 1969; Sambrook et al., 1968). Sedimentation then separates the DNA chains according to their size. When HeLa or Chinese hamster cells are given very short pulses of radioactive DNA precursors and the DNA analyzed in this manner, it is found that newly synthesized DNA exists as short chains (Painter, 1968; Taylor et al., 1968; Schandl and Taylor, 1969). Similar results have been obtained with L cells (Fig. 18). The L cell DNA produced during a 20-second pulse has a sedimentation coefficient smaller than 34 S. By one minute these short pieces have become larger and sediment around 34 S. During a 10-minute pulse most incorporation occurs into strands having sedimentation coefficients larger than 34 S but smaller than 100 S. When cells are pulsed for 10 minutes and given a chase of several hours or more (6 hours in Fig. 18) with unlabeled thymidine, the pulse-labeled DNA becomes converted into much larger DNA having a sedimentation coefficient of approximately 100 S. Estimates indicate that this 100 S DNA is 10 to 20 times larger than the DNA produced during one- to three-minute pulses and perhaps 100 times larger than that produced during a 20-second pulse. Although the mechanism of these conversions has not been completely analyzed, it seems reasonable to assume that chain elongation involves both DNA polymerase and ligase action (Introduction; Schandl and Taylor, 1969; Taylor, 1968b).

2. In Vivo Metabolic Requirements

Suspension cells were given a 10-minute pulse with H^3-deoxycytidine prior to the addition of FUdR (2 μM) with a chase consisting of a 100-fold excess of unlabeled deoxycytidine. As Figure 19 (top) shows, half of the daughter chains apparently can undergo further elongation to normal 100 S DNA during the 2.9 hour chase even with FUdR present. However, some of the chains, about half, do not undergo normal elongation to 100 S DNA when precursor addition is inhibited

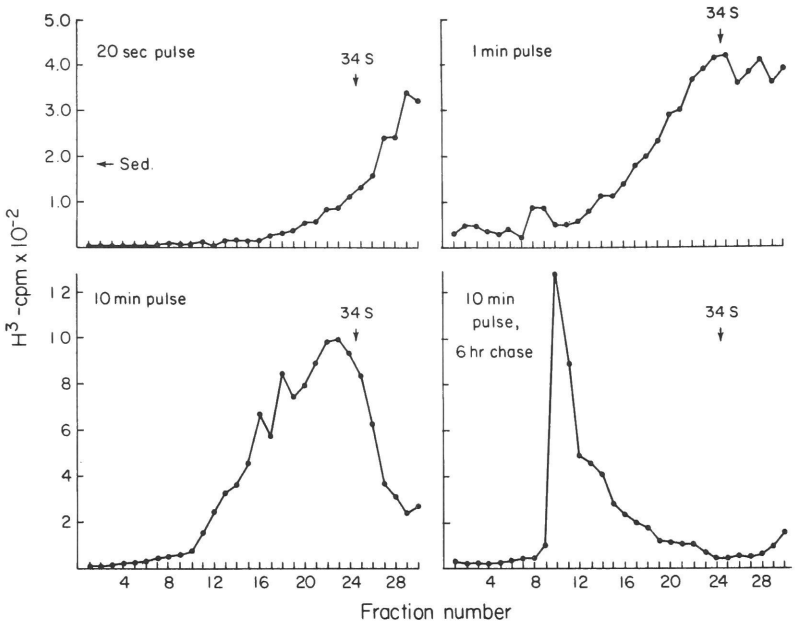


Figure 18. The growth of DNA chains *in vivo*. Labeled DNA from L cells was subjected to rate zonal analysis on alkaline sucrose gradients. Cells were pulsed with 10 μc/ml of H^3-TdR and chased with a 100-fold excess of unlabeled TdR. The number of cells per gradient was 5 x 10^5 (top) or 1 x 10^5 (bottom).

by FUdR. The elongation which does occur under FUdR inhibition probably results from ligase action. This finding will receive further attention in the Discussion Section.

In another experiment, suspension cells were pulsed for 3 minutes with radioactive deoxycytidine and then given FUdR. A 100-fold excess of unlabeled deoxycytidine was added as a chase and after 2.9 hours the DNA was analyzed on alkaline sucrose gradients. It was found that normal chain elongation does not occur under the conditions of starvation for endogenous TTP (Fig. 19, bottom). As the sedimentation profile indicates, no pulse-labeled DNA was found in the normal 100 S position after a 2.9-hour chase when FUdR was present.

In yet another experiment, cells were pulsed for 3 minutes with radioactive thymidine and deoxycytidine under conditions where protein synthesis was totally inhibited by cycloheximide treatment (50 μg/ml added when pulse was initiated). A 100-fold excess of unlabeled thymidine and deoxycytidine was added for a chase of 2.9 hours and the DNA was analyzed on alkaline sucrose gradients (Fig. 20). The resulting profile (broken line) shows that there was normal chain elongation with the production of DNA sedimenting at 100 S. The pattern seen in inhibited cells is also found under conditions where DNA synthesis is more completely inhibited by pretreatment for 10 minutes with cycloheximide before pulsing. Puromycin treatment gives a result identical to that of cycloheximide treatment.

Résumé

The effects of FUdR treatment on DNA chain elongation *in vivo* indicate that continued precursor incorporation (i.e. polymerization) is needed for normal elongation to occur. On the other hand, results of treatment with cycloheximide show that normal elongation can occur without protein synthesis. This seems to indicate that protein synthesis is not needed for ongoing polymerization *in vivo*. These results are compatible with the hypothesis presented in the Introduction, i.e. that protein synthesis is essential to a regulatory process *in vivo*, namely initiation of synthesis on new replication sections.

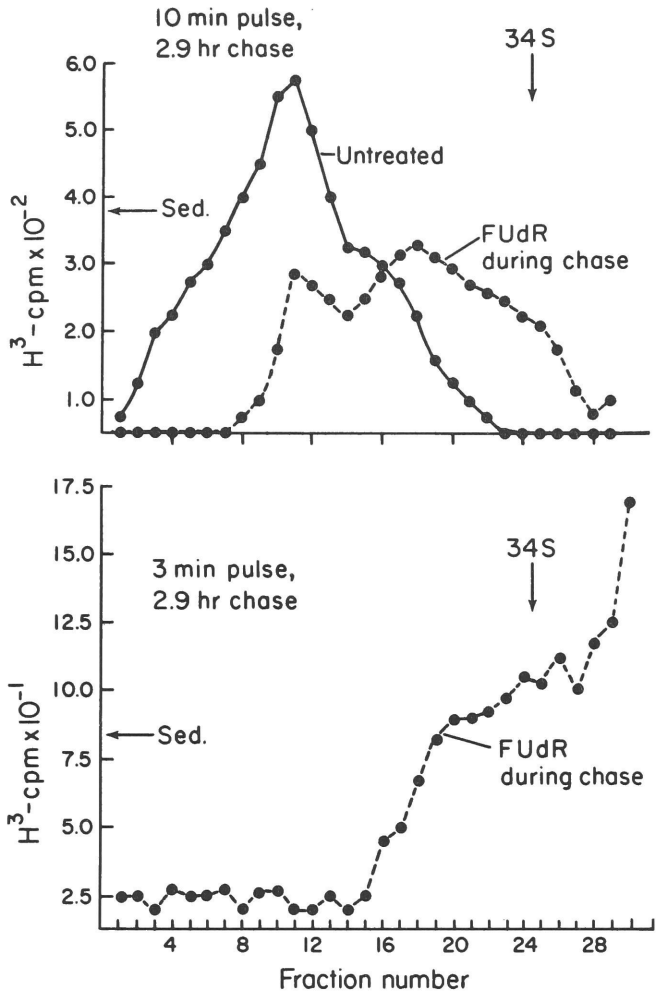


Figure 19. Effect of FUdR treatment upon DNA chain growth in vivo. The labeled DNA from drug-treated L cells was subjected to a rate zonal analysis in alkaline sucrose gradients. Cells (top, 2 x 10^5/gradient; bottom, 4 x 10^5/gradient) were pulsed with H^3-deoxycytidine at 10 μc/ml. Chase was effected with a 100-fold excess of unlabeled deoxycytidine. Where indicated, FUdR was added to a final concentration of 2 μM during the chase.

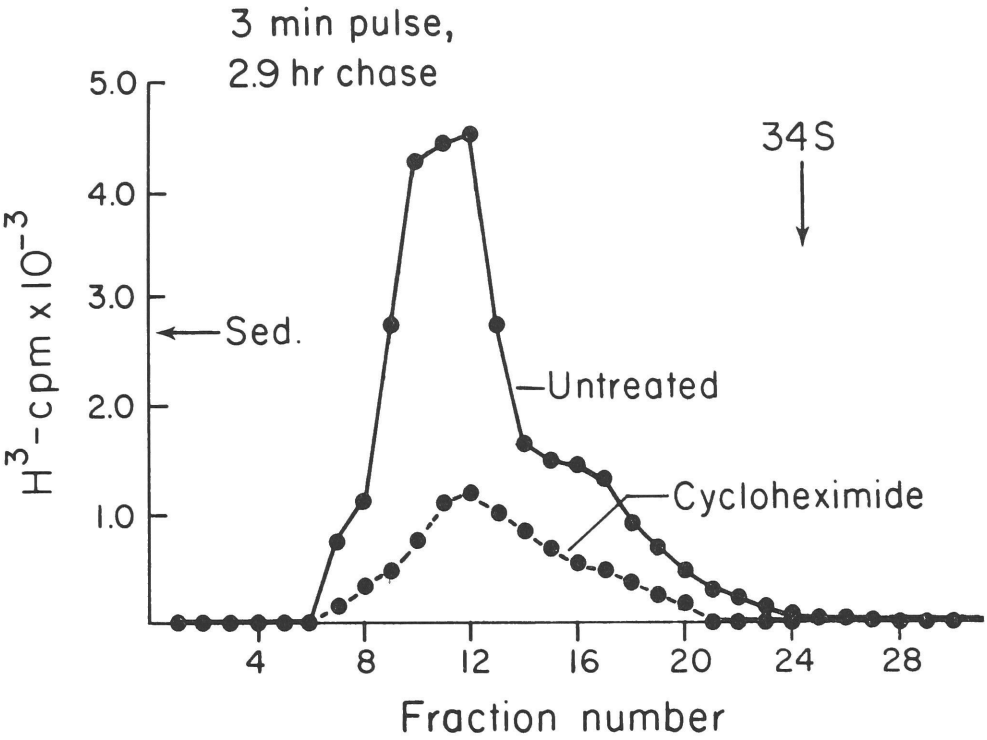


Figure 20. Effect of cycloheximide treatment upon DNA chain growth in vivo. Labeled DNA from drug-treated L cells was subjected to rate zonal analysis in alkaline sucrose gradients. Cells (1 x 10^5/gradient) were pulsed with 10 μc/ml of H^3-thymidine plus 10 μc/ml of H^3-deoxycytidine. Chase was effected with a 100-fold excess of unlabeled thymidine and deoxycytidine. Cycloheximide was added to 50 μg/ml at time of initiation of pulse.

3. Growth During Viral Inhibition of DNA Synthesis

Suspension cells were infected with each of the three viruses and pulsed for various periods. The elongation of DNA chains was measured using alkaline sucrose gradients (Figs. 21, 22, 23). Aliquots of cells were analyzed after 3- and 10-minute pulses, and after a 10-minute pulse with a 2.9-hour chase. Infected cells were pulsed at times after infection when inhibition was apparent but not so marked as to interfere with the radioactivity assay of DNA from gradients. Pulses were initiated at 3.5 hours p.i. in NDV- or mengovirus-infected cells and at 9 hours p.i. in reovirus-infected cells. Since nearly equal aliquots of cells were used, inhibition of DNA synthesis is shown by lowered specific activities, and thus by depressed curves for the gradients from infected cells.

In the experiment presented in Fig. 21, the radioactivity in the DNA from NDV-infected cells is greatly reduced compared to that from uninfected cells. Nevertheless, normal chain elongation occurs in the small amount of DNA which is synthesized. The DNA synthesized in infected cells is comparable in size to that synthesized in uninfected cells at each point of analysis. After a 10-minute pulse with a 2.9-hour chase, the DNA of infected cells sediments as rapidly as that from uninfected cells, that is, at 100 S. Similar patterns of normal elongation were found for mengovirus- and reovirus-infected cells (Figs. 22, 23). This normal pattern of elongation has been found in other experiments conducted later in infection when inhibition of cellular DNA synthesis was more marked.

Résumé

It appears that DNA elongation is normal in all three viral infections. Virus-induced inhibition of DNA synthesis does not affect chain elongation, as does a precursor block with FUdR. Virus-induced inhibition of DNA synthesis is reminiscent of cycloheximide inhibition in which ongoing polymerization is also not affected. We may conclude that the inhibition of cellular DNA replication during viral infection is not likely to be due to a block in precursor production or polymerization, since the time course of chain elongation is similar to that

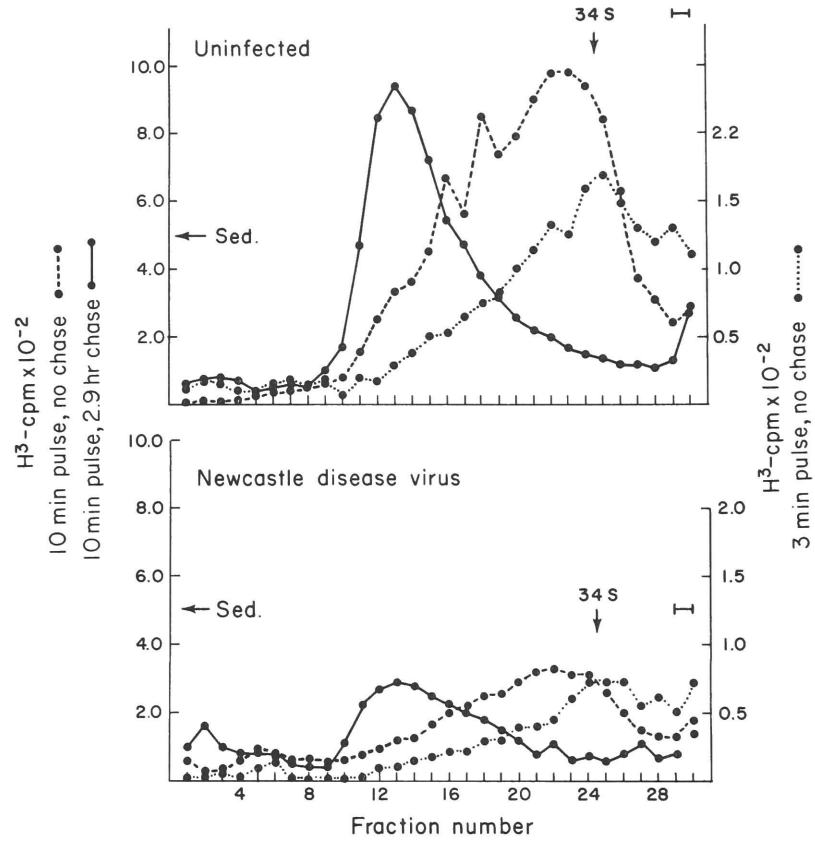


Figure 21. DNA chain growth in Newcastle disease virus-infected L cells. Labeled DNA from uninfected and infected cells (1 x 10^5/ gradient) was subjected to rate zonal analysis in alkaline sucrose gradients. Pulses, 10 μc/ml of H^3-TdR, were all initiated at 3.5 hr p.i. Chases were effected with a 100-fold excess of unlabeled TdR. (Input multiplicity of 200 PFU/cell)

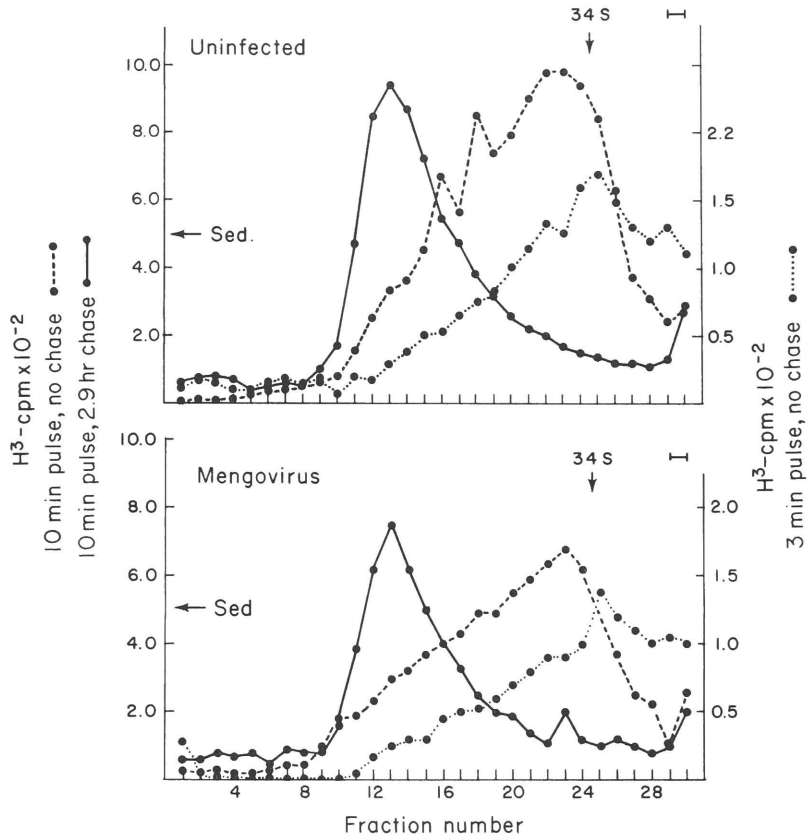


Figure 22. DNA chain growth in mengovirus-infected L cells. The conditions were similar to those of Figure 20 except that mengovirus was used instead of NDV.

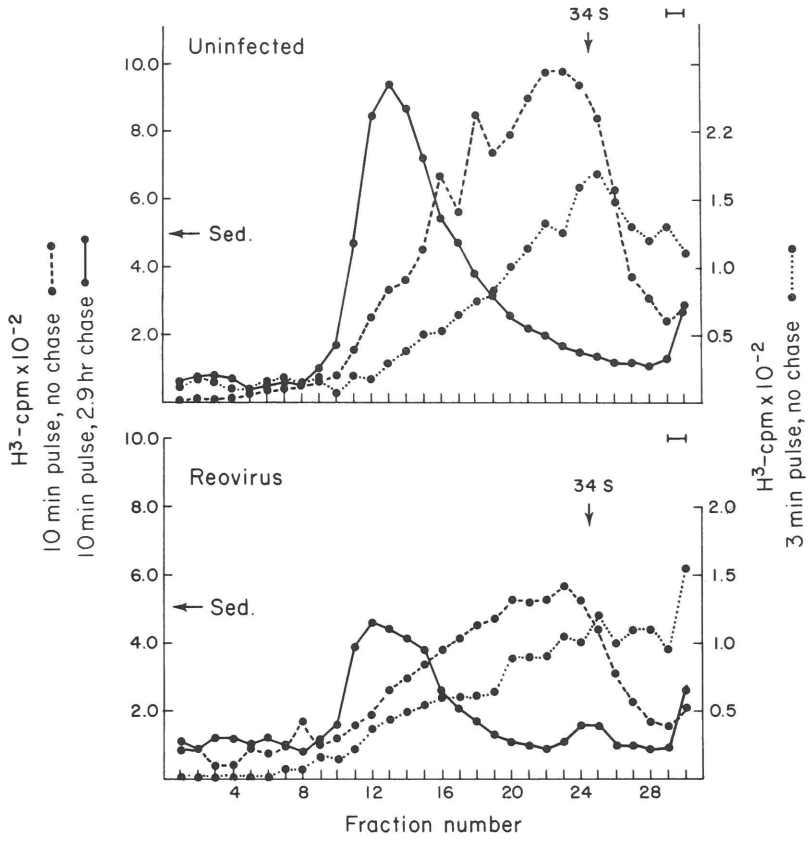


Figure 23. DNA chain growth in reovirus-infected L cells. The conditions were similar to those of Figure 20 except that pulses were initiated at 9.5 hr p.i. (Input multiplicity of 100 PFU/cell)

in uninfected cells. It would also seem that ligase function is not impaired since normal chain elongation probably involves this enzyme.

The single-stranded DNA in infected cells examined in these gradients corresponds in size to that in uninfected cells. This would seem to indicate that the DNA of infected cells does not possess more single-strand breaks than the DNA of uninfected cells.

I. DNA Synthesis in Isolated Nuclei

Since our interest concerns a nuclear inhibitory effect on DNA synthesis during the replication of cytoplasmic RNA viruses, we attempted to develop an assay system to detect the presence (or lack) of substances affecting DNA synthesis in the cytoplasm of infected cells. Previous investigations have shown that isolated rabbit thymus nuclei (Friedkin and Wood, 1956), hen erythrocyte nuclei (Thompson and McCarthy, 1968), and HeLa cell nuclei (Friedman and Mueller, 1968) can incorporate added precursors into DNA.

1. Properties of the Nuclear System from L Cells

Isolated nuclei of L cells can carry out a small amount of DNA synthesis under appropriate conditions. Nuclei isolated in 0.025 M sucrose-0.003 M calcium chloride incorporate radioactive thymidine triphosphate or other precursors into DNA (Table XII). We have found that this incorporation differs from that in our previously described (Materials and Methods) DNA polymerase assays in three respects. Assays of nuclear activity do not require denatured, exogenous DNA template, while our DNA polymerase assays do. L cell nuclei carry on linear incorporation for only 25-30 minutes, whereas DNA polymerase extracts carry on such incorporation for over 90 minutes. Hypotonic conditions do not influence DNA polymerase assays on L cell extracts, but completely eliminate incorporation into isolated L cell nuclei, in spite of the fact that the nuclei are not disrupted.

Previous studies have shown that DNA synthesis in isolated hen erythrocyte nuclei (Thompson and McCarthy, 1968) and HeLa cell nuclei (Friedman and Mueller, 1968) is stimulated 15-fold and 3-fold respectively by addition of appropriate cytoplasmic fractions. Addition of

TABLE XII

Properties of the Nuclear System

	Counts per minute
1. Time Course of Thymidine Incorporation by Cell Homogenates	
5 minutes	240
10 minutes	470
15 minutes	660
20 minutes	870
25 minutes	940
30 minutes	940
40 minutes	950
2. Relationships Between Concentration of Cell Fractions and Thymidine Incorporation	
Homogenate	800
Homogenate at 1/2 dilution	400
Homogenate at 1/4 dilution	270
Nuclei	230
Nuclei at 1/2 dilution	220
Nuclei plus undiluted cytoplasm	670
Nuclei plus cytoplasm at 1/2 dilution	320
3. Effect of Dialysis on the Cytoplasmic Factor Stimulating Thymidine Incorporation into Isolated Nuclei	
Nuclei	590
Nuclei plus undialyzed cytoplasm	2240
Nuclei plus dialyzed cytoplasm	430
Nuclei plus dialysate (1/2 dilution)	1270
4. Effects of DNAase on Thymidine Incorporation by Intact and Homogenized Cells	
Intact cells (H^3-TTP)	100
Homogenized cells (H^3-TTP)	1400
Intact cells plus DNAase (H^3-TTP)	130
Homogenized cells plus DNAase (H^3-TTP)	760
Intact cells (H^3-TdR)	95300
Homogenized cells (H^3-TdR)	1310
Intact cells plus DNAase (H^3-TdR)	97100
Homogenized cells plus DNAase (H^3-TdR)	600
5. Effects of Deoxynucleosides on Thymidine Incorporation by Cell Homogenates	
Homogenate (H^3-TdR)	2900
Homogenate (H^3-TdR) plus 100 μM CdR	1800
Homogenate (H^3-TdR) plus 100 μM AdR	2580
Homogenate (H^3-TdR) plus 100 μM GdR	2880
Homogenate (H^3-TdR) plus AdR plus GdR	2650
Homogenate (H^3-TdR) plus AdR plus GdR plus CdR	1700

(continued on next page)

TABLE XII (Continued)

	Counts per minute
6. Incorporation of Labeled Deoxynucleosides by Cell Homogenates	
A. H^3-deoxycytidine (5 μc/ml, 14.7 c/mM)	1700
H^3-thymidine (5 μc/ml, 25 c/mM)	1740
H^3-deoxycytidine plus H^3-thymidine (5 μc/ml each of above)	2900
B. H^3-deoxyadenosine (5 μc/ml, 1.2 c/mM)	1900
H^3-thymidine triphosphate (5 μc/ml, 15.7 c/mM)	3000
7. Effects of Several Miscellaneous Compounds on Thymidine Incorporation into Cell Homogenates	
A. Homogenate	1740
Homogenate plus dithiothreitol	1400
B. Homogenate	1060
Homogenate plus cycloheximide	1030
Homogenate plus 5-fluoro-2'-deoxyuridine	1130

cytoplasm also stimulates incorporation of thymidine into L cell nuclei; the effect is proportional to the amount of cytoplasm added (Table XII, sec. 2). The cytoplasmic substance responsible for the stimulatory effect in our L cell system is dialyzable and stable to freezing (Table XII, sec. 3).

Thymidine triphosphate can enter isolated nuclei but not intact cells (Table XII, sec. 4). Hence, use of radioactive TTP as a DNA precursor excludes the possibility that all incorporation occurs in the few percent of undisrupted cells which are found in all preparations of nuclei. Isolated L cell nuclei differ in permeability to proteins as well, i.e. DNAase attacks DNA in isolated nuclei but not in intact cells (Table XII, sec. 4). The level of DNA synthesis in isolated nuclei, as measured by incorporation of radioactive thymidine, is only 1% of that of intact cells (Table XII, sec. 4). There is no stimulation of incorporation upon the addition of the other three deoxyribonucleoside precursors (Table XII, sec. 5). Our results (Table XII, sec. 5, sec. 6) suggest that the nuclear precursor pool is large enough to support the small amount of synthesis carried out without the addition of exogenous precursors. Apparently this is also the case in the isolated nuclear system from rabbit thymus (Friedkin and Wood, 1956), but not in HeLa cell nuclei (Friedman and Mueller, 1968), which require all four DNA precursor bases.

2. Infected Cells and Nuclear Synthesis

Even though the level of DNA synthesis in these nuclei is low, studies of nuclei from infected cells can tentatively answer several questions. It can be determined whether there is an inhibitory substance in the cytoplasm where a virus is replicating. In addition, one can determine whether there is as much stimulatory activity in infected cell cytoplasm as in normal cell cytoplasm (Table XII, part 2).

Experiments were performed on suspension cells 5.5 hours after infection with NDV or mengovirus (Table XIII) and 9.5 hours after infection with reovirus (Table XIV). In all cases, the level of synthesis in the total cell extract reflected the level found in the

TABLE XIII

DNA Synthesis in Isolated Nuclei[a]

	H^3-TTP Incorporated in 20 min. into DNA (CPM)					
	Total Cell Extract	Nuc.	Cyt.	Nuc. + Cyt.	Inf. Nuc. + Uninf. Cyt.	Inf. Cyt. + Uninf. Nuc.
Uninfected	2900	645	175	2015		
Newcastle disease virus	670	325	125	540	685	1860
Mengovirus	430	315	110	305	310	1440

[a]At 5.5 hours post-inoculation of suspension cells, 1 x 10^7 cells were disrupted in 0.25 M sucrose-0.003 M $CaCl_2$ using a tight-fitting Dounce homogenizer.

TABLE XIV

DNA Synthesis in Isolated Nuclei[a]

	H^3-TTP Incorporated in 20 min. into DNA (CPM)					
	Total Cell Extract	Nuc.	Cyt.	Nuc. + Cyt.	Inf. Nuc. + Uninf. Cyt.	Inf. Cyt. + Uninf. Nuc.
Uninfected	840	270	30	670		
Reovirus	350	210	30	340	290	630

[a]At 9.5 hours post-inoculation of suspension cells, 5 x 10^6 cells were disrupted in 0.25 M sucrose-0.003 M $CaCl_2$ using a tight-fitting Dounce homogenizer.

intact cells. The cytoplasm from infected cells did not markedly inhibit DNA synthesis in uninfected cell nuclei. In fact, the addition of infected cell cytoplasm to uninfected cell nuclei stimulates nuclear incorporation nearly as well as does addition of uninfected cell cytoplasm. For example, uninfected cell nuclei incorporate 645 counts/min, but the addition of cytoplasm from NDV-infected cells stimulates incorporation to 1860 cpm (Table XIII), even though the infected cell cytoplasm was taken from cells in which DNA synthesis was 90% inhibited.

Résumé

In our L cell system of isolated nuclei, inhibition appears to be a process reflected in the nucleus of infected cells, not in the cytoplasm. Infected cell cytoplasm does not contain a soluble inhibitor of nuclear DNA synthesis. However, it does possess nearly normal amounts of the cytoplasmic stimulatory substance. Further work may improve the efficiency of this system to a level more like that found *in vivo*. The synthesis carried out in these isolated nuclei may, in fact, be related to the short-term, residual DNA synthesis occurring in cells after protein synthesis has been completely inhibited with drugs such as puromycin (Fig. 6).

IV. DISCUSSION

A. The Inhibition of Cellular DNA Synthesis in RNA Virus Infection

In this work we have tried to determine which component of the *in vivo* DNA-synthesizing system is defective after cellular DNA synthesis becomes inhibited in RNA virus infection. The viruses used in this study belong to three different RNA virus groups. It is therefore likely that the present discussion can be generalized to a wide range of analogous virus-cell systems.

1. The Integrity of Cellular Template DNA during Infection

During viral infection cellular template DNA might be degraded either by a cellular nuclease released from lysosomes or by a virus-coded nuclease. Such degradation, whether by a cellular or viral nuclease, should be detectable in the DNA isolated from infected cells. However, sucrose gradient analysis of cellular DNA isolated from L cells infected with mengovirus, NDV, and reovirus has not revealed any loss of structural integrity in DNA. There is no change in the size of native double-stranded DNA or of denatured single-stranded DNA at times in the infection when cellular DNA synthesis has been inhibited 80-90%. The DNA isolated by current techniques may not represent the true *in vivo* size. Nevertheless, it is very large ($1\text{-}2 \times 10^8$ daltons), and any virus-induced degradation would probably be detected. It thus appears that there is no degradation of cellular template DNA associated with virus-induced inhibition of DNA synthesis in the three systems which have been investigated.

2. The Polymerization of Deoxyribonucleotides into DNA

A number of enzymes involved in DNA synthesis display *in vitro* activities commensurate with the *in vivo* proliferative levels of cells in tissue or culture. The activities of these enzymes tend to reflect the level of DNA synthesis in cells. As mentioned earlier, DNA polymerase is one such enzyme (Bollum and Potter, 1959; Smellie, 1961; Lieberman *et al.*, 1963; Kara and Weil, 1967). The kinases producing TTP from TdR or TMP also are at very low levels in non-dividing cells, but are at much higher levels in cells that are in rapid proliferation

(Bollum and Potter, 1959; Gray et al., 1960; Weissman et al., 1960). Another enzyme involved in endogenous TTP production, dCMP deaminase, shows a similar pattern of activity related to cell proliferation (Maley and Maley, 1960). These enzymes are necessary for DNA synthesis, but may not be part of the mechanism which controls initiation of DNA synthesis.

It is noteworthy that normal levels of these enzymes are found in virus-infected cells in which DNA synthesis is 80% inhibited. In vitro activities of the enzymes mentioned above have been measured in extracts of L cells infected with mengovirus, NDV, or reovirus, and in no case has evidence been obtained of significantly reduced activity. Thus it is unlikely that virus-induced inhibition of DNA synthesis results from a direct effect upon enzymes in the pathway of DNA synthesis. Results of studies on the rate of chain elongation during in vivo synthesis also support this conclusion.

The ultimate product of in vivo DNA synthesis as revealed by alkaline sucrose gradient analysis is large (100 S) DNA. However, the relationship of this species of DNA to the autoradiographic replication section (Huberman and Riggs, 1968) is not yet clear. Nevertheless, the size of the large (100 S) DNA chains indicates that they must derive from a number of replication sections (ca. 2-6) which are coordinately active in DNA synthesis.

Size changes involved in DNA chain growth can be observed when newly synthesized DNA is removed from the template DNA by alkaline denaturation and subjected to sucrose gradient analysis. During short pulses, radioactive DNA precursors are incorporated into very small DNA chains. These chains gradually increase in size during a chase period until they reach a size of ca. 100 S. This pattern of chain elongation is more uniform than would be expected as the cells are distributed throughout the S phase and are replicating all regions of the DNA complement. It may be speculated that this uniform and reproducible pattern is created as follows (Fig. 24): first, there are more short chains of independently polymerizing DNA on both template strands in the region of the growing fork than

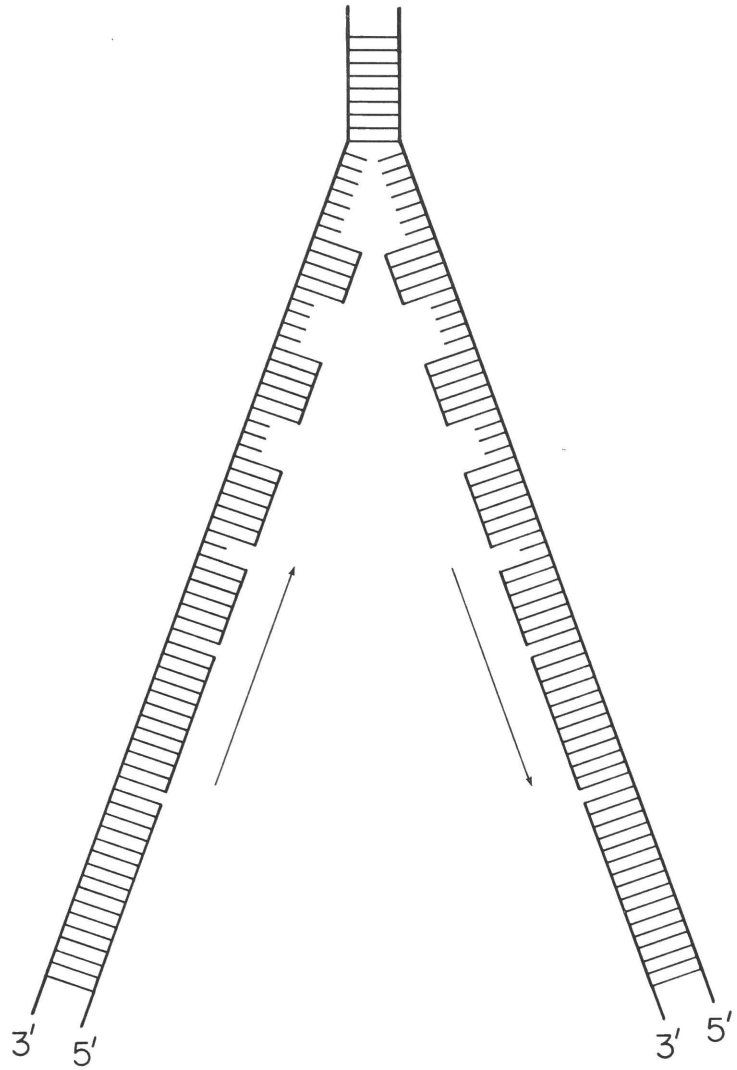


Figure 24. Modified scheme for the discontinuous replication of high molecular weight DNA (see Fig. 1). The phosphodiester backbone is represented by the darker (vertical) lines and the purine and pyrimidine bases by the lighter, short (horizontal) lines.

was postulated for the Okazaki model in the Introduction. (Fig. 1). These very short chains are not immediately joined together after their synthesis is complete, but form a pool. The joining process occurs with a time-dependent probability. As time passes, more and more short chains are linked into chains of increasing length. In this scheme, DNA polymerase polymerizes the short chains, but they are joined by polynucleotide ligase. Moreover, polynucleotide ligase cannot fill a nucleotide gap (Weiss et al., 1968a,b), such as might be formed if polymerization of the shortest chains is not completed. Hence, if polymerization of the shortest chains were suddenly blocked, chain elongation would cease as soon as all adjacent completed chains were joined.

To subject this hypothesis to a test, alkaline sucrose gradient analysis of pulse-labeled DNA has been carried out under conditions where polymerization was inhibited by precursor starvation. L cells were pulse-labeled with radioactive deoxycytidine and then chased under conditions where TTP production was blocked with FUdR. Starvation for precursor TTP tended to block further chain elongation, although after a 10-min pulse some chains have formed which can be joined in a process insensitive to FUdR. Presumably this insensitive process is the ligase catalyzed joining of adjacent completed chains. This evidence is in agreement with the hypothesis that chain elongation involves precursor polymerization and, more importantly, that completion of smaller chains is required before they can be joined together.

This approach has been applied to the analysis of virus-induced inhibition of cellular DNA synthesis. Chain elongation was measured in infected cells after onset of virus-induced inhibition of DNA synthesis. There was no decrease in the rate at which elongation occurred. In infected cells the DNA produced in 3 minutes has the normal size of ca. 20 million daltons. By 10 minutes the DNA has grown to ca. 40-60 million daltons and after a 2.9-hour chase it has been incorporated into DNA chains ranging in size between 100 and 200 million daltons. Since the labeled DNA isolated from uninfected L cells

after a 20-second pulse has a molecular weight of ca. 2-3 million daltons, a normal 10-fold increase in size takes place during the first three minutes of labeling of infected cells.

Our evidence thus indicates that a normal course of chain elongation is maintained in L cells infected with mengovirus, NDV, and reovirus. This should only occur if ongoing polymerization is unaffected during the virus-induced inhibition. Thus, viral inhibition of DNA synthesis probably does not involve either precursor depletion or interference with DNA polymerase and polynucleotide ligase. This is in agreement with measurements of the activities of several selected enzymes active in precursor synthesis and of DNA polymerase in extracts from infected cells.

3. Interference with In Vivo Regulation, Particularly Initiation

Since mengovirus, NDV, and reovirus infection affects neither the size of template DNA nor the enzymatic processes of polymerization, there is left, by inference, the possibility of a disturbance in the regulation of DNA synthesis in infected cells. In the Introduction we presented evidence supporting a model for the control of *in vivo* DNA synthesis. Regulation was postulated to involve the initiation of polymerization at the origins of replication sections. It was suggested that initiator proteins are synthesized in the cytoplasm on particularly unstable messenger-RNA, and then transported to the nucleus where their function is to initiate replication of appropriate replication sections. The initiation of DNA synthesis could be blocked by interference at any step in this sequence of events. Inhibition of DNA synthesis occurring in this manner would not affect ongoing polymerization upon active replication sections, but would block the initiation of synthesis in new sections.

Our studies with chemical inhibitors of protein synthesis demonstrate the necessity for continual protein synthesis to maintain DNA synthesis. Moreover, our results indicate that protein synthesis is not required for ongoing chain elongation. When L cells are treated with inhibitory concentrations of cycloheximide, short chains of pulse-labeled DNA are still converted to much larger chains of near

normal lengths in the absence of protein synthesis. These data support our hypothesis that protein synthesis is required to initiate replication at the origin of a replication section, but not to maintain DNA synthesis within a section already initiated.

Since Newcastle disease virus and mengovirus infections cause inhibition of L cell protein synthesis, this provides a basis for inhibition of cellular DNA synthesis by interference with the initiation mechanism. This view is strengthened by evidence obtained concerning the rate of chain elongation and the levels of enzyme activities. Thus the available data favor the concept that mengovirus- and NDV-induced inhibition of cellular DNA synthesis involves a decreased rate of initiation of replication sections and not a decreased rate of synthesis within already active sections.

The inhibition of cellular DNA synthesis during reovirus infection is not associated with detectable inhibition of protein synthesis and therefore probably involves a different mechanism from that operative in NDV and mengovirus infections. Nevertheless, it again seems to be initiation, and not ongoing polymerization, that is affected by viral processes. At present we can only speculate as to how initiation may be inhibited in reovirus-infected cells. Initiation of DNA synthesis would be deleteriously affected if transport from nucleus to cytoplasm of messenger-RNA for initiator protein were hindered or if transport of initiator protein from cytoplasm to nucleus were hindered. The dense perinuclear ring of reovirus aggregates might in some manner interfere with these transport mechanisms. The possibility cannot be excluded, however, that initiator protein synthesis is specifically inhibited during reovirus infection. Such a specific inhibition could occur if the translation complex involved in initiator protein synthesis were exquisitely responsive to, and perhaps regulated by, changes in the cytoplasmic milieu.

In synchronized cells infected with reovirus there is somewhat more DNA synthesis in pre-infected cells during the first hour after release from FUdR block than expected. Perhaps there is sufficient accumulation of initiator protein during the period of the block to support synthesis under otherwise inhibitory conditions. As mentioned

in the Introduction, some previous investigations have shown that initiator proteins can accumulate under conditions where DNA precursor polymerization is blocked with FUdR. More thorough investigation of this phenomenon in reovirus-infected synchronized cells would be necessary to determine the validity of this explanation.

4. Viral-induced Inhibitory Substances Directly Affecting DNA Synthesis

Mengovirus, Newcastle disease virus, and reovirus 3 replicate in the cytoplasm of L cells. Cytoplasmic inhibitory substances might be produced during infection which could diffuse to the nucleus and adversely affect DNA synthesis.

In our studies, we have found that the cytoplasmic fraction from uninfected or infected cells stimulates nuclear DNA synthesis in nuclei isolated from uninfected cells. No evidence has been obtained for the presence of a cytoplasmic inhibitory substance in infected cells. If such substances do exist, they should have been present in the cytoplasm of infected cells late in infection when the extracts were prepared. The rate of DNA synthesis in isolated nuclei reflects the *in vivo* rate but on a reduced scale. Nuclei making less DNA *in vivo* in turn make less DNA *in vitro*. A more efficient nuclear system for DNA synthesis would increase the probability of detecting inhibitory substances if such exist.

There is an early stimulation of histone synthesis in cells infected with the picornaviruses, Maus-Elberfeld (ME) virus (Holoubek and Rueckert, 1964) and poliovirus (Sokol *et al.*, 1965). Moreover, histones can inhibit DNA polymerase activity in *in vitro* assays (Gurley *et al.*, 1964). Hence, excess histone production might interfere with the enzymatic process of replication of DNA. It should be noted, however, that in ME and poliovirus infections, the stimulation of protein synthesis early in infection involves not only the histone fractions, but all nuclear proteins. Moreover, the synthesis of all of these nuclear proteins declines concurrent with the onset of inhibition of cellular DNA synthesis. It is therefore possible that the inhibition of protein synthesis is again the primary effect, and

inhibition of DNA synthesis a secondary consequence. In addition, our cell fractionation studies on mengovirus-infected L cells do not reveal any early stimulation of histone synthesis such as was reported for these closely related picornaviruses. Nor is there any early stimulation of histone synthesis during reovirus infection. Therefore, the inhibition of cellular DNA synthesis in RNA virus infection is not likely to be a direct result of an early stimulation of histone synthesis.

5. Conclusions

The control of cellular DNA replication may well be effected at the level of initiation of replication sections. Initiation may require the presence and continual synthesis of a particular "initiator" protein. Mengovirus, Newcastle disease virus, and reovirus 3 do not inhibit ongoing polymerization of precursor deoxynucleotide triphosphates. The enzymatic capacity to polymerize is unimpaired even when there is much in vivo inhibition of synthesis. Our findings indicate that all three viruses inhibit the initiation step in the DNA synthetic process. Mengovirus and NDV inhibit overall protein synthesis and this might in turn lead to a decrease in the synthesis of initiator protein. Reovirus 3 infection may result in interference with the transport of m-RNA for initiator protein into the cytoplasm, with the translation of this m-RNA, or with the transport of the initiator protein produced into the nucleus.

B. The Inhibition of Cellular DNA Synthesis in DNA Virus Infection

Work from a number of laboratories shows that infection with certain DNA viruses (Table I) leads to inhibition of cellular DNA synthesis. A thorough analysis of the mechanisms of inhibition has not been carried out in DNA-virus systems. Nevertheless, an attempt will be made to consider DNA virus-induced inhibition of cellular DNA synthesis in the light of the concepts developed in this thesis.

1. Degradation of Cellular Template DNA

Two exonucleases appear after poxvirus infection of HeLa cells (Eron and McAuslan, 1966). The first nuclease appears in infected cells 2 hours after infection. It digests only denatured, single-stranded DNA (Jungwirth and Joklik, 1965). The second nuclease is evident 10 hours post-infection. It degrades native, double-stranded DNA. Assays for both nucleases are carried out on cell extracts using added substrate DNA. There is no basis to postulate that cellular chromosomal DNA is degraded by these enzymes just because they are present in infected cells. In the L line of mouse fibroblasts, digestion of cellular DNA to mononucleotides is not detectable until cell lysis occurs between 15.5 and 48 hours post-infection (Kit and Dubbs, 1962), yet inhibition of cellular DNA synthesis is detectable 1 hour after infection and is complete 2-3 hours later (Joklik and Becker, 1964). Since the inhibition of cellular DNA synthesis appears 6 hours before the nuclease which could attack double-stranded cellular DNA and 11 hours before any obvious degradation, inhibition would seem unrelated to degradation.

Infection of mammalian cells with herpes simplex virus results in the appearance of a new alkaline DNAase which functions exonucleolytically (Keir and Gold, 1963; Morrison and Keir, 1968). This activity becomes evident 4-6 hours after infection; however, inhibition of cellular DNA synthesis occurs by 2 hours post-infection. A related herpes virus, pseudorabies, causes inhibition with identical kinetics (Kaplan and Ben-Porat, 1963). During infection there is no breakdown of prelabeled cellular DNA even after complete inhibition of synthesis. It would appear that the inhibition of cellular DNA synthesis is not a consequence of breakdown of cellular DNA in herpesvirus and poxvirus infections.

2. Interference with Polymerization Processes

Precursor competition might lead to reduced cellular DNA synthesis in situations where the rate of viral DNA synthesis approaches the rate of normal cellular synthesis. During productive infections with adenoviruses or polyoma virus there is very little change in the overall

rate of DNA synthesis (Ginsberg et al., 1967; Doerfler, 1969; Sheinin, 1967). As infection proceeds, the decreasing rate of cellular DNA synthesis is offset by a corresponding increase in the rate of viral DNA synthesis. In these instances, viral DNA production may be depriving cellular DNA replication of precursors and possibly of polymerase. Current evidence is however inconclusive and permits no statements about the likelihood of such competition.

The purified fiber antigen of the adenovirus particle inhibits cellular DNA, RNA, and protein syntheses 20-25 hours after addition to human KB cells (Levine and Ginsberg, 1967). Furthermore, fiber antigen inhibits cellular DNA polymerase in vitro (Levine and Ginsberg, 1968). However, it is unlikely that fiber antigen formed during infection directly inhibits DNA synthesis since cellular DNA replication becomes inhibited 6-10 hours before antigen production can be detected (Ginsberg et al., 1967).

3. Interference with In Vivo Regulation, Particularly Initiation

Cellular protein synthesis is rapidly inhibited within one hour following infection of HeLa cells with the cytoplasmic poxvirus, vaccinia (Salzman and Sebring, 1967; Moss, 1968). Inhibition of cellular DNA synthesis (Joklik and Becker, 1964) occurs concurrently with the inhibition of cellular protein synthesis. According to our previous discussion, it appears highly likely that the inhibition of cellular DNA synthesis may be due to the inhibition of initiator protein synthesis. It remains to be demonstrated that initiation is the step actually inhibited in this case.

4. Conclusions

DNA viruses should inhibit cellular DNA synthesis in one of the three general ways we have previously outlined. The small amount of available evidence cannot support interference via template degradation in any instance where this possibility has been explored. Certain nuclear viruses (adenovirus and polyoma virus) might possibly interfere with cellular DNA synthesis at the precursor level, but there is no direct evidence to substantiate such a mechanism. The

cytoplasmic poxviruses (vaccinia) might indirectly inhibit cellular DNA synthesis by blocking the synthesis of cellular proteins and thus the synthesis of initiator protein. In summary, we do not in fact know what step in cellular DNA replication is affected in any system where DNA virus infection leads to inhibition of cellular DNA synthesis. The concepts presented in this thesis could provide an outline for more complete studies with such DNA virus systems.

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