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# The Diversion of Macromolecular Synthesis in L-Cells Towards Ends Dictated by Mengovirus

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THE DIVERSION OF MACROMOLECULAR SYNTHESIS IN L-CELLS

TOWARDS ENDS DICTATED BY MENGOVIRUS

A thesis submitted to the Faculty of The Rockefeller Institute  
in partial fulfillment of the requirements  
for the degree of Doctor of Philosophy

by

David Baltimore, B.A.

||

*Acceptable for publication.*

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## PREFACE

The last few years have seen the beginnings of biochemical and physiological studies on animal virus multiplication. Work of this kind with bacterial viruses had begun in the early 1950's and provided much information before research on animal viruses was initiated. The delay in the study of animal systems was due to the great difficulties which attended the development of methods of cell culture and of quantitative virology. The solution of these problems, through the painstaking work of T. Puck, H. Eagle, R. Dulbecco and their associates, has made possible the recent rapid advance in our knowledge of the more intimate details of cell and virus growth (cf. volume 27 of the Cold Spring Harbor Symposium on Quantitative Biology).

The techniques which were developed during the study of bacteria and bacteriophages and the large body of data and theory which was accumulated, have contributed greatly to the rapidity with which our understanding of mammalian systems has increased over the last few years. In the case of the details of viral nucleic acid metabolism, the pioneering work of S.S. Cohen and A. Kornberg and the many studies which followed on bacterial viruses have provided much knowledge about the enzymes involved in bacteriophage multiplication and the physiological changes wrought in bacteria by infection (Cohen, 1961; Kornberg, 1961). In attempting to provide comparable information about mammalian viruses, we must of necessity follow their footsteps.

The following presentation is divided into four main sections. The first is an introduction which provides some necessary and some incidental background to the experimental sections. The discovery of Mengovirus and its characterization are covered in order to place the biochemical evidence in a framework of historical continuity. This discussion is by no means complete but is meant to be sufficient to provide some understanding of the place of this virus relative to some of its closest relatives, especially poliovirus. The introduction then goes on to discuss at some length the structure of the virus and its multiplication in two different cell types. Finally, the literature on the time course of virus precursor synthesis is reviewed.

It was deemed advisable to separate the experimental work into two sections since there are two quite different topics under examination. The first experimental section deals with studies on the Mengovirus-induced inhibition of cellular RNA synthesis. The inhibition is shown to be due to inactivation of the DNA-enzyme complex responsible for cellular RNA synthesis. In the second experimental section, evidence is presented concerning the enzymatic mechanism of the synthesis of viral RNA. The results indicate that virus infection causes the production of a new, cytoplasmic RNA polymerase with properties which are consistent with its being the enzyme responsible for viral RNA synthesis. Each experimental section has its own introduction where relevant problems are discussed. The fourth major division of the thesis integrates the conclusions of the three earlier sections into a general model of the multiplication of Mengovirus and related viruses. In this final discussion, certain aspects of virus multiplication are discussed which were not dealt with earlier and some very recent experiments are mentioned.

## ACKNOWLEDGMENTS

Although many others deserve mention, I should especially like to thank Drs. J. Hurwitz, J.T. August, N. Zinder, J. Darnell and I. Tamm, who contributed greatly to the progress of my ideas and experiments. I should also like to express my deepest appreciation to the faculty and students of The Rockefeller Institute who aided the advancement of my knowledge and abilities both directly and by the provision of an atmosphere of dedication. To Drs. L. Mindich, J. Schwartz and J.T. August I should like to express my gratitude for their assistance, respectively, in the sedimentation studies on DNA mentioned in Chapter II, the electrophoresis experiments discussed in Chapter III and the preparation of  $\alpha$ - $P^{32}$ -ATP which was utilized in some of the experiments of Chapter III.

My most profound gratitude is reserved for Richard Franklin. It is to him that I dedicate this thesis, since without his guidance and friendship it could never have been started nor carried through. His flexibility, dynamism, thoughtfulness and knowledge provided an ever-present example and guide to a brash neophyte.

## GLOSSARY

Acridine orange - A cytochemical stain used to reveal nucleic acids. When excited with ultraviolet light the acridine-RNA complex has a red fluorescence; when the dye is bound to DNA the fluorescence is green.

Actinomycin - An antibiotic which specifically stops DNA-dependent RNA synthesis.

Activity of an enzyme - This phrase is used often in this thesis in referring to an RNA polymerase. In this case it refers specifically to a measurement of the amount of RNA synthesis catalyzed by a given preparation per unit weight of protein per unit time as determined by incorporation of a radioactively labeled nucleoside monophosphate from a labeled nucleoside triphosphate under defined conditions.

Ascites cell - A tumor cell line which grows as single suspended cells in the peritoneal cavity of the mouse.

Dependent - This word is used in two specialized senses. The phrase "DNA-dependent" is used to denote a system which requires the presence of DNA for the system to work. This usually means that the system uses the DNA as a template for the synthesis of RNA. The word is also used in a phrase such as "activity is dependent on all four nucleotides". In this case it means that for the system to show maximal activity all four nucleotides must be present.

Fluorescent antibody - An antibody which has been coupled to a dye which fluoresces when activated with ultraviolet light. This material is used as a cytochemical stain.

Hemagglutination - Certain viruses cause red blood cells to clump and this reaction can be used to quantitate the amount of virus in a given preparation. A titration of virus by this method is known as hemagglutination titration.

Hybridization - Nucleic acids of a given base sequence will combine with nucleic acids having a complementary base sequence to form a duplex molecule. This process is known as hybridization.

**Incorporation** - This word is used to refer to the enzyme-catalyzed process whereby mononucleotides are polymerized into a nucleic acid molecule.

**Monolayer culture** - A method of growing animal cells where the cells grow attached to glass in a sheet which is one cell thick.

**Polymerase** - An enzyme which forms long chain molecules from monomer units.

**Polysome** - A number of ribosomes held together by a strand of messenger RNA. The polysomes have been shown to be the site of all protein synthesis in the cell.

**Pulse label** - When a culture is exposed to a radioactive precursor molecule for a short length of time and then the culture is killed and the radioactivity which has been incorporated during the short period of time is determined, the process is referred to as a pulse label. Pulse labels are used to determine the rate of synthesis of a given macromolecule.

**Puromycin** - A drug which selectively stops cellular protein synthesis.

**Self-absorption** - The absorption by a given sample of its own radioactive decay products before they can be counted.

**Sucrose gradient** - The sedimentation velocity of a given molecular species is often determined by layering a solution containing the molecules over a solution containing sucrose. The sucrose solution is contained in a centrifuge tube and the concentration of sucrose varies linearly from the bottom to the top of the tube (often 20% at the bottom going to 5% at the top). The sucrose is used to prevent convection currents from forming and so the molecules can sediment smoothly down the tube in a sharp band.

**Tris** - An abbreviation for the buffer Tris(hydroxymethyl)aminomethane.

## ABBREVIATIONS

ATP - Adenosine triphosphate

CpCpA - The terminal nucleotide sequence on s-RNA consisting of cytidylate-cytidylate-adenylate

CPM - counts per minute

CTP - Cytidine triphosphate

DNA - Deoxyribonucleic acid

DNAase - Deoxyribonuclease

EMC - Encephalomyocarditis

FPA - Fluorophenylalanine

GTP - Guanosine triphosphate

OD<sub>260</sub> - Optical density determined at 260 mμ

PCA - Perchloric acid

RNA - Ribonucleic acid

RNAase - Ribonuclease

s-RNA - Soluble or transfer RNA

UTP - Uridine triphosphate



## ABSTRACT

Mengovirus is an RNA-containing lipid-free virus about 27 m $\mu$  in diameter. It is pathogenic for mice, causing encephalomyelitis. Mengovirus grows in L-cells, a cultured cell line derived from mouse fibroblasts. Infected L-cells begin to produce new virus about 3 hours post-infection, and multiplication ceases after about 6 hours.

Soon after infection of L-cells by Mengovirus, the rate of cellular RNA synthesis begins to decline and by 2.5 hours post-infection the rate is only 10% of normal. During the next 3 hours of the growth cycle, the rate of RNA synthesis in the infected cells increases. The synthesis is now localized in the cytoplasm, as opposed to normal cellular RNA synthesis which occurs in the nucleus. These two phases of the RNA metabolism of infected cells have been investigated at the enzymological level.

The DNA-dependent RNA polymerase of mammalian cells, which is responsible for cellular RNA synthesis, has not been obtained free of DNA and so can only be studied in an aggregate of DNA and protein known as the aggregate enzyme. To determine whether the virus-induced inhibition of cellular RNA synthesis is due to a direct effect on this synthetic system, the activity of the aggregate enzyme from Mengovirus-infected L-cells was measured. Incorporation of radioactively labeled ribonucleotides into RNA was used as a measure of the activity of the aggregate enzyme.

The activity of the aggregate enzyme isolated from infected cells is markedly diminished; by 2 hours post-infection it is only 20% of the control value. This decrease in activity is not due to deoxyribonuclease or ribonuclease in the enzyme preparations. The ability of Mengovirus to inhibit the aggregate enzyme is eliminated by ultraviolet light inactivation of the virus, thus implicating the viral nucleic acid in the inhibitory process. The virus is unable to suppress nuclear RNA synthesis in the presence of puromycin, an inhibitor of protein synthesis. Fluorophenylalanine is also able to protect the cell against the virus-induced decrease in RNA synthesis. The effects of these two drugs indicate that the inhibitor is a protein made under the direction of the viral genome.

The new cytoplasmic RNA synthesis is insensitive to actinomycin and represents, at least in part, the synthesis of RNA which is later incorporated into virus particles. On this basis, it seemed likely that the infected cell contained a new cytoplasmic RNA-synthesizing system responsible for viral RNA synthesis.

A new RNA polymerase has been isolated from infected cells and certain of its properties have been studied. It incorporates each of the four ribonucleotides into an acid-insoluble product and the incorporation of any one nucleotide is dependent on the presence of the others. The product of this enzyme system contains ribonucleotides in internal phosphodiester linkages and is hydrolyzed by alkali, thus proving that the product is RNA. The nearest neighbor frequencies of the nucleotides in the product do not depend on the ratio of nucleotides in the reaction mixture. The RNA polymerase is insensitive to actinomycin and is thus not dependent on DNA. The appearance of this enzyme system in infected cells follows the kinetics of virus production.

In conjunction with data from the literature, the above findings allow the postulation of a general model for the multiplication of Mengovirus.

## I. GENERAL INTRODUCTION

### A. The History and Classification of Mengovirus, a Columbia-SK Virus

In 1948 Dick et al. (1948a) reported the isolation of a hitherto undescribed virus. A paralyzed Rhesus monkey, two strains of mosquitoes and a mongoose all provided material containing a filterable agent which readily produced paralysis in mice. The isolates were immunologically indistinguishable, and since they were found in the Mengo district of Buganda, Uganda, and since the symptoms in mice were those of encephalitis, the virus was named Mengo encephalitis or simply, Mengovirus.

The virus, which was lethal when inoculated into mice, produced only rare paralysis in guinea pigs and monkeys and had no effect on rabbits (Dick, 1948). By filtration, the virus was estimated to be 10-15  $\mu$  in diameter, the value then accepted for poliovirus. Dick reported that he contracted a disease due to Mengovirus (Dick et al., 1948b), but there is little other evidence to support the idea that the virus is pathogenic for human beings.

Jungeblut and Sanders (1940) had earlier isolated an agent from a stock of the Yale-SK strain of poliovirus which produced paralysis and death in mice, and which they called a "murine strain of poliovirus". Jungeblut and Dalldorf (1943) found a second agent in association with an outbreak of human poliomyelitis which they named MM virus. The virus was apparently not the etiologic agent of the disease in this case. A gibbon and a chimpanzee provided Helwig and Schmidt (1945) with a third virus which was shown to be neurotropic in mice by Warren and Smadel (1946), and which has been called encephalomyocarditis virus (EMC). A fourth related virus, known as ME virus, was isolated in Germany in 1949 by R. Gonnert (quoted in Franklin et al., 1959).

These four viruses and Mengovirus are indistinguishable by immunologic criteria. Cross-neutralization tests (Dick, 1949; Warren et al., 1949), cross-immunity tests (Warren et al., 1949) and cross-complement-fixation tests (Warren et al., 1949) showed that sera made in response to infection by either Mengo, EMC, MM or Columbia-SK had identical titers when tested against all four viruses. By cross-neutralization, ME virus was found to be indistinguishable from these four viruses (Hausen and

Schafer, 1962). The five viruses have therefore been classified together as the Columbia-SK group of viruses. The only known difference between the strains is that found by Dales and Franklin (1962), who demonstrated that EMC causes a more rapid breakdown of cell permeability than Mengovirus. Since the time of virus release is apparently correlated with changes in permeability, Mengovirus will form crystals in the cytoplasm of the infected cell, while EMC is apparently released from the cell before crystallization occurs (Dales and Franklin, 1962).

The Columbia-SK viruses show no immunologic cross-reactivity with any strains of poliovirus, even the Lansing and Yale-SK strains which have been adapted to grow in mice (Dick, 1949). The original designation of Columbia-SK as a murine poliovirus has thus been dropped (National Foundation for Infantile Paralysis, 1948). Theiler (1934; 1937) had reported isolation of a virus which produced paralysis in mice. Three especially virulent strains of this virus (GDVII, FA and TO) have been distinguished (Theiler and Gard, 1940; Olitsky, 1939) and partially characterized (Theiler and Gard, 1940; Gard and Pedersen, 1941). The viruses are approximately the same size as Columbia-SK and polio. Theiler's viruses do not, however, cross-react at all with Columbia-SK strains (Dick, 1949) and thus have been classified separately from Columbia-SK and polio (von Magnus et al., 1955).

Virus classification is at best a difficult task, but recent attempts at systemization have had the salutary effect of pointing up the great similarities among viruses with widely differing immunologic properties and host ranges (Andrewes et al., 1961; Franklin, 1962b; Lwoff et al., 1962; Melnick et al., 1963). Although the original proposal that all small (25-30 m $\mu$ ), ether-insensitive, RNA viruses be called Naniviruses (nanus = a dwarf; Andrewes et al., 1961) received little support, the more recent proposal of the term Picornavirus (Pico = small, RNA, virus; Melnick et al., 1963) as a designation for these viruses has met with some acceptance. This term will be used here to denote this class of viruses, which have not only morphological similarities but also biochemical homologies.

An extensive review of the literature on the Columbia-SK viruses has appeared (Jungeblut, 1958) and a compact discussion is available (Warren, 1952).

## B. Studies on the Structure of Columbia-SK Viruses and their RNA

After the initial isolation of Columbia-SK virus (Jungeblut and Sanders, 1940), an attempt was made to purify and characterize the virus (Bourdillon, 1944). The partially purified virus from infected mouse brains was found to have a sedimentation coefficient of 130S (Bourdillon, 1943-44), and crude electron micrographs indicated that the particle had a diameter of 25-30  $\mu$  (Jungeblut and Bourdillon, 1943). Later work with virus purified by protamine precipitation of contaminants, ultracentrifugation, and enzyme digestion, gave preparations of EMC with a sedimentation coefficient of 148-159S and a size, as determined by electron microscopy, of  $27 \pm 7$   $\mu$  (Weil et al., 1952). Using more modern techniques of microscopy, Dales and Franklin (1962) have confirmed this size estimate.

Faulkner et al. (1961) have produced very pure EMC virus by chromatography on calcium phosphate (brushite) columns. Suspensions of this purified virus crystallize, although the crystals are very fragile and not suitable for fine structure analysis by X-ray diffraction. The diameter of the virus, as determined by X-ray diffraction of wet crystals, is 30  $\mu$ . These virus preparations show a sedimentation coefficient of 160S which, assuming a density of 1.37, gives a particle weight of  $1 \times 10^7$ .

The preparations of Faulkner et al. (1961) have a ratio of infectious particles to total particles (as counted in the electron microscope) of 1:10. The virus has about 30% RNA by weight, no DNA, and no phospholipid (Faulkner et al., 1961). The RNA base ratios are close to those reported by Schaffer et al. (1960) for poliovirus (GMP:AMP:CMP:UMP = 24:27:23:26), and the amino acid composition of the coat protein is similar to, although definitely distinguishable from, that reported for poliovirus by Levintow and Darnell (1960). From the percentage of RNA and the particle weight of the virus, it can be estimated that a single particle contains  $3 \times 10^6$  molecular weight units of RNA.

Using a fixed partition cell in the ultracentrifuge, Burness et al. (1963) have estimated a sedimentation coefficient of  $161 \pm 3.3$ S for the infectious virus in crude homogenates of EMC-infected Krebs II ascites cells. The hemagglutinating activity of the same preparations sedimented in a single peak with a sedimentation coefficient of 164 4.5S; this indicates that all particles which hemagglutinate contain RNA. The infectious RNA

extracted from the virus sedimented at  $37 \pm 0.5S$ , which would correspond to a molecular weight of  $3.1 \times 10^6$ , according to Gierer's (1958) formula derived for TMV-RNA. Montagnier and Sanders (1963a) have isolated P-32-labeled EMC virus by the method of Faulkner et al. (1961), and have found a value of 37S for the sedimentation coefficient of the RNA. In sucrose gradients with 0.02M salt, the viral RNA sediments significantly faster than the 28S component of the host cell ribosomal RNA; at lower ionic strength, the viral RNA sediments more slowly than this ribosomal RNA.

The value of  $3.1 \times 10^6$  molecular weight units for the RNA of EMC virus compares well with the value of  $3 \times 10^6$  indicated by Faulkner et al. (1961) to be the amount of RNA contained in a single virus particle. Together, these data demonstrate that the RNA of EMC virus is a single molecular unit with a length of 8-9,000 nucleotides.

Hausen and Schafer (1962) have obtained somewhat lower values for the physical constants of ME virus. The virus was purified by banding in CsCl (density = 1.34) and crystallized. They found a sedimentation coefficient of 152S, which, when the measured diffusion was taken into account, gave a particle weight of  $5.7 \times 10^6$ . The diameter of the negatively stained virus was 24 mμ in the electron microscope. The isolated RNA sedimented at 27.5S in 0.02 M phosphate buffer, pH 7.2, giving a molecular weight of about  $2 \times 10^6$  by Gierer's (1958) formula. The reason for the discrepancy between these results and those mentioned above is not clear. The preparations of Hausen and Schafer (1962) had a physical particle to infectious unit ratio of about 1,000, which might indicate that their virus preparations were significantly damaged.

#### C. The Multiplication of Columbia-SK Viruses in Cultured Mammalian Cells; Biological and Cytological Studies

The details of the multiplication of Columbia-SK viruses have been studied in two very different cell systems. In one case, the virus is grown in rapidly dividing monolayer or suspension cultures of L-cells (a mouse-derived fibroblastic cell, Sanford et al., 1948); in the other it is propagated in non-growing cultures of Krebs II ascites cells, which are themselves propagated in mice.

A growth curve of Mengovirus in monolayer cultures of L-cells is

depicted in Figure 1 (taken from Franklin, 1962a). Identical development of the virus is found in suspension cultures of L-cells (Brownstein and Graham, 1961; Baltimore and Franklin, 1962a). Figure 1 shows that although intracellular viral development is complete within 6 hours, the virus is only slowly released from the infected cells. On the average, a single cell releases 1,000 - 10,000 infectious virus particles per cell, or up to 100,000 total particles.

The second procedure for virus propagation was first developed by Sanders (Sanders, 1957; Sanders et al., 1958), and later adopted by Work and colleagues (Martin et al., 1961). Krebs II ascites cells are grown in mice, the ascitic fluid harvested, and the cells suspended in a maintenance medium. The cells have a low rate of metabolism and do not divide after being put into in vitro culture. These cells can then be infected and allowed to continue in maintenance medium until they disintegrate due to the infection. Infectious virus appears in these ascites cells between 4 and 8 hours after infection (Sanders, 1960; Martin et al., 1961), which is significantly later than in L-cells. About 500 infectious particles are produced per cell. Although the yield of virus is less per cell than from L-cells, the fact that the cells grow to a very high density in mice and can be infected at high density in culture, makes this a more efficient method for the production of large quantities of virus. The procedure is, however, very erratic, and difficult to control.

In all recent studies, virus is titrated either in monolayers of L-cells under agar (Franklin et al., 1959; Brownstein and Graham, 1961; Franklin, 1961), or in Krebs II ascites cells suspended in agar (Martin et al., 1961). In both cases a single virus particle produces localized degeneration of cells in the otherwise complete sheet, or suspension, of cells. The degenerated area is visualized by allowing the intact cells to accumulate the dye neutral red. Dead cells are not stained by the dye and the unstained area is known as a plaque. Mengovirus agglutinates sheep red blood cells (Jungeblut, 1958) and the method of hemagglutination titration has been used to determine virus concentrations (Franklin, 1962a; Martin et al., 1961).

The effects of Columbia-SK viruses on L-cells have been studied by phase contrast microscopy (Barski and Kishida, 1961), electron microscopy

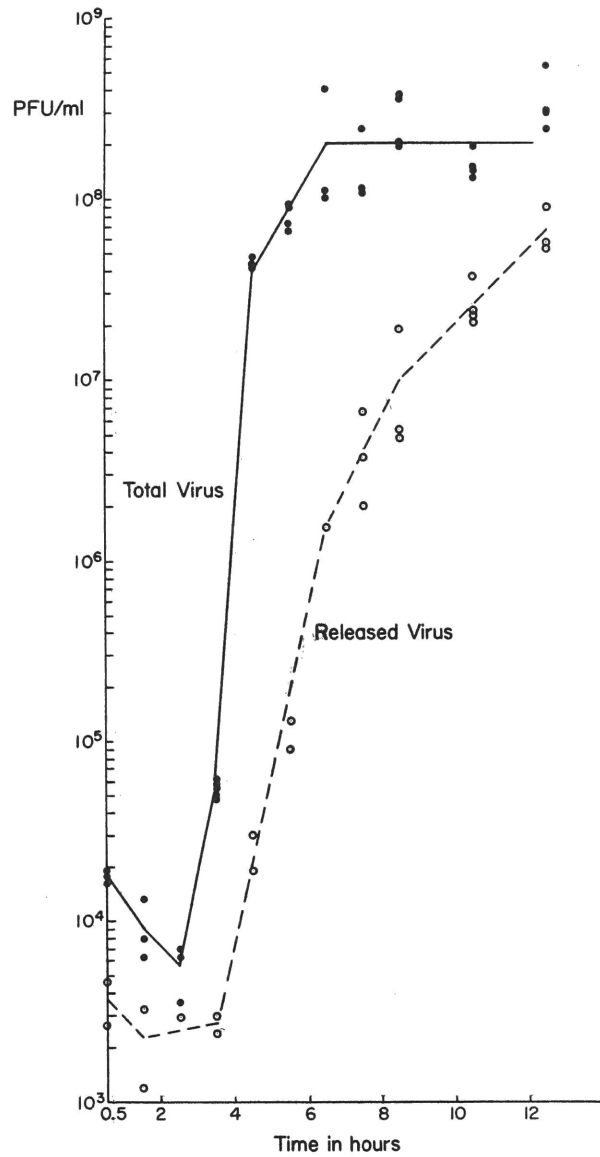


Fig. 1. The growth of Mengovirus in monolayers of L-cells (taken from Franklin, 1962a).



(Dales and Franklin, 1962; Hinz et al., 1962) and cytochemical methods (Franklin, 1962a). The major conclusions from the morphological work are that the earliest changes in infected cells are a subtle granulation or clumping of the chromatin (Hinz et al., 1962; Dales and Franklin, 1962; Franklin, 1962a) and a hyperplasia of membranous structures in the cytoplasm (Hinz et al., 1962; Dales and Franklin, 1962). No morphological studies of virus synthesis have been possible, as the small size of the virus makes it difficult to distinguish virus particles from ribosomes.

After Mengovirus synthesis is complete, the virus often crystallizes in the cytoplasm of the cell; this is not true for EMC virus grown in the same cell line (Dales and Franklin, 1962).

Barski and Kishida (1961), using time lapse phase microscopy, noted that all infected cells undergo a "retraction of the nucleus ... accompanied by ... an abrupt increase in refringence of the nuclear membrane". This change often takes place within a period of 5 minutes in any one cell, but its occurrence is spread over a period from 4 - 11 hours post-infection. Virus maturation may be an exceedingly rapid phenomenon in any one cell, and could be responsible for this dramatic change. The conclusion of their study, that "marked and rapid modifications of the physiochemical state of the nucleoplasm and cytoplasm were evidently taking place", is intriguing.

Franklin (1962a) has studied infected L-cells by staining with acridine orange and fluorescent antibody. His study showed that about 3 hours after infection, an intense red fluorescence becomes evident in a perinuclear area, due to binding of acridine orange to RNA. One area around the nucleus is not involved. This may be the centrosphere, or Golgi region, in which a large accumulation of small vesicles is evident (Dales and Franklin, 1962). From 6 - 11 hours after infection, bright red staining inclusions become evident. The fluorescence of these inclusions is not affected by ribonuclease, as opposed to the perinuclear staining, which is largely sensitive to the enzyme. The inclusions are probably the crystals of virus which are seen in the electron microscope (Dales and Franklin, 1962) and are visible in the phase microscope as dense bodies (Franklin, 1962a).

The studies with virus-specific fluorescent antibody indicate that

antigen begins to appear at 2 hours post-infection in the perinuclear cytoplasm of some of the cells. The negligible background of this method allows detection of very small quantities of antigen. All cells have detectable antigen by 4 hours. Later in the infective cycle, cells show discrete intensely staining inclusions which are, again, probably virus crystals.

#### D. Time Course of Synthesis of the RNA and Protein of Columbia-SK Viruses

Krug and Franklin (1963) determined the time in the viral growth cycle of the synthesis of the RNA and protein which are incorporated into Mengovirus particles. They used a modification of the method of Darnell and Levintow (1960) which involves adding radioactive precursors of RNA or protein to cells at various times after infection. The infection is then allowed to continue until virus maturation has ceased. The specific activity of purified virus from cultures labeled for different lengths of time is then a measure of the time of virus RNA or protein synthesis. Krug and Franklin (1963) found that the RNA and protein of the mature particles are both synthesized about 0.5 hours prior to viral maturation. This result agrees with that found by Darnell et al. (1961) for poliovirus RNA and protein synthesis. Penman, Becker and Darnell (unpublished results) have recently shown that a proportion of the radioactivity incorporated into actinomycin-treated, poliovirus-infected HeLa cells during a 15 minute exposure to uridine-C<sup>14</sup> is found in whole virus. Since actinomycin stops all host RNA synthesis (see Section IIA), this experiment shows that there need be no more than a 15 minute lag between poliovirus RNA synthesis and the encapsulation of the RNA into whole virus particles. Whether or not this is also true of Mengovirus RNA has yet to be shown, but the general similarity in the growth of the two viruses argues in favor of only a short lag between RNA synthesis and the maturation of the particle.

Martin and Work (1962) found a lag of 1-1.5 hours between EMC viral RNA synthesis in ascites cells and its maturation into whole virus. The Darnell and Levintow (1960) method, which they employed, is dependent for its accuracy on the time required for the equilibration of nucleic acid precursors between the infected cells and their medium. Since Martin and Work (1962) showed that the equilibration time is not constant throughout

infection, their estimate of a 1-1.5 hour lag is open to question. The conflict between their result and that of Krug and Franklin (1963) may thus be either real or due to different times of equilibration. It would not be unreasonable for there to be a real difference in the time of encapsulation in ascites cells as compared to L-cells.

Viral RNA synthesis has also been studied by measuring infectious RNA production. This method assumes that the extraction procedure is equally efficient for viral RNA in whole virus particles and for RNA prior to its encapsulation. But since this assumption can apparently not be made when Krebs cells are used (Sanders, 1960), the claim that EMC viral RNA synthesis precedes virus maturation by up to 4 hours (Sanders, 1960) is not justified. This criticism also applies to the 1-1.5 hour lag which was reported to occur between ME virus RNA synthesis in L-cells and its encapsulation (Hausen and Schafer, 1961).

The cytochemical studies of Franklin (1962a) indicated that viral RNA accumulates in the cytoplasm of the infected cell, but the methods used could not ascertain the locus of viral RNA synthesis. Franklin and Rosner (1962) investigated this question with striking and significant results. A 30 minute pulse of tritiated uridine given to uninfected cells is incorporated into a nuclear, acid-insoluble, ribonuclease sensitive product, as localized by autoradiography. By one hour after infection the rate of nuclear synthesis has fallen dramatically, and by 4 hours massive accumulation in the cytoplasm is evident. Similar results were obtained with 5 minute pulses (Hausen, 1962), 4 minute pulses (Franklin and Baltimore, 1962) and pulses as short as 0.5 minutes (Scholtissek et al., 1962). These findings clearly demonstrate that viral RNA synthesis is a cytoplasmic process. From the data obtained with 4 minute pulses, it can be calculated that if viral RNA were synthesized in the nucleus, it would have to be transported to the cytoplasm within 2 seconds. Such a rapid transport is very unlikely, especially since the RNA of normal cells requires at least an hour for transport into the cytoplasm (Franklin and Baltimore, 1962).

The only evidence on the intracellular site of synthesis of Mengo-virus protein comes from work with fluorescent antibody staining (Franklin, 1962a), which showed that antigen is found only in the cytoplasm. This

method can, however, locate only sites of accumulation of antigen, and not the locus of synthesis.

At one time it was proposed that a precursor of the finished viral particle, the "virosome", was present in the cytoplasm of infected cells (Huppert and Sanders, 1958; Bellett and Burness, 1961). This proposal was based on a faulty method of extraction of viral RNA (Colter et al., 1962) and has since been retracted (Bellett et al., 1962).

## II. THE INHIBITION OF HOST CELL RNA SYNTHESIS BY MENGOVIRUS

### A. Introduction

The results of the autoradiographic experiments of Franklin and Rosner (1962) mentioned above (Section ID; Franklin and Rosner, 1962; Hausen, 1962) were the starting point of the work discussed in this thesis. The alteration of cellular RNA synthesis, indicated by the finding of a decreased nuclear incorporation of uridine after infection, suggested a virus-induced inhibition of cellular RNA synthesis. There are several possible explanations of their results: (1) an inhibition of the RNA synthetic system of the cell, (2) a decreased availability of RNA precursors to the synthetic site, or (3) a rapid degradation of host cell RNA. The work reported in this section indicates that the first hypothesis, that of decreased synthetic ability, is the correct one.

The descriptions of DNA-dependent RNA polymerases of bacterial and mammalian origin by Hurwitz et al. (1960), Stevens (1960), and Weiss (1960), and the later characterizations of the reaction catalyzed by these enzymes (see Weiss, 1962, for a review of this material) have provided an understanding of the mechanism of cellular RNA synthesis. Of most interest to the present study is the RNA polymerase from mammalian cells. The existence of this polymerase was first demonstrated by Weiss (1960), but, unfortunately, it has never been purified to the extent that RNA synthesis can be controlled by added DNA. The enzyme is so firmly attached to the cellular DNA that all methods for separation of enzyme and DNA which have been tried so far have inactivated the enzyme (this statement is based on personal communications from numerous investigators, especially T. Nakamoto). For this reason, all of the data on the mechanism of the reaction have been obtained with RNA polymerase purified from bacteria.

Chamberlin and Berg (1962) and Furth et al. (1962) have purified the bacterial RNA polymerase to the point where it is completely dependent on added DNA for activity. The enzyme catalyzes the formation of a phosphodiester linkage between the 3' and 5' positions of the ribose moieties of mononucleotides. The substrates for the reaction are 5'-nucleoside triphosphates, and one pyrophosphate is released per nucleotide polymerized (Furth et al., 1962). The RNA which is synthesized in vitro from a given

DNA is an antiparallel complementary copy of the base sequence of both strands of the DNA (Weiss and Nakamoto, 1961a, b; Geiduschek et al., 1961; Hurwitz et al., 1962a). Single-stranded DNA will function as a template for the reaction (Hurwitz et al., 1962a). The RNA polymerase, therefore, has the properties which one might expect of an enzyme responsible for the synthesis of an RNA which could carry the informational specificity of DNA. Since all RNA in the normal cell appears to be synthesized as a complementary copy of one strand of DNA (Spiegelman, 1963; see Section IIIA for a discussion of this point), it is presumed that this enzyme is responsible for all cellular RNA synthesis; however, no direct evidence on this point has as yet been adduced.

When a cell is exposed for a short time to a tritiated nucleic acid precursor (e.g., uridine), almost all of the label incorporated into an acid-precipitable form is in the nucleus (see Section IIIA for references and detailed discussion). This incorporation is completely eliminated by actinomycin D (Reich et al., 1961, 1962; Franklin and Baltimore, 1962; Franklin, 1963), an antibiotic which binds to DNA (Kawamata and Imanishi, 1961) and specifically inhibits DNA-dependent RNA synthesis in vitro (Hurwitz et al., 1962b; Goldberg and Rabinowitz, 1962; Baltimore and Franklin, 1962a). Nuclear RNA synthesis in mammalian cells is therefore dependent on DNA, and thus is probably catalyzed by the RNA polymerase described above.

In order to investigate the activity of the RNA polymerase from Mengovirus-infected L-cells, the purification procedure and assay system of Goldberg (1961) was adopted. The method was later modified to allow more rapid and reproducible assay of activity.

Most of the work discussed here has been published (Baltimore and Franklin, 1962a; Franklin and Baltimore, 1962).

## B. Materials and Methods

Biological Techniques. L-cells (strain 929, originally obtained from Microbiological Associates) were grown in suspension culture on a roller machine which can accommodate 250 ml capped centrifuge bottles (Siminovitch et al., 1957; Gwatkin et al., 1957). The bottles contained 100-150 ml of medium with cells at a density of  $1-5 \times 10^5$  per ml. When

the cell titer reached  $5 \times 10^5$  per ml, the cells were harvested by centrifugation, and were then either used for an experiment, or resuspended in medium at  $1 \times 10^5$  per ml and allowed to grow again to  $5 \times 10^5$  per ml. The generation time of the cells was 18 - 24 hours, and the medium was changed every second day by centrifugation and resuspension.

The cells were propagated in Eagle's medium containing non-essential amino acids and salts for spinner culture (Eagle, 1959), and 7% fetal calf serum (Microbiological Associates, Bethesda, Maryland).

A description of the strain of Mengovirus used and its interaction with stationary (monolayer) cultures of L-cells has been published (Franklin, 1961, 1962). The intracellular growth curve of the virus in monolayer culture is depicted in Figure 1; a similar development of virus has been observed in L-cells in suspension.

Suspensions of cells were infected with virus as follows: bottles containing 150 ml of cells at a density of  $5 \times 10^5$  per ml were centrifuged, and the resulting pellet was resuspended in 2 ml of stock Mengovirus suspension (titring  $1-2 \times 10^9$  plaque-forming units per ml). Thus, each cell was exposed to 10 or more infectious virus particles, which insured a rapid infection of all cells. After incubation for 30 minutes at room temperature with intermittent shaking, medium prewarmed to  $37^\circ\text{C}$  was returned to the bottles, and the cells were incubated at  $37^\circ\text{C}$  on the roller machine until harvest time. In these experiments, times after infection refer to times after the addition of medium.

Preparation of the DNA-Dependent RNA Polymerase. For a single assay point in an experiment, from  $5 \times 10^8$  to  $2 \times 10^9$  of either control or infected cells were harvested by centrifugation and washed twice in 0.25 M sucrose with 0.001 M magnesium chloride (sucrose-Mg). Washing and all subsequent steps were performed at  $4^\circ\text{C}$ . All reagents contained 0.005 M mercaptoethanol.

The following procedures are essentially those of Weiss (1960) and of Goldberg (1961). To prepare the enzymatically active fraction, the packed cells were suspended in 2 volumes of sucrose-Mg, and 3 ml aliquots were mixed with one gram of glass beads (Superbrite 110-500, Minnesota Mining and Manufacturing Company; the beads were washed by agitation for

one day in molar NaOH and then one day in molar HCl. This was repeated three times and finally the beads were agitated in distilled water until the pH returned to neutrality.). The cells were homogenized in the smallest cup of a Virtis "45" homogenizer for 3 minutes at a setting of 70. The cup was continually immersed in an ice bath. The nuclei were then spun down at 600 x g for 8 minutes, washed twice in 0.25 M sucrose, and disrupted osmotically by the addition of 0.05 M Tris buffered at pH 7.2. A solution of 2 M KCl was added to the lysate to a final concentration of 0.4 M, and the resulting precipitate of DNA and protein was wound up on a glass rod. This DNA-protein aggregate was washed twice in 0.4 M KCl buffered with 0.05 M Tris-HCl, pH 7.2, and resuspended in 0.05 M Tris buffer, pH 7.9, with the aid of a Dounce homogenizer. Following Weiss (1960), this preparation will be referred to as the aggregate enzyme.

Aggregate enzyme preparations from either uninfected or infected cells have approximately equal quantities of DNA and protein. RNA is present at about one-third the concentration of DNA.

Preparation of Nuclei. Since preparation of the aggregate enzyme as described above is a laborious procedure requiring large numbers of cells, a simpler method for the isolation of the enzyme system was developed. About  $10^8$  cells were taken up in 20 ml of distilled water and the cells were homogenized in a Dounce homogenizer by 10 strokes of the loose-fitting pestle and 20 strokes of the tight-fitting pestle. This produced nuclei virtually free of cytoplasm as demonstrated by phase contrast microscopy (Figure 2; this field was chosen to show the maximum amount of cytoplasm which can adhere to nuclei). To the 20 ml of homogenate was added 5 ml of a 5 times concentrate of sucrose-Mg; the nuclei were sedimented in the centrifuge at 600 x g, washed once in sucrose-Mg, and suspended in about 4 ml of sucrose-Mg. The properties of these nuclei are described in Section IIC.

Assay of Aggregate Enzyme. The complete reaction mixture for assay of the aggregate enzyme contained the following constituents in a total volume of 0.5 ml: 50  $\mu$ moles Tris-HCl, pH 7.9; 1.5  $\mu$ moles manganese chloride; 10  $\mu$ moles sodium fluoride; 2.5  $\mu$ moles mercaptoethanol; 0.1  $\mu$ C ATP-C<sup>14</sup> (2.05 or 3.5  $\mu$ C/mg); 60  $\mu$ g each of GTP, UTP and CTP; 0.05 ml of ammonium sulfate saturated at 4°C; and 0.2 ml of aggregate enzyme (approximately



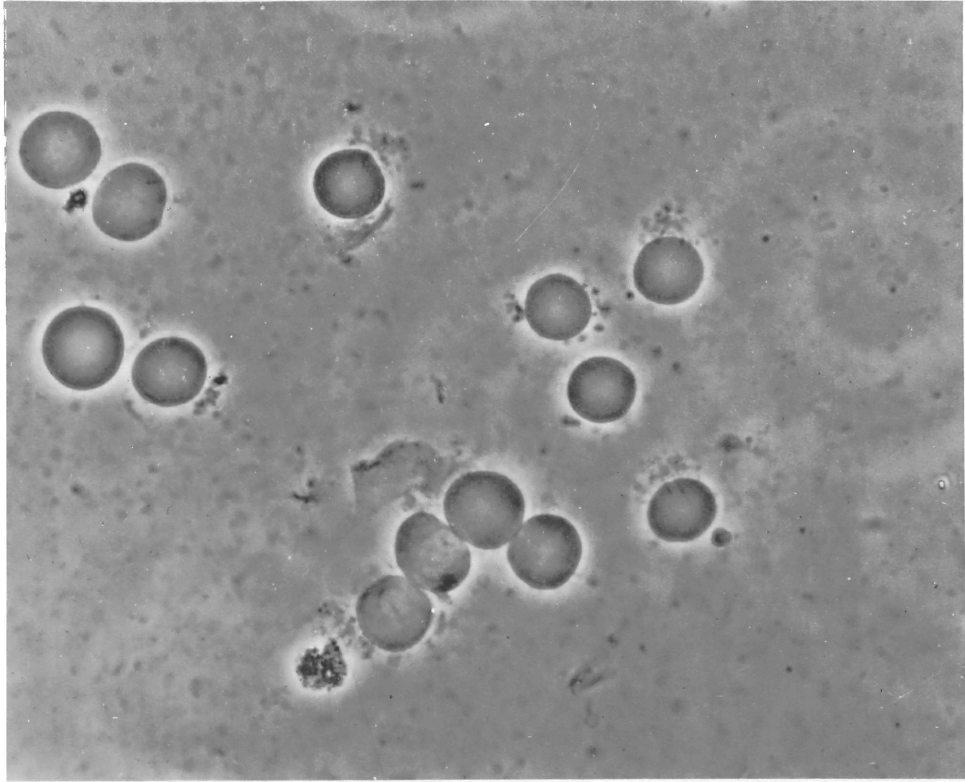


Fig. 2. Phase-contrast photomicrograph of L-cells homogenized in distilled water with the Dounce homogenizer. The round structures are nuclei.

0.30 mg). After incubation for 20 minutes at 37°C, the reaction tube was cooled to 4°C and 0.5 ml of 0.1 M sodium pyrophosphate was added in order to release adsorbed ATP (Kammen et al., 1961). One mg of bovine serum albumin was added as carrier and 3 ml of 0.5 M perchloric acid were added in order to precipitate nucleic acids and proteins. The precipitate was collected by centrifugation, washed three times with 0.5 M perchloric acid and once with ethanol-ether (1:1), dissolved in hydroxide of hyamine (Packard Instrument Company), and counted in a Packard Tri-Carb liquid scintillation counter.

Incorporation Studies: Coverslip Technique. It has been possible to obtain data on the rate of synthesis of cellular and viral macromolecules by a modification of the method of autoradiography, such that the direct counting of cell layers replaces photographic techniques. A total of  $2.5 \times 10^5$  cells were inoculated onto 60 mm x 15 mm petri dishes containing 2 coverslips (18 mm square, Corning no. 2) and the cells were allowed to grow for 1 to 3 days. All further procedures were carried out in a 37°C room. In an experiment designed to measure the rate of RNA synthesis after infection, the following schedule was followed. The growth medium was removed from the appropriate number of plates, and at zero time, 0.2 ml of a virus suspension was inoculated onto the plates. The plates were then returned to a CO<sub>2</sub> incubator, and after a 15 minute adsorption period, the virus suspension was removed and replaced by 1.9 ml of Eagle's medium without serum. At appropriate times, 0.1 ml of a solution of 5 µC/ml uridine-H<sup>3</sup> (2 C/m mole) plus a 1000-fold concentration of unlabeled thymidine were added to a plate. Thymidine was used to prevent the uridine from entering DNA. After 15 minutes of further incubation at 37°C, the medium was removed from the plate which was then flooded with ice-cold acetic alcohol (1 part glacial acetic acid to 3 parts absolute ethanol) and left in the cold for at least 10 minutes.

The coverslips were removed from the plate and placed in 70% ethanol. After standing overnight in the cold, the coverslips were placed in holders (Chen Type staining rack, A.H. Thomas Co.), extracted twice at 4°C for 15 minutes with 0.5 M perchloric acid, washed 4 times with water and dehydrated with alcohol-ether (1:1) followed by ether. After drying, each coverslip was placed in a 2 inch aluminum planchet, dried further with an infrared

lamp, and counted in a Nuclear Chicago windowless gas-flow counter. Although the efficiency of tritium detection was low, the high specific activity of the labeled compounds provided enough disintegrations to give reasonable counting rates. The monolayer of cells adherent to the coverslip was sufficiently constant in thickness to give reproducible results without the application of a self-absorption correction. Even though the exposure to uridine was for 15 minutes, data were plotted as a point located at the time of addition of labeled compound.

Protein synthesis has been studied with this technique using tritiated leucine (5  $\mu$ C/petri dish; 5 C/mmmole). Ribonuclease digestion (60 minutes at 37°C, 10  $\mu$ g/ml) or 0.5 M perchloric acid extraction (37°C, 1 hour) was employed to extract amino acids bound to s-RNA.

The rate of synthesis in non-infected control cells was measured in every experiment. The 15 minute virus adsorption period in 0.2 ml of medium did not affect protein or RNA syntheses, even though the cells were usually maintained in at least 2 ml of medium. Control cells were therefore not treated with 0.2 ml of medium. The effects of inhibitors such as p-fluorophenylalanine (FPA) and puromycin were studied by incorporating them into the medium. When the effects of FPA were under study, the medium contained no phenylalanine for either control or experimental cultures. This did not affect protein or RNA synthesis in control cells over the time of the experiment.

Determination of Protein Concentration. Protein concentrations were determined by the method of Lowry et al. (1951). Fraction V bovine albumin powder (Armour Pharmaceutical Company, Kankakee, Illinois) was used as a standard.

Materials. ATP-8-C<sup>14</sup> was obtained from Schwarz BioResearch, Inc., Orangeburg, N.Y. Non-labeled ribonucleoside triphosphates were obtained from Sigma Chemical Company, St. Louis, Mo. DNase and RNase were purchased from Worthington Biochemical Corporation.

### C. Results

Characteristics of the Enzyme. The aggregate enzyme preparation described here has many of the characteristics of the DNA-dependent RNA polymerase of mammalian cells described by Goldberg (1961). The elimination

of nucleoside triphosphate incorporation by the addition of DNase (Table 1) shows that the enzymatic activity is dependent on DNA. The product is hydrolyzed by a 16 hour treatment at 37°C with 0.3 N NaOH. That the incorporation of nucleotides is into internal linkages, and is not solely terminal addition to preformed RNA molecules, was shown by treating the product at pH 6.8 for 1 hour at room temperature with sodium periodate (0.001 M) and cleaving the oxidized terminal residue by incubation for 1 hour at 60°C in pH 9.9 glycine buffer (Preiss et al., 1959). This procedure released little of the incorporated radioactivity (Table 1).

The preparations possess an incorporating activity per milligram of "aggregate" protein very similar to that found by Goldberg (1961). The enzyme shows a four-fold decrease in activity when manganese is replaced by magnesium, and is activated by high salt concentrations, as reported by Goldberg (1961).

There is no indication of polyadenylic acid formation, as was reported by Chamberlin and Berg (1962) for a purified DNA-dependent incorporating system from E. coli. The addition of C<sup>14</sup>-ATP without the other triphosphates led to no appreciable incorporation (Table 1), in contrast to the significant incorporation found by Chamberlin and Berg (1962).

Enzymatic activity is proportional to the concentration of added protein (Figure 3; this includes data from four different experiments).

Effect of Virus Infection. The aggregate enzyme isolated from Mengovirus-infected cells has much less activity per unit of protein than that isolated from uninfected cells (Table 2). Since preparation of the aggregate enzyme requires large numbers of cells, only isolated points in the infectious cycle have been studied in any one experiment. It is evident that activity falls soon after infection and that the inhibition is permanent. The residual activity is sensitive to actinomycin. The incorporation of nucleotides into RNA catalyzed by the enzyme is linear with time for at least 60 minutes, whether the enzyme is from normal or infected cells (Figure 4); the rate of RNA synthesis, however, is much lower in preparations from infected cells, usually 20-30% of control activity.

Studies with isolated nuclei. The activity of the aggregate enzyme can be studied with nuclei prepared by the distilled water technique described

TABLE 1

Characteristics of the Aggregate Enzyme from L-Cells

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	$\mu\text{moles C}^{14}\text{-ATP}$ incorporated per mg protein
Complete	455
+ DNase, 10 $\mu\text{g/ml}$	< 15
- UTP, - GTP	< 15
after periodate oxidation	380
after 0.3 N NaOH hydrolysis	< 15

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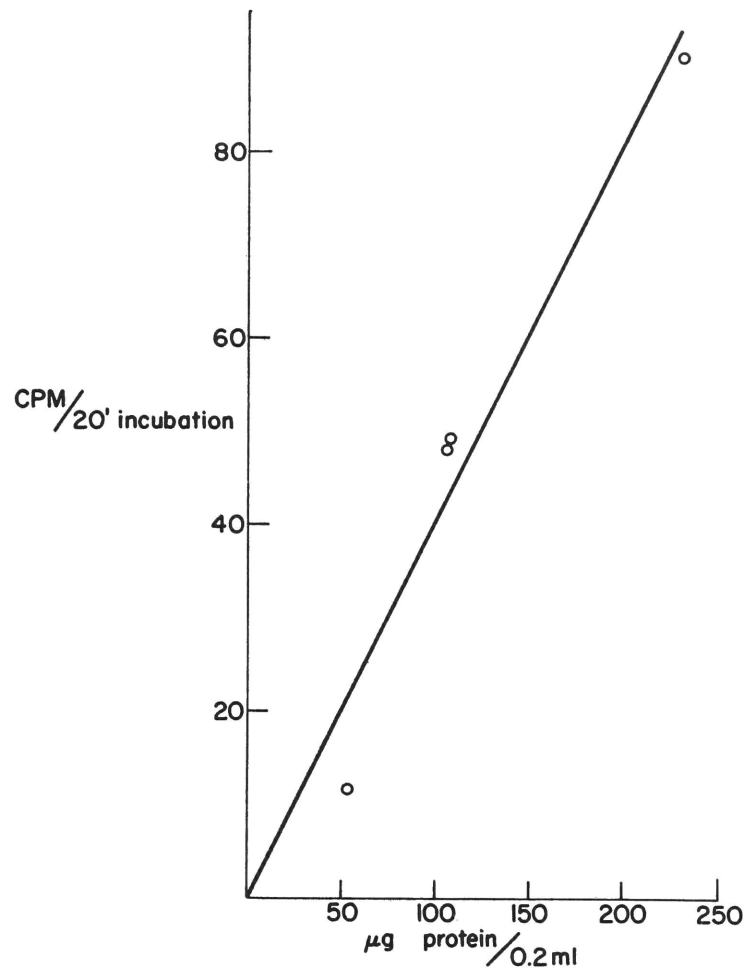


Fig. 3. Relation between protein concentration and the activity of the aggregate enzyme isolated from L-cells.

TABLE 2

C<sup>14</sup>-ATP Incorporation by the Aggregate Enzyme Isolated  
from Mengovirus-Infected L-Cells

Exp. no.	Hr. after infection	μmoles C <sup>14</sup> -ATP incorporated per mg protein
6	0	440
	2	358
	4	282
	9	104
15	0	446
	4	182
	8	46
23	0	375
	2	44
	3	77

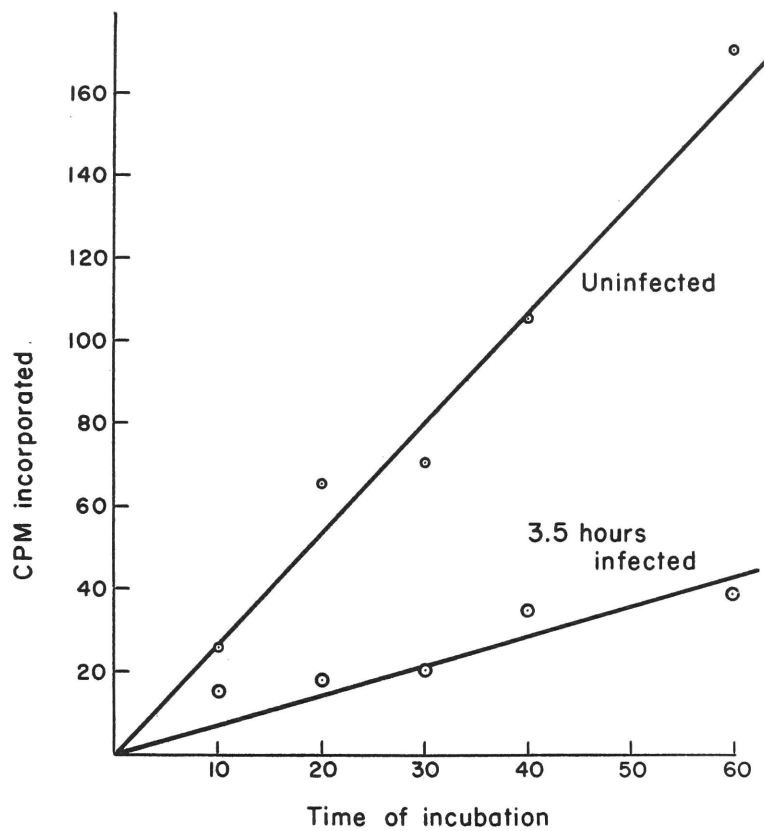


Fig. 4. Rate of incorporation of ATP-C<sup>14</sup> into acid-insoluble product, catalyzed by aggregate enzyme from control and infected L-cells. The two preparations had equal concentrations of protein.



in Section IIB. These nuclei are permeable to nucleoside triphosphates, and their properties are identical to the properties of the isolated aggregate enzyme (Table 3). They show the same dependence on manganese, activation by high ionic strength, sensitivity to actinomycin and DNase, and dependence on UTP and GTP.

Figure 5 shows the time-course of the inhibition of aggregate enzyme activity in nuclei isolated from infected cells. Also shown is the decrease in the rate of uridine and thymidine uptake in infected cells, as measured by the coverslip technique. The curves of uridine uptake and aggregate enzyme activity are quite similar, indicating that the inhibition of aggregate enzyme is responsible for the decreased uridine uptake. The increased rate of uridine uptake starting at 3 hours will be dealt with in Section III. DNA synthesis is also inhibited by virus infection, but much less precipitously than RNA synthesis. The rapidity of the inhibition of RNA synthesis is noteworthy; infectious virus production begins only at 3 hours post-infection, well after the inhibition of nuclear RNA synthesis is complete.

Nature of the Inhibition of the Aggregate Enzyme. Inhibition of aggregate enzyme activity could be due to a specific or nonspecific effect on the enzyme, or to a change or degradation of primer DNA. In order to study this latter possibility, DNA was isolated by phenol extraction of the nuclei from uninfected L-cells and cells infected for 4 and 8 hours, and the sedimentation coefficient and melting profile of the DNA's were determined. For sedimentation analysis, each preparation was centrifuged in 0.1 M NaCl, 0.1 M phosphate buffer, pH 7.2, at a concentration of 20 µg/ml. Ultraviolet absorption optics were used with the Spinco Model E centrifuge. The sedimentation constants ( $S_{20}$ ) of the control and 4- and 8-hour-infected preparations were 23, 29 and 30, respectively, indicating that infection causes no major degradation of DNA. The same preparations were diluted three-fold into distilled water and slowly heated to 95°C while absorption at 260 mµ was measured. Each preparation showed a melting temperature ( $T_m$ ) of approximately 82°C, and no change in the shape of the melting curve was observed. Thus, there seems to be little or no gross change in the hydrodynamic properties, size, or secondary structure of the extractable DNA, even at the end of the infectious cycle

TABLE 3

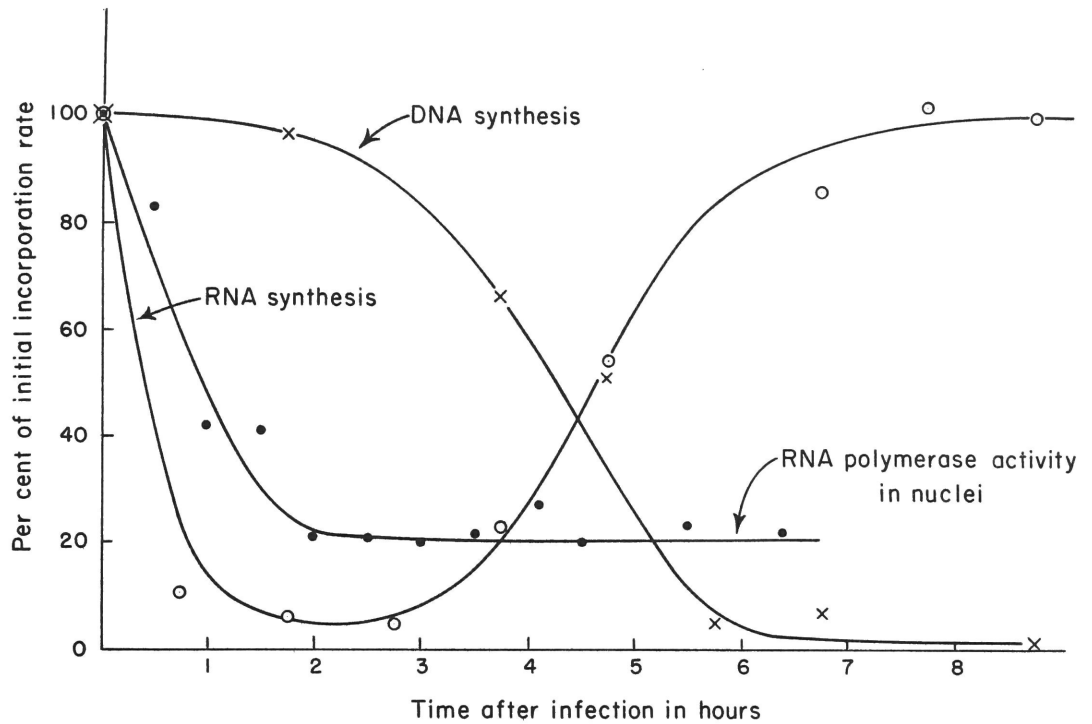
Characteristics of L-Cell Nuclei Isolated by Distilled Water  
Homogenization

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	cpm $^{14}\text{C}$ -ATP incorporated per mg protein
Complete	485
- UTP, - GTP	55
+ DNase, 10 $\mu\text{g/ml}$	56
+ Actinomycin, 8 $\mu\text{moles/ml}$	< 20
- Manganese chloride, + 1.5 $\mu\text{moles}$ magnesium chloride	170
- $(\text{NH}_4)_2\text{SO}_4$	12

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Incubation was for 10 minutes at 37° C. Other conditions as in text except that the ethanol-ether washed pellet was dissolved in formic acid, placed on planchets and counted in a windowless gas flow counter.



**Fig. 5.** Comparison of the activity of aggregate enzyme and of the rate of cellular RNA synthesis during the growth cycle of Mengovirus. Closed circles depict aggregate enzyme activity, open circles the rate of uridine uptake in whole cells, crosses the rate of DNA synthesis in whole cells.

when there is little measurable aggregate enzyme activity.

In order to test for degradation of primer DNA, highly polymerized calf thymus DNA was added to aggregate enzyme preparations from control cells and from cells infected for 2 and 6 hours. The concentration of added DNA in the reaction mixture was 300 µg/ml. The added DNA actually caused a decrease of about 20 per cent in the incorporation of ATP, possibly due to the binding of manganese. Since added DNA did not increase enzymatic activity in the control preparations, the enzyme system from both control and infected cells is probably "saturated" with respect to DNA. The possibility that virus infection causes some DNA breakdown was tested in experiments where cells were prelabeled with thymidine- $H^3$  (Franklin and Baltimore, 1962). Infection of prelabeled cells caused no loss of acid precipitable radioactivity up to 8 hours post infection.

In order to determine if the decreased activity of the aggregate enzyme after infection was due to increased nuclease activity, aggregate enzyme preparations from control cells and from cells infected 4 and 8 hours were assayed for DNase and RNase activity. Aliquots of aggregate enzyme were incubated with  $10^{-3}$  M EDTA, 0.02 M phosphate buffer (pH 7), and 70 µg rat liver microsomal RNA in 0.5 ml for RNase assay, and with  $10^{-3}$  M magnesium chloride, 0.002 M phosphate buffer (pH 7), and 150 µg DNA in 0.5 ml for DNase assay. After 60 minutes, acid insoluble material was precipitated with 0.5 M perchloric acid and the OD<sub>260</sub> of the supernatant was determined. No DNase activity could be detected. The slight amount of RNase activity was the same in both infected and control preparations. Furthermore, the relative amount of RNA in the isolated aggregate enzyme did not change systematically throughout the course of infection. Also, cytochemical and biochemical studies did not suggest any marked change in the amounts of RNA present in the nucleus or in the whole cell until rather late in infection (Franklin, 1962a; Franklin and Baltimore, 1962). According to these preliminary data, therefore, it does not seem likely that activation of an enzyme which may destroy either the DNA template or the RNA product could be responsible for the inhibition.

Holland (1962b) has demonstrated that the DNA of aggregate enzyme which has been inhibited by poliovirus is still able to act as a primer for the E. coli RNA polymerase, even without extraction of the DNA from

the aggregate. This fact indicates that the viral inhibition of host RNA synthesis is not directed against the DNA, although it is still possible that an inhibitor binds to the DNA only in small regions but that these are the regions that the cellular RNA polymerase uses as a template.

There is considerable evidence that the inhibition of aggregate enzyme is not the result of a general inhibition of all cellular enzymatic activity. DNA polymerase, alkaline phosphatase, and acid phosphatase activities, for instance, show no comparable inhibition (Franklin and Baltimore, 1962). DNA polymerase itself has not been assayed, but in vivo incorporation studies show no decrease in DNA synthesis until well after RNA synthesis is inhibited (Franklin and Baltimore, 1962). Thus, the data would suggest that the inhibition is due to a direct effect on one, or at most, a few enzymes.

The inhibition of nuclear RNA synthesis by Mengovirus could be due to a direct effect of some component of the infecting virus or to a compound synthesized in the host cell after infection. Also, since the virus stocks used in these experiments were not purified, but were simply the clarified lysates of infected cells, there could have been an inhibitory substance in the stocks, apart from the infectious virus particles. If a product of virus infection is involved, a likely possibility would be a protein. To test these various possibilities, two types of experiments were performed. In the first, ultraviolet light inactivated virus stocks were used to infect cells in order to find out if there was an inhibitory substance in the stock which would resist radiation damage, and thus would not be nucleic acid. In the second type of experiment, inhibitors of protein synthesis were tested to see if they could prevent the inhibition.

Experiments to determine whether the protein coat or a non-infectious substance in the virus stocks is responsible for the inhibition were carried out as follows: Virus was crudely purified by one cycle of sedimentation in the ultracentrifuge (2 hours, 105,000 x g) and resuspension at  $2 \times 10^9$  plaque-forming units per ml in 0.02 M phosphate buffer, pH 7.4. Some of this virus was then inactivated by ultraviolet light to 0.1% survivors and used to infect cells at a multiplicity of about 50 particles of inactive virus per cell. Nuclei isolated after infection under these conditions showed no impairment of synthetic ability (Table 4), as opposed to nuclei

TABLE 4

Effect of UV-Irradiated Mengovirus on RNA Synthesis  
by L-cell Nuclei

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	cpm/mg protein
No virus	190
Normal virus (3 hr after infection)	71
UV-virus (3 hr after infection)	210

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Standard assay conditions (complete incubation mixture) was used. Incubated 20 minutes at 37° C.

isolated from cells infected with the unirradiated virus stock. Thus, ultraviolet light inactivation of the virus particle prevents it from inhibiting cellular RNA synthesis, indicating an involvement of the nucleic acid of the virus in this process. Once this was established, studies with puromycin and p-fluorophenylalanine (FPA) were initiated.

Puromycin is a powerful inhibitor of protein synthesis, both in cell-free systems (Yarmolinsky and de la Haba, 1959; Nathans and Lipmann, 1961) and in intact Ehrlich ascites cells (Rabinovitz and Fisher, 1962). Puromycin, therefore, was used to investigate whether protein synthesis is required to initiate inhibition of RNA polymerase.

Puromycin stops the growth of L-cells, the multiplication of Mengo-virus in these cells, and protein synthesis (Figure 6). A concentration of 25  $\mu\text{g/ml}$  of puromycin suppresses all virus growth, while 100  $\mu\text{g/ml}$  are required to inhibit all protein synthesis. At 10  $\mu\text{g/ml}$ , puromycin inhibits cell growth, while 5  $\mu\text{g/ml}$  significantly decreases the growth rate. Soon after treatment of cells with puromycin there is a dramatic decrease in their ability to incorporate leucine- $\text{H}^3$  into an acid insoluble form (Figure 7). RNA synthesis is also affected rapidly, but the degree of inhibition by puromycin is much less than the inhibition of protein synthesis (17% inhibition at 25  $\mu\text{g/ml}$ ; 53% inhibition at 100  $\mu\text{g/ml}$ ).

Although 25  $\mu\text{g/ml}$  of puromycin does not affect the virus-induced inhibition of RNA synthesis, 200  $\mu\text{g/ml}$  completely prevents the decline (Figure 8). This concentration of the drug also depresses the rate of normal RNA synthesis; there is a rapid loss of 50% of RNA synthetic activity and then a further gradual decline. For this reason the cells were pre-treated for 0.5 hours before infection. Since virus infection decreases the rate of RNA synthesis much more extensively than puromycin alone, the protection afforded by the drug is evident. To further demonstrate this protection, the puromycin was removed from one set of infected cells in the same experiment, with the result seen in Figure 9. Within 2 hours there is a fall of the rate of RNA synthesis to the level of untreated cells, which proves that the puromycin does not cause any nonreversible effects relative to this virus-induced process.

Partial prevention of the virus-induced inhibition of RNA synthesis

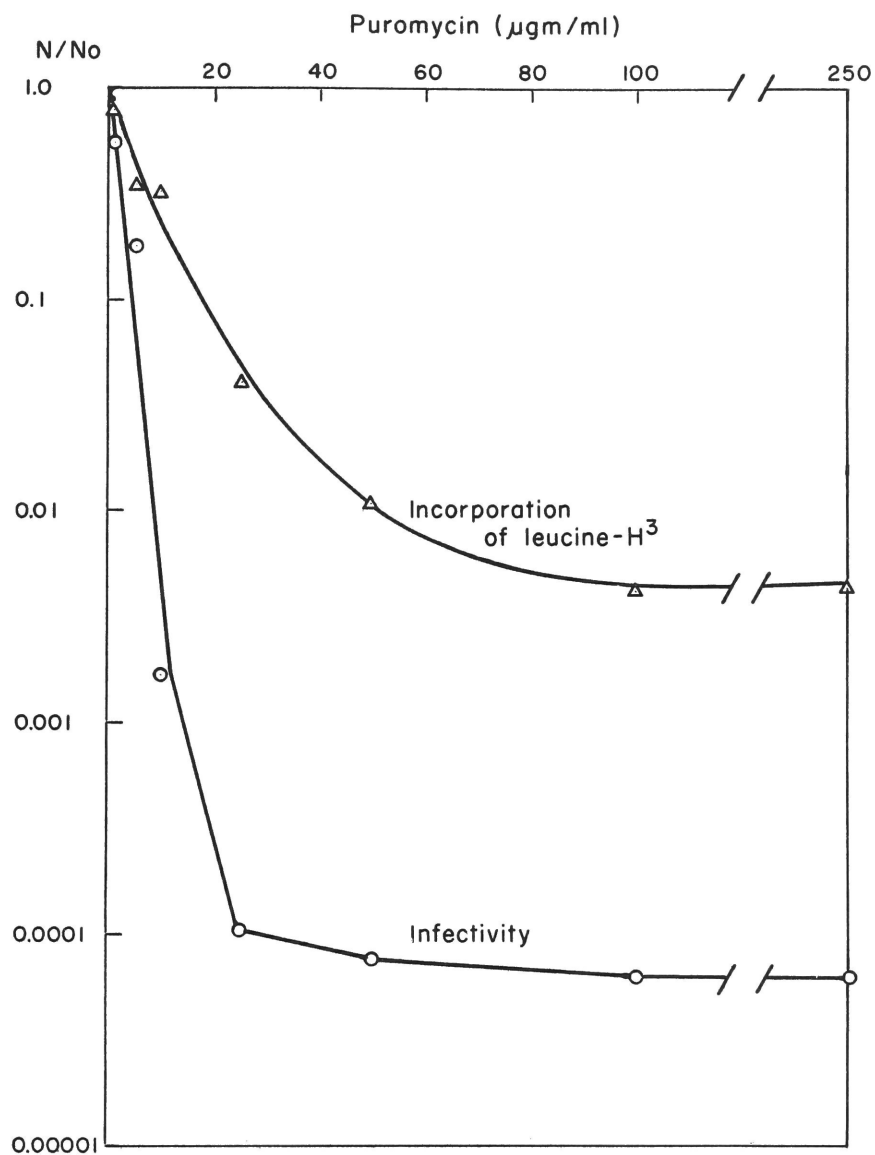


Fig. 6. Effect of puromycin on protein synthesis in non-infected L-cells and on the production of infectious virus in Mengovirus-infected L-cells. Protein synthesis measured with leucine- $H^3$ .



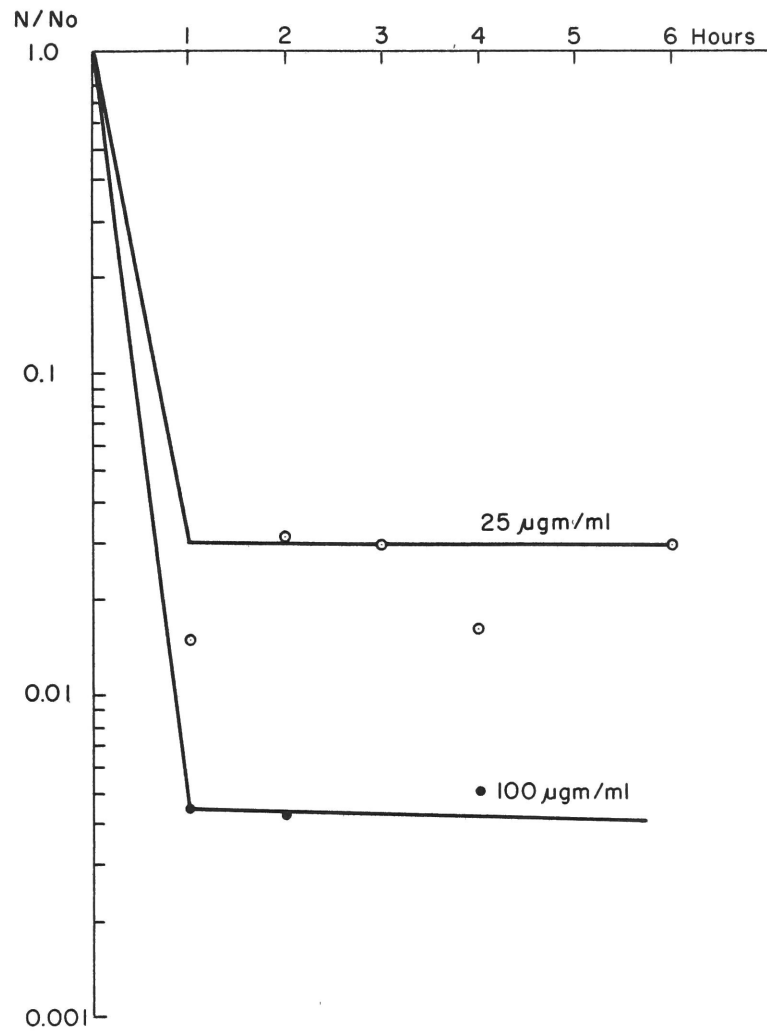
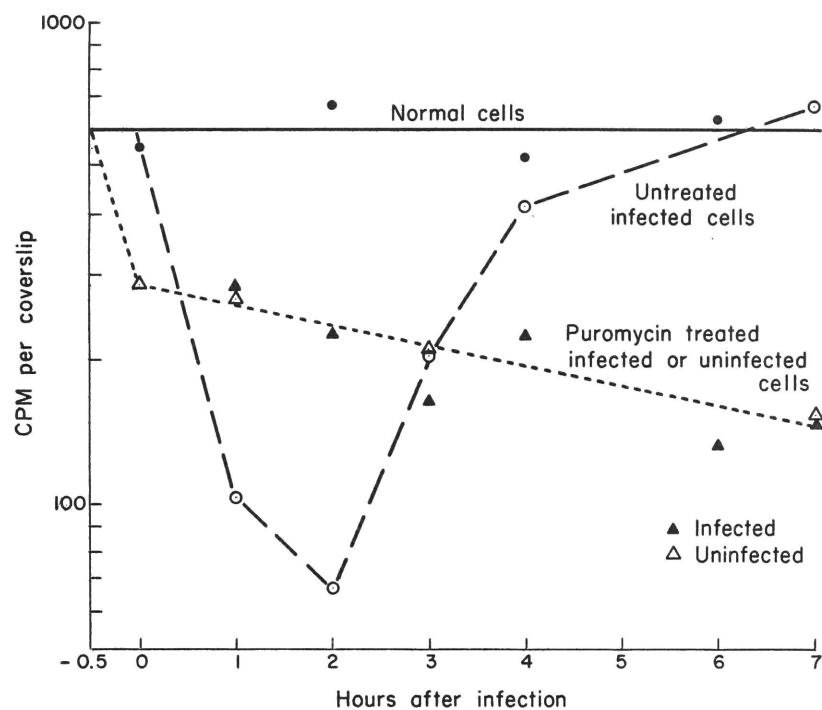
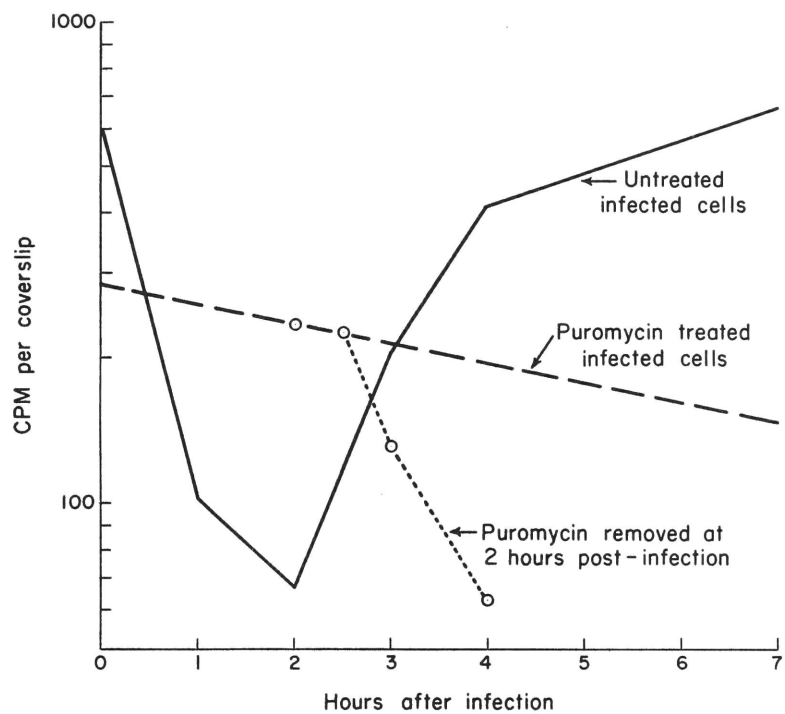


Fig. 7. Effect of puromycin at two concentrations on the rate of protein synthesis in uninfected L-cells.



**Fig. 8.** Prevention by puromycin of the virus-induced inhibition of cellular RNA synthesis.



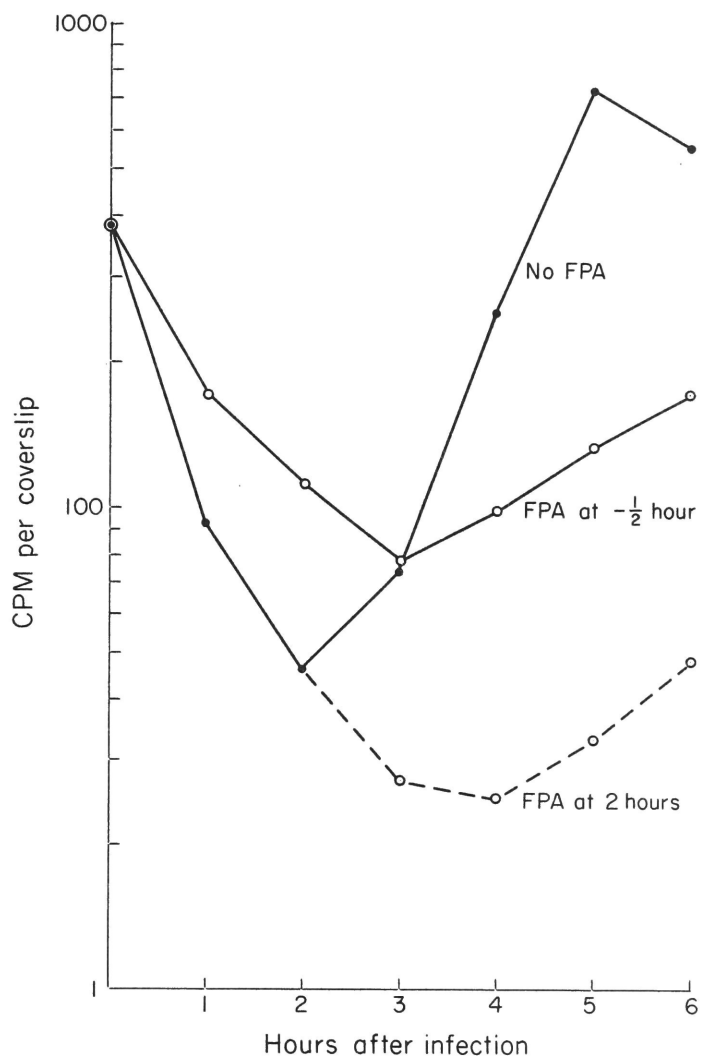
**Fig. 9.** Reversal of puromycin effect on the virus-induced inhibition of cellular RNA synthesis.

can also be achieved with 500 µg/ml of FPA. This concentration of the analogue will inhibit all virus formation and also synthesis of viral RNA and protein (Baltimore and Franklin, 1963b). Even at this concentration, FPA has little or no effect on the rate of cellular RNA synthesis in uninfected cells. When cells are infected after a 0.5 hour pretreatment with FPA, a decline in the rate of RNA synthesis is evident, but its slope is shallower and its extent less than in untreated, infected cells (Figure 10). When the FPA is added at 2 hours post-infection, a further decline is evident, and it is this situation which can most profitably be compared to that which exists when FPA is added before infection. The protection against the virus-induced decline of RNA synthesis is evident from these two curves. Both, however, show a secondary rise after 3 - 4 hours. Provisionally, this rise is interpreted as a reversal of the inhibition of nuclear RNA synthesis. Since the amount of the rise is much lower if FPA is added at 2 hours post-infection, it could not represent viral RNA synthesis.

#### D. Discussion

The above data show that infection of L-cells with Mengovirus causes a marked decrease in the activity of the DNA-dependent RNA polymerase (aggregate enzyme). These results correlate well with the autoradiographic studies, which show a marked decrease of nuclear incorporation after infection (Franklin and Rosner, 1962). It should be emphasized that the enzyme inhibition seen in these studies occurs before mature virus or viral RNA is demonstrable.

Studies of the biochemical events associated with the multiplication of EMC virus in mouse ascites cells (Martin and Work, 1961) can also be correlated with the present findings. In studies with EMC virus, synthesis of RNA and DNA in infected and control cells was followed using orotic acid-C<sup>14</sup> label; after exposure to the tracer, cells were homogenized and fractionated. It was shown that nuclear RNA synthesis decreases rapidly after infection, following approximately the kinetics seen in the present study. For example, at 4 hours after infection, only 20 per cent of control activity was found in nuclear RNA. These studies further showed that DNA synthesis was normal up to about 4 hours after infection, decreasing slowly thereafter (Martin and Work, 1961).



**Fig. 10. Protection by p-fluorophenylalanine against the virus-induced inhibition of cellular RNA synthesis.**

Neither the synthesis of cellular DNA nor its integrity are necessary for the replication of Mengovirus (Simon, 1961; Reich and Franklin, 1961). Employing actinomycin-inhibited cells, Reich et al. (1961, 1962) showed that DNA function, as expressed by nuclear RNA synthesis, is not a prerequisite for the multiplication of Mengovirus. The present study has demonstrated that processes occurring during virus infection lead, in fact, to inhibition of cellular DNA-dependent RNA synthesis.

These observations may help to explain how a virus kills a cell. Inhibition of nuclear RNA synthesis may be expected to interfere with protein synthesis and lead to eventual cell death. On the other hand, this is by no means the complete explanation of viral cytopathogenesis, since an actinomycin-inhibited L-cell, which is also deprived of all nuclear RNA synthesis, undergoes much less drastic morphological changes (Franklin, 1962a; Reich et al., 1961, 1962).

On the basis of the above data, only negative statements can be made about the mechanism of the inhibitory process. It does not involve an extensive change in the structure of the DNA, although localized changes are not excluded. Inhibition does not involve an increase in aggregate enzyme-associated RNase activity.

The demonstration that ultraviolet-irradiated virus does not cause inhibition precludes the viral coat protein or another protein contaminating the virus stocks as the inhibitory agent. The experiments with puromycin and FPA implicate a newly synthesized protein in the inhibitory reaction since these compounds can prevent or mitigate the virus-infected decline. The inhibitor is, therefore, a protein or a product of the action of a protein (enzyme).

### III. THE MENGOVIRUS RNA POLYMERASE

#### A. Introduction

The synthesis of RNA in mammalian cells was discussed briefly in Section IIA. In order to provide a background for the experimental work of the present section, the thesis that all RNA synthesis in uninfected cells takes place on a DNA template in the nucleus will be defended. With this point established, the fact that viral RNA is made by a process independent of DNA becomes more significant.

The finding that actinomycin D can abolish almost all RNA synthesis in the cell (see Section IIA) is potent evidence that DNA is involved as a template in the synthesis of all cellular RNA. The further demonstration that the small (about 1%) residual incorporation after actinomycin treatment is into the terminal positions of a species of RNA with a sedimentation coefficient of 4S (Franklin, 1963; Merits, 1963) indicates that this residuum is due to the turnover of the CpCpA terminal sequence of s-RNA. This turnover is known to occur independently of DNA, is insensitive to actinomycin (Hurwitz et al., 1962b), and occurs in the cytoplasm (Tamaoki and Muller, 1962).

Other types of experiments are also relevant to the theory of the nuclear origin of RNA. Most autoradiographic and cell fractionation studies have been interpreted as support for the theory, as have the experiments on hybridization of RNA to DNA. These two experimental approaches are discussed below, and are followed by a general discussion of the problem.

The initial uptake of RNA precursors into the RNA of cells has been shown in many systems to be by a process localized almost entirely in the nucleus (cf. reviews by Zalokar, 1961; Prescott, 1961). Experiments with centrifuged hyphae of Neurospora (Zalokar, 1960), cultured mammalian cells (Goldstein and Micou, 1959; Siskin and Kinoshita, 1961; Perry, 1961; Feinendegen et al., 1960, 1961), salivary gland cells from Drosophila (Taylor, 1953; McMaster-Kaye and Taylor, 1958; McMaster-Kaye, 1960), Tetrahymena (Prescott, 1961), bacteria (Caro and Forro, 1961), plants (Woods and Taylor, 1959; Woods, 1959) and animals (Amano and Leblond, 1960)

all led to the conclusion that the initial polymerization of RNA precursors after a short period of labeling is a nuclear event. In order to retain the hypothesis of cytoplasmic localization of the synthesis of a significant amount of RNA, the above data require the postulation of a large cytoplasmic pool of RNA precursors. Perry (1961) has shown that the maximal rate of labeling of nuclear RNA is reached within 5 minutes after addition of the radioactive precursor, while cytoplasmic labeling requires at least 2 hours to reach a peak. Furthermore, cytoplasmic label shows little accumulation until 30 minutes, while nuclear labeling begins immediately. These results are most consistent with a model of nuclear synthesis and transfer of whole molecules to the cytoplasm.

Harris and co-workers are the major opponents of the views expressed above. They interpret their studies on RNA synthesis in both multiplying and non-multiplying cells as indicating that nuclear RNA is rapidly turning over, but is not transferred to the cytoplasm as intact molecules (Harris, 1959; Watts and Harris, 1959; Harris and Watts, 1962; Harris et al., 1963; Harris, 1963a, b). It is worth considering in some detail their experiments on RNA synthesis in HeLa cells. The paper of Harris and Watts (1962) is difficult to evaluate directly, since the data are given in terms of specific activity. Any attempt to test the hypothesis of a nuclear to cytoplasmic movement of RNA must use total activity, not specific activity, since there is much more cytoplasmic RNA than nuclear RNA. Harris and Watts (1962) estimate that there is four times more cytoplasmic RNA than nuclear RNA and if their data is converted to total activity by use of this number it becomes consistent with the theory of nuclear to cytoplasmic movement of RNA. In a later paper (Harris et al., 1963), results are plotted in terms of total activity. The critical experiment of this paper is the exposure of HeLa cells to adenine- $C^{14}$  for 10 minutes and then the suspension of the cells in a medium containing a large excess of unlabeled adenosine and guanosine. The labeled adenine is rapidly taken up into the nuclear RNA and only slowly begins to appear in cytoplasmic RNA during the time in unlabeled medium. This kind of result could be taken to mean that nuclear RNA is slowly being transferred to the cytoplasm, but the authors' interpretation is different. They point out that more radioactivity is lost from the nuclei than appears in the cytoplasm, and thus conclude



that all the nuclear RNA is being degraded to acid-soluble products, while cytoplasmic RNA is being synthesized directly from the acid-soluble pool. On the basis of this experiment, there is no way to decide between their conclusion and the model of nuclear to cytoplasmic flow of large molecular weight RNA.

The conclusions in a third paper from this group (Harris, 1963a) are much more difficult to refute than the earlier ones. Here cells were given a 10 minute exposure to adenine- $C^{14}$  and then were transferred to non-radioactive medium containing a concentration of actinomycin sufficient to prevent any further incorporation of adenine into RNA. Over the next 5 hours after this treatment, 80% of the labeled nuclear RNA breaks down to acid-soluble products, while no label is transferred to the cytoplasm. If it is assumed that actinomycin does not interfere with an hypothetical nuclear to cytoplasmic flow of RNA, then this experiment proves that such a flow does not occur. It also indicates that actinomycin directly prevents the synthesis of cytoplasmic RNA. The assumption that a nuclear to cytoplasmic transfer of RNA should not be prevented by actinomycin is reasonable, but a recent result of Girard, Penman and Darnell (in press) throws doubt upon it. When HeLa cells are exposed to a 30 minute pulse of uridine- $C^{14}$ , most of the radioactive RNA has a sedimentation coefficient of 45S or 35S in a sucrose gradient (Scherrer et al., 1963) and a base composition closer to that of ribosomal RNA than to that of the DNA. If actinomycin is added at this time, the labeled RNA is converted to the 16S and 28S sizes of ribosomal RNA (Scherrer et al., 1963). Girard, Penman and Darnell (in press) have shown that in actinomycin-treated cells some of this 16S and 28S RNA is transferred to the cytoplasm, while the bulk of it remains in the nucleus. Such a result is consistent with the contention that actinomycin treatment prevents the passage of much of the recently synthesized ribosomal RNA (the bulk of cytoplasmic RNA) from nucleus to cytoplasm, although the drug does not prevent the intranuclear transformation of the 45S and 35S ribosomal RNA precursors to their ultimate size. Thus, a fundamental assumption used by Harris (1963a) is thrown into doubt and again his contention that nuclear RNA is not the precursor of cytoplasmic RNA is weakened.

A discrepancy between the results of Scherrer et al. (1963) and

Harris (1963a) has not yet been discussed. The latter author found that 80% of the rapidly labeled RNA is degraded to acid soluble products by what appear to be two kinetically separable processes. About 30% is degraded within the first hour by a rapid process and another 50% disappears via a slower route over the next 4 hours. Scherrer et al. (1963) found that about 25% of the rapidly labeled RNA is degraded to acid soluble products. In their experiments, points were taken at shorter intervals and they found that the fraction decayed within 10 minutes after actinomycin addition, with no further decrease in the next 3 hours. One reason for this discrepancy might be that Scherrer et al. (1963) labeled for 30 minutes, while Harris labeled for 10 minutes. The longer period of label used by Scherrer et al. could allow the labeled RNA to stabilize against the slow degradation process of Harris and the two fractions of rapidly decaying RNA are comparable in amount.

In summary, Harris' work provides no compelling reason to doubt that RNA is made in the nucleus and then transferred to the cytoplasm. His work does, however, indicate that the proof of this hypothesis is by no means complete. The most important positive contribution of this work is the demonstration of the phenomenon of intranuclear degradation of at least part of the rapidly labeled RNA, especially in non-multiplying cells (Watts and Harris, 1959). This has been noted by others (McMaster-Kaye, 1960), but its significance is yet to be determined. Harris has very recently adduced evidence that this intranuclear degradation is mediated by an enzyme with the properties of a polynucleotide phosphorylase (Harris, 1963b).

The hypothesis of DNA mediation in the synthesis of all cellular RNA has been strengthened by a completely different type of experimental approach. Spiegelman and his associates have shown, in an elegant series of experiments, that there are regions of bacterial DNA which have a base sequence complementary to messenger RNA (Spiegelman, 1961), ribosomal RNA (Yankofsky and Spiegelman, 1962a, b, 1963) and soluble RNA (Giacomoni and Spiegelman, 1962; Goodman and Rich, 1962). In all cases, specific hybridization between the RNA species in question and a segment of the bacterial DNA was demonstrable. This fact virtually necessitates that these RNA species be copies from a DNA template. It is hard to imagine

that such base sequence homology, which is species-specific, could be fortuitous.

In summary, certain facts relative to the theory of the nuclear origin of cytoplasmic RNA are quite clear. (1) Cell RNA is made by a process which is sensitive to actinomycin, a drug whose only known action is the inhibition of DNA-dependent RNA synthesis. (2) When a cell is exposed to a radioactive precursor, incorporation into an acid-insoluble product is localized in the nucleus for the first 20 minutes and afterward this label begins to accumulate in the cytoplasm. (3) There exists a system for the intranuclear degradation of at least 30% of the RNA labeled during a 10-30 minute exposure to a radioactive RNA precursor. (4) When a cell which has been exposed to uridine-C<sup>14</sup> for 30 minutes (and thus has no labeled 28S and 16S RNA, but has labeled 35S and 45S RNA) is treated with actinomycin, there is no further uptake of uridine, but the previously incorporated labeled material is transformed to 16S and 28S. The 45S and 35S RNA's have a base composition with a high content of guanine and cytosine. This composition is close to that of ribosomal RNA (especially the 28S) and dissimilar to that of DNA. (5) There exist regions of the DNA with a base sequence complementary to that of all the known RNA fractions in the cell.

When certain reasonable inferences are drawn, the above facts allow the derivation of a model for cellular RNA synthesis. The consequences of denying these inferences will be considered later. The demonstrated base sequence homology of all known RNA's (i.e., ribosomal, soluble or transfer and messenger) and the actinomycin sensitivity of the synthesis of these fractions implies that DNA is the template for all cellular RNA synthesis. The results of uptake experiments with radioactive RNA precursors is consistent with this inference and the time relations found by Perry (1961) and by Franklin and Baltimore (1962) indicate that 30 to 60 minutes is required before the bulk of the newly synthesized RNA reaches the cytoplasm. A significant fraction of the RNA synthesized during a short exposure of the cell to a radioactive precursor is rapidly degraded in the nucleus to acid-soluble products. Ribosomal RNA, which is the bulk of cellular RNA, is derived from 35S and 45S precursors which are converted to 16S and 28S in the nucleus.

Harris and co-workers apparently consider the hybridization experiments as irrelevant and the actinomycin data as inconclusive. Using, then, only uptake experiments, they are forced to kinetic arguments, always a treacherous approach (cf. Singh and Koppelman, 1963 and Harris' following answer). Unfortunately these arguments cannot be evaluated in their own right because a significant piece of information is wholly lacking: there have been no measurements of the size and equilibration times of the pools of RNA precursors. Thus, one can set these parameters at any convenient level and can further erect barriers, especially between nucleus and cytoplasm, which allow the postulation of separate pools for separate synthetic reactions. In this way, the fact that radioactive precursors are first incorporated into RNA in the nucleus ceases to be critical, one merely postulates a large pool of cytoplasmic precursors which only slowly equilibrates with the nuclear pool. In the absence of the necessary data, and having the results of other experimental approaches to draw on, it becomes unprofitable to speculate along such lines any further. Until compelling results appear, we can consider the thesis of the nuclear origin of the major components of cellular RNA as proved and go on to consider the case of viral RNA synthesis.

In the light of the above discussion, the finding that viral RNA synthesis is a cytoplasmic process (Franklin and Rosner, 1962; Hausen, 1962) is clearly indicative of the very special nature of viral RNA synthesis. Simon's (1961) demonstration that viral RNA synthesis can occur in a cell which is unable to polymerize new DNA, along with the findings of the resistance of viral RNA synthesis to mitomycin (Reich and Franklin, 1961) and actinomycin D (Reich et al., 1961; 1962; Franklin and Baltimore, 1962), strongly suggest that viral RNA is made by a process which is independent of DNA. Since viral RNA is faithfully reduplicated in the infected cell, there must be a system for copying it. The above conclusion that DNA is not involved leaves one with the hypothesis that the infected cell must have a mechanism for the direct replication of the nucleotide sequence of RNA, a process which apparently is not carried out in the uninfected cell.

This line of reasoning provided the stimulus and rationale for a search for an RNA-dependent RNA polymerase in Mengovirus-infected cells.

The success of this search is documented below. Much of this work has been published (Baltimore and Franklin, 1962b, 1963b).

## B. Materials and Methods

Viral RNA Polymerase Assay. The following assay has been found to give optimal results and was used throughout with minor modifications as indicated. The reaction mixture (0.5 ml) contained 20  $\mu$ g phosphoenolpyruvate kinase (PK), 5  $\mu$ moles phosphoenolpyruvate (PEP), 30  $\mu$ moles Tris-HCl buffer (pH 8.1), 5  $\mu$ moles magnesium acetate, 1  $\mu$ g actinomycin, and ATP, UTP, GTP, and CTP, one of which was labeled with  $C^{14}$ . Two-tenths ml of an enzyme preparation was added to each tube (0.5 mg of protein). After incubation at 35°, usually for 10 minutes, the mixtures were rapidly chilled and 0.5 ml of 0.1 M sodium pyrophosphate added (Kammen et al., 1961), followed by 5 ml of 0.5 M perchloric acid (PCA). After 10 minutes in ice, the suspension was centrifuged and the precipitate washed 3 times with PCA and once with ethanol-ether (1:1) by centrifugation and resuspension. The acid-insoluble material was dissolved with 1 ml of concentrated formic acid, transferred to metal planchets, dried, and counted in a windowless gas-flow counter. In some experiments, samples which were unincubated gave high levels of radioactivity (about 200 cpm); this value could be reduced to less than 15 cpm by the following procedure, which did not affect the acid-insolubility of the product of the enzymatic incorporation of nucleotides. After the initial acid precipitation, 0.3 ml of 0.5 M NaOH was added, followed rapidly by 0.5 ml of 0.1 M sodium pyrophosphate and 4 ml of 0.5 M PCA. After 10 minutes the suspension was centrifuged and the precipitate washed twice more with PCA, washed once in ethanol-ether, dissolved in formic acid, and counted as before. All radioactivity measurements were corrected by subtraction of the counting rate of an unincubated control sample which never exceeded 10% of incubated samples. No correction for self-absorption was made.

Electrophoresis experiments. Reaction conditions were identical with those used for  $C^{14}$  incorporation studies, except for the use of  $\alpha$ - $P^{32}$ -ribonucleoside triphosphates (about 10,000 cpm/ $\mu$ mole) in the place of the  $C^{14}$ -ribonucleoside triphosphates. After the ethanol-ether wash, the precipitate was hydrolyzed for 18 hours at 37° in 0.3 ml of 0.3 N KOH. The resulting solution was acidified to pH 1 with 10 N perchloric acid,

centrifuged, and the insoluble proteins and potassium perchlorate discarded. The supernatant solution was taken to pH 3.5 with 1 N KOH, clarified by centrifugation, and dried overnight at 37° under a continuous air flow. The residue was taken up in 10-20  $\mu$ liters of buffer, applied to Whatman 3 MM paper, and electrophoresis carried out in 0.1 M ammonium formate buffer, pH 3.5 for 4.5 hours at 38 volts/cm (Markham and Smith, 1952). The nucleotides were detected by ultraviolet absorption (the ribosomal RNA in the enzyme preparation supplying the bulk of the optical density) and autoradiography on x-ray film. There was exact correspondence of optical density and radioactivity; parallel marker spots for the 2'(3') mononucleotides allowed classification of the spots as AMP, CMP, UMP and GMP. The ultraviolet absorbing regions were cut out, counted directly in a windowless gas-flow counter and the results expressed as percent of total.

Materials.  $C^{14}$ -nucleoside triphosphates were purchased from Schwarz BioResearch, Inc. Unlabeled nucleoside triphosphates, pyruvate kinase, phosphoenolpyruvate and myokinase were obtained from Sigma Chemical Company. RNase and DNase were products of Worthington Biochemical Corporation.

$\alpha$ - $P^{32}$ -UTP was a gift from Dr. J. Hurwitz. Dr. T. August kindly provided  $\alpha$ - $P^{32}$ -GTP and  $P^{32}$ -labeled cyanoethylphosphate.  $AMP^{32}$  was prepared by the method of Tener (1959) and converted to  $\alpha$ - $P^{32}$ -ATP using myokinase, phosphoenolpyruvate and pyruvate kinase (Hurwitz and Bresler, 1961).

### C. Results

Enzyme Preparation. Two methods were used for preparation of cell fractions which incorporate ribonucleotides into an acid-insoluble form. Cells infected for 5 hours were homogenized either in 0.25 M sucrose with  $10^{-3}$  M  $MgCl_2$  (sucrose-Mg) or in demineralized water followed by addition of NaCl to 0.5 M. The former method, which causes little nuclear breakage, was used in early work on this system, while the latter, which yields more active preparations, has been used for later studies. Both methods are described below.

(a) Sucrose homogenization. Procedures described in Section IIB for the homogenization of cells in a VirTis homogenizer were used except

that mercaptoethanol was eliminated since it was shown to be unnecessary. Less than 5% nuclear breakage occurred as determined by measurements of DNA liberated into cytoplasmic fractions. After nuclei were removed by centrifugation, the cytoplasmic preparations were either used directly or fractionated further by differential centrifugation. For assay, centrifuged fractions were resuspended in sucrose-Mg at 5-10 mg of protein per ml, using a Dounce homogenizer when necessary.

(b) Water homogenization. Infected cells were centrifuged in the cold at  $200 \times g$ , resuspended in sucrose-Mg, recentrifuged and either frozen at  $-20^{\circ}$  or used directly. All further procedures were carried out at  $0-4^{\circ}$ . The cell pellet was suspended in 20-40 volumes of cold water and homogenized with 5-10 strokes of the tight-fitting pestle of a Dounce homogenizer (Kontes Glass Co., 40 ml size). Such preparations were routinely examined by phase contrast microscopy and the homogenization was terminated when the preparation consisted almost entirely of cell debris and nuclei free of adherent cytoplasm. If 10 homogenization strokes were not sufficient, more water was added and a few more strokes applied. The disrupted cell suspension was then adjusted to a final concentration of 0.1 M Tris-HCl buffer (pH 7.6) and  $5 \times 10^{-3}$  M  $MgCl_2$ . One-tenth volume of 5 M NaCl was added and the preparation mixed by rapid pipetting. After 5-10 minutes at  $0^{\circ}$ , the viscous suspension was diluted two-fold with water to reduce its viscosity; this resulted in fine aggregates of nucleoprotein. After centrifugation at  $600 \times g$  for 10 minutes to remove nuclei, whole cells and the aggregated nucleoprotein, the supernatant solution was centrifuged at  $78,000 \times g$  in the Spinco Model L ultracentrifuge for 1.5 - 2.0 hours. The resulting supernatant solution was discarded, the surface of the intact pellet and the tube rinsed once with sucrose-Mg, and the pellet resuspended in sucrose-Mg with an all-glass homogenizer to give a suspension containing 2.5 - 10 mg of protein/ml. Such suspension incorporate in 10 minutes about 400  $\mu$ moles of  $GMP-C^{14}$  per mg of protein as compared with the sucrose homogenates which incorporate about 100  $\mu$ moles under optimal conditions. These particulate preparations from infected cells, which will be referred to as an "enzyme preparation", could be stored at  $-20^{\circ}$  C with retention of 75% of activity after a week."

The incorporation of ribonucleotides catalyzed by the enzyme proceeds

linearly for approximately 10 minutes. Between 15 and 30 minutes the reaction rate falls off, and the reaction ceases by 60 minutes. In one experiment, the incorporation of GMP-C<sup>14</sup> into an acid-insoluble form in 5, 10, 15 and 20 minutes was 0.11, 0.19, 0.24, and 0.31  $\mu$ moles, respectively. The rate of the reaction is proportional to enzyme concentration in the range of 2 - 0.5 mg protein per 0.5 ml of reaction mixture. In a typical experiment, the addition of 1.8, 1.2 and 0.6 mg of protein resulted in the incorporation into an acid-insoluble form of 0.26, 0.15 and 0.07  $\mu$ moles of GMP-C<sup>14</sup>, respectively, in 10 minutes.

Viral RNA polymerase activity and its properties. The enzyme preparation from infected cells incorporates about 10 times more of each of the four monophosphates (AMP, GMP, CMP and UMP) than a similar extract from uninfected cells (Table 5). Incorporation of each nucleoside monophosphate is at least partially dependent on the presence of the others. For maximal GMP incorporation, the presence of all 4 ribonucleoside triphosphates is essential (Table 6). The omission of a single nucleoside triphosphate markedly reduced GMP incorporation; similar results with ATP-C<sup>14</sup> have been obtained. The ribonucleotide incorporating activity is actinomycin, DNase and puromycin insensitive and dependent on the presence of an ATP generating system; corresponding deoxynucleoside triphosphates do not replace their ribonucleoside counterparts (Table 7). The reaction is not affected by the addition of up to 0.4 M NaCl nor is it sensitive to 0.02 M fluoride or 4 mM phosphate. While higher concentrations of phosphate will inhibit the incorporation of GMP up to 30%, the lack of inhibition by 4 mM phosphate clearly differentiates the enzyme from polynucleotide phosphorylase. All of the incorporated radioactivity can be sedimented in one hour at 105,000 x g, which suggests that the radioactivity is associated with a relatively large particle.

pH and ionic requirements. Incorporation is maximal between pH 7.8 and 9.5 and falls to 50% at pH 7.2. The reaction is completely dependent on added magnesium ions and shows an optimum at about 2 mM (Figure 11); there is no inhibition by magnesium until the concentration becomes greater than 50 mM. Manganese ions markedly inhibit the reaction in the presence of optimal magnesium (Figure 12). Even 0.2 mM MnCl<sub>2</sub> inhibits the reaction by 40%, and, at equimolar concentrations of manganese and magnesium ions,



TABLE 5

Comparison of C<sup>14</sup>-Nucleoside Triphosphate Incorporation  
by Enzyme Preparations from Uninfected  
and Mengovirus-Infected L-Cells

		$\mu\mu$ moles/mg protein	
		Infected	Control
ATP-C <sup>14</sup>	complete	120	13.8
"	less UTP, GTP	51.5	20.3
GTP-C <sup>14</sup>	complete	55	5.9
"	less CTP, ATP	7.8	7.5
UTP-C <sup>14</sup>	complete	50.5	3.9
"	less ATP, GTP, CTP	14.1	3.9
CTP-C <sup>14</sup>	complete	73.5	20.8
"	less ATP, CTP, UTP	27.9	19.0

The usual reaction mixture was employed with either 96  $\mu\mu$ moles of ATP-C<sup>14</sup> (690 cpm/ $\mu\mu$ mole) plus 120  $\mu\mu$ moles each of the other triphosphates; 53  $\mu\mu$ moles GTP-C<sup>14</sup> (3,380 cpm/ $\mu\mu$ mole) plus 30  $\mu\mu$ moles each of the others; 25  $\mu\mu$ moles of UTP-C<sup>14</sup> (7,250 cpm/ $\mu\mu$ mole) with 30  $\mu\mu$ moles each of the others; or 174  $\mu\mu$ moles of CTP-C<sup>14</sup> (1,680 cpm/ $\mu\mu$ mole) with 120  $\mu\mu$ moles each of the other triphosphates. One enzyme preparation made by sucrose homogenization was employed.

TABLE 6

The Effect of Omission of Single Triphosphates  
on GTP-C<sup>14</sup> Incorporation

---

	<u>cpm</u>
Complete	414
- ATP	159
- CTP	172
- UTP	74
- CTP, - UTP	65

---

Complete reaction mixture contained 73  $\mu$ moles of GTP-C<sup>14</sup> (2,100 cpm/ $\mu$ mole) and 60  $\mu$ moles of each of the other ribonucleotides and was incubated for 15 minutes.

TABLE 7

Properties of GTP-C<sup>14</sup> Incorporation by the Viral RNA Polymerase

<u>Exp</u>		<u>cpm</u>
1	Complete	2440
	+ 10 µg actinomycin	2510
2	Complete	532
	+ 50 µg DNase	526
3	Complete	761
	- PEP, - PK	352
4	Complete	1146
	- UTP, - CTP	50
	- UTP, - CTP, + TTP, + CTP	58
5	Complete	596
	+ 4 mM phosphate	622

Experiments involved different batches of enzyme incubated with the following amounts of GTP-C<sup>14</sup> in µmoles: experiments 1 and 3, 73 (2,100 cpm/µmole); experiments 2 and 5, 53 (3,380 cpm/µmole) and experiment 4, 32 (5,080 cpm/µmole). The concentration of unlabeled nucleotides was adjusted to be approximately equal to that of the GTP-C<sup>14</sup>. For experiment 2 both samples were preincubated at 37° for 5 minutes with either DNase or water and 0.003 M MgCl<sub>2</sub> before addition of the components of the reaction mixture.

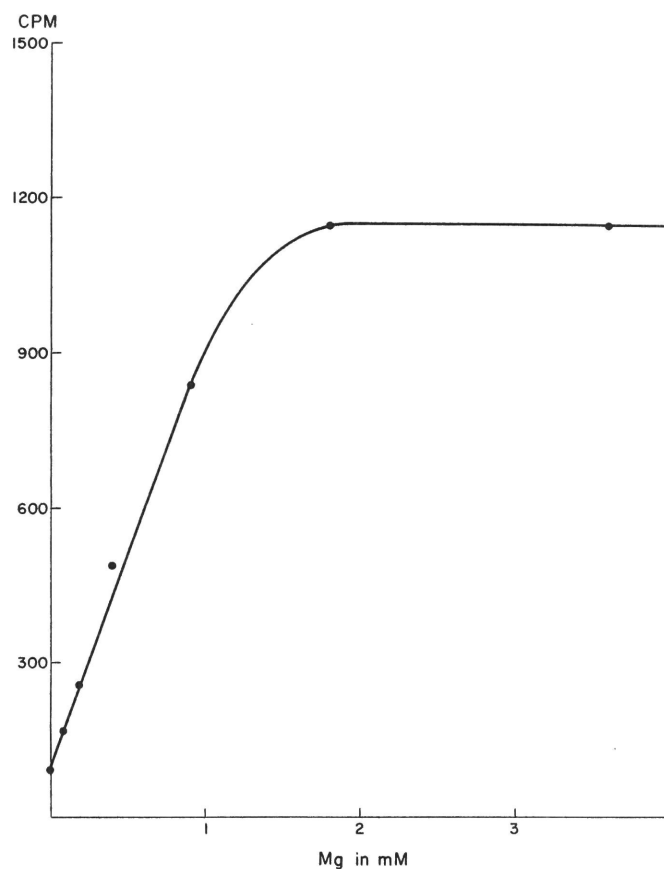


Fig. 11. Effect of  $Mg^{++}$  concentration on the activity of the viral polymerase. Reaction terminated after 10 minutes incubation with various concentrations of magnesium acetate under conditions described in Table 6. Enzyme preparation contained no added magnesium.

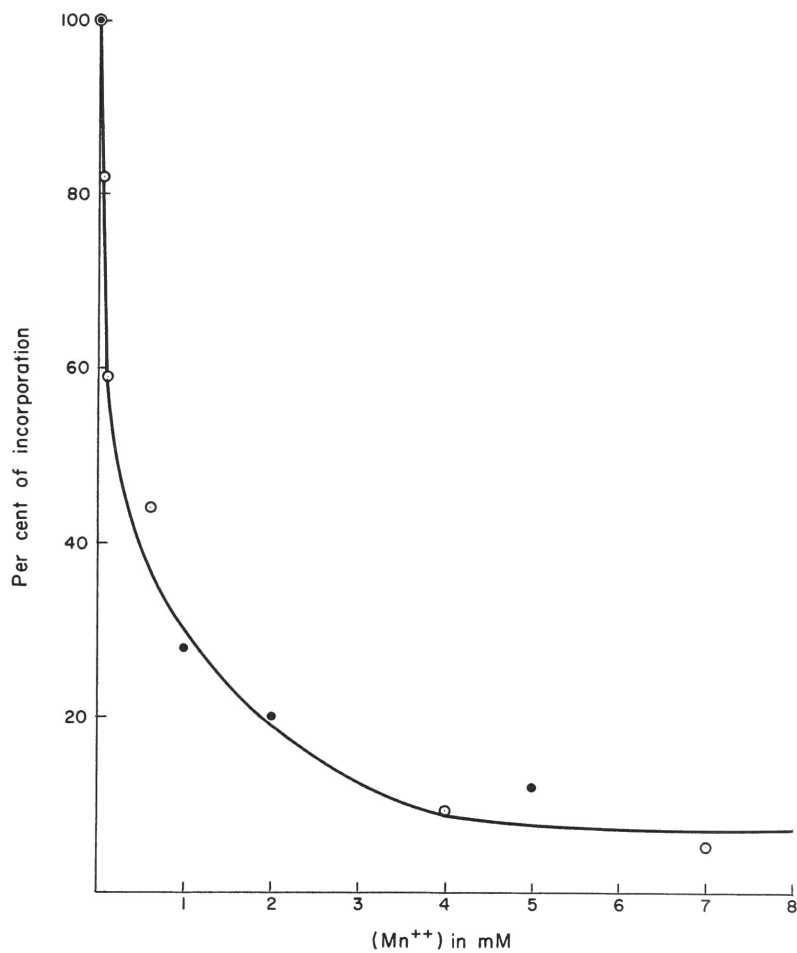


Fig. 12. Effect of  $Mn^{++}$  concentration on the activity of the viral polymerase. To the reaction mixture described in Table 6, with 1.8  $\mu$ moles of magnesium, was added manganese chloride at various concentrations. Closed and open circles refer to separate experiments.

over 90% inhibition occurs.

Nature of the product. The acid-insoluble radioactivity is completely solubilized by incubation with 0.3 M NaOH at 37° for 18 hours. When GTP-C<sup>14</sup> or ATP-C<sup>14</sup> was used as precursor and the alkaline hydrolysate of RNA fractionated by electrophoresis, all of the incorporated radioactivity originally in ATP was recovered in the AMP region of the paper. When C<sup>14</sup>-GMP was incorporated, only 3% of the counts were recovered in guanosine after alkaline degradation of the product, the rest being in GMP (Table 8). Since the guanosine spot was not entirely separated from the origin under these conditions, the few counts in the spot could be material which was not completely hydrolyzed. These results support the conclusion that essentially all of the incorporated radioactivity is in internal phosphodiester linkages rather than in terminal positions.

When guanosine-P<sup>32</sup>-PP, adenosine-P<sup>32</sup>-PP or uridine-P<sup>32</sup>-PP (CTP not tested) were incubated with an enzyme preparation and the product hydrolyzed in base and subjected to electrophoresis, radioactivity was found in all four mononucleotides (Table 9). Furthermore, the nearest neighbor frequencies to AMP so derived are little affected by a 2.5 fold increase in the amount of the unlabeled triphosphates, which suggests that these nearest neighbor frequencies are not determined randomly. Similar results were found with RNA labeled with GMP<sup>32</sup>.

With washed enzyme preparations from uninfected cells, incorporation of GMP is almost negligible, while ATP shows significant incorporation. This difference is also observed between ATP and GTP with washed enzyme preparations. Significant AMP incorporation is evident (up to 20% of "complete") in the absence of one or all of the unlabeled triphosphates, while GMP incorporation falls to less than 5% under these conditions. For these reasons, a preparation of enzyme was incubated with  $\alpha$ -P<sup>32</sup>-ATP in the absence of the other triphosphates and the resulting product was hydrolyzed in alkali and subjected to electrophoresis. The nearest neighbor of AMP under these conditions was predominantly AMP, to a lesser extent CMP, and rarely UMP or GMP (Table 9, last line).

Effect of ribonuclease on reaction and product. When 10  $\mu$ g of RNase is added to the reaction mixture, a variable decrease in activity is observed which never represents more than 50% (Table 10). Even 100  $\mu$ g

TABLE 8

Distribution of Radioactivity in an Alkaline Hydrolysate  
of  $C^{14}$ -labeled Viral RNA Polymerase Product

Precursor	AMP	GMP	Guanosine	Adenosine
ATP- $C^{14}$	165	-	-	< 3
GTP- $C^{14}$	-	204	6	-

Reactions mixtures containing either 169  $\mu$ moles of ATP- $C^{14}$  (1,960 cpm/ $\mu$ mole) plus 240  $\mu$ moles of each of the other three ribonucleotides or 32  $\mu$ moles of GTP- $C^{14}$  (5,080 cpm/ $\mu$ mole) plus 120  $\mu$ moles of each of the other three ribonucleotides were incubated for 15 minutes and prepared for electrophoresis (see Section III B). No other radioactivity except that which is indicated was demonstrable. Data are given in cpm.

TABLE 9

## Nearest Neighbor Analysis of Viral RNA Polymerase Product

Labeled substrate	Distribution of Radioactivity (as % of total)			
	Up	Gp	Ap	Cp
Guanosine-P <sup>32</sup> -PP	33.7	20.0	30.1	16.2
Uridine-P <sup>32</sup> -PP	36.2	14.1	21.1	28.5
Adenosine-P <sup>32</sup> -PP*	16.3	17.5	33.5	32.6
Adenosine-P <sup>32</sup> -PP+	15.5	16.1	35.1	33.2
Adenosine-P <sup>32</sup> -PP†	7.7	3.6	61.4	27.4

\* = 120 µg each of CTP, UTP and GTP

+ = 300 µg each of CTP, UTP and GTP

† = no added unlabeled triphosphate



TABLE 10

Effect of Ribonuclease on the Product of the  
Viral RNA Polymerase

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(1) 10 µg RNase added to reaction tube	
	<u>cpm</u>
(a) ATP-C <sup>14</sup>	
Complete	252
+ RNase	182
(b) UTP-C <sup>14</sup>	
Complete	1063
+ RNase	623
(c) GTP-C <sup>14</sup>	
Complete	1146
+ RNase	854
(2) RNase treatment of product	
(a) In PCA precipitate	
Control	850
+ RNase	49
(b) Phenol extract of reaction mixture	
Control	97
+ RNase	18

---

### Legend for Table 10

In experiment 1, (a) employed a sucrose homogenization enzyme preparation while (b) and (c) used water homogenization. ATP-C<sup>14</sup> and UTP-C<sup>14</sup> incubations as in Table I, GTP-C<sup>14</sup> incubation as in Table III.

In experiment 2 (a) a 3 times PCA washed, ethanol-ether extracted sample was dried and then dispersed in 0.4 ml of 0.01 M Tris-HCl (pH 7.2) and readjusted to pH 7.2 with NaOH. One-tenth ml of a 200 µg/ml solution of crystalline ribonuclease was added, the tube left at 37° for 30' and then chilled. 0.5 ml of 0.1 M sodium pyrophosphate was added followed by 5 ml of 0.5 M PCA. The resulting precipitate was washed once with PCA, once with ethanol-ether, and counted. The control sample was similarly treated except that 0.1 ml of Tris buffer replaced the RNase solution. In experiment 2 (b) a 30 minute incubated reaction mixture was directly extracted at 60° with phenol plus 0.5% sodium dodecyl sulfate (Scherrer and Darnell, 1962). An aliquot of the phenol extract was incubated in 0.6 ml with 0.2 M Tris (pH 7.4) and 20 µg of ribonuclease at 37° for fifteen minutes. The control was identically treated except for the absence of enzyme. After incubation, 1 mg of bovine serum albumin was added, and the samples treated as in experiment 2 (a).

of RNase are unable to abolish incorporation, although their effect is greater than that of 10 µg. On the other hand, if acid-precipitated products or products extracted with hot phenol plus sodium dodecyl sulfate (Scherrer and Darnell, 1962) are treated with ribonuclease, they are largely sensitive to the enzyme (Table 10).

Kinetics of enzyme appearance. When infected cells are harvested at various times after infection and the incorporation of GMP assayed with the sedimented enzyme fraction, detectable incorporation is evident at 3 hours and maximal activity occurs at 5-6 hours (Figure 13). At all time points beyond "0", omission of CTP and UTP from the reaction mixture reduces GMP-C<sup>14</sup> incorporation by 70%.

#### D. Discussion

Since the product of the reaction catalyzed by the enzyme isolated from infected cells is sensitive to alkali and RNase, and contains labeled mononucleotides in internal phosphodiester linkages, and since each of the tested nucleoside triphosphates is incorporated next to all of the other mononucleotides, the enzyme under consideration has the properties of an RNA polymerase. Since an ATP-generating system is required for maximal activity, it would appear that the precursors are ribonucleoside triphosphates rather than the ribonucleoside diphosphates. The lack of inhibition by phosphate differentiates the enzyme from polynucleotide phosphorylase. The enzyme system synthesizes, in vitro, in 15 minutes, between 10<sup>3</sup> and 10<sup>4</sup> viral equivalents of RNA per cell equivalent of material added. This is approximately equal to the maximal rate of viral RNA synthesis in infected cells. The viral RNA polymerase activity may be in the cytoplasm of the infected cell since sucrose homogenization releases much of the enzyme while breaking few nuclei. The homogenization procedure, however, might release the enzyme from the nuclei so a definitive statement on the localization of the enzyme cannot be made.

The fact that the nearest neighbor frequencies of the product are unchanged by a 2.5-fold increase in the level of unlabeled nucleoside triphosphates (see Table 9) suggests that there is a factor in the enzyme preparation which is determining these ratios. Since the lack of inhibition by actinomycin or DNase precludes DNA as a template in the reaction (Hurwitz et al., 1962b), the existence of an RNA template is strongly suggested.

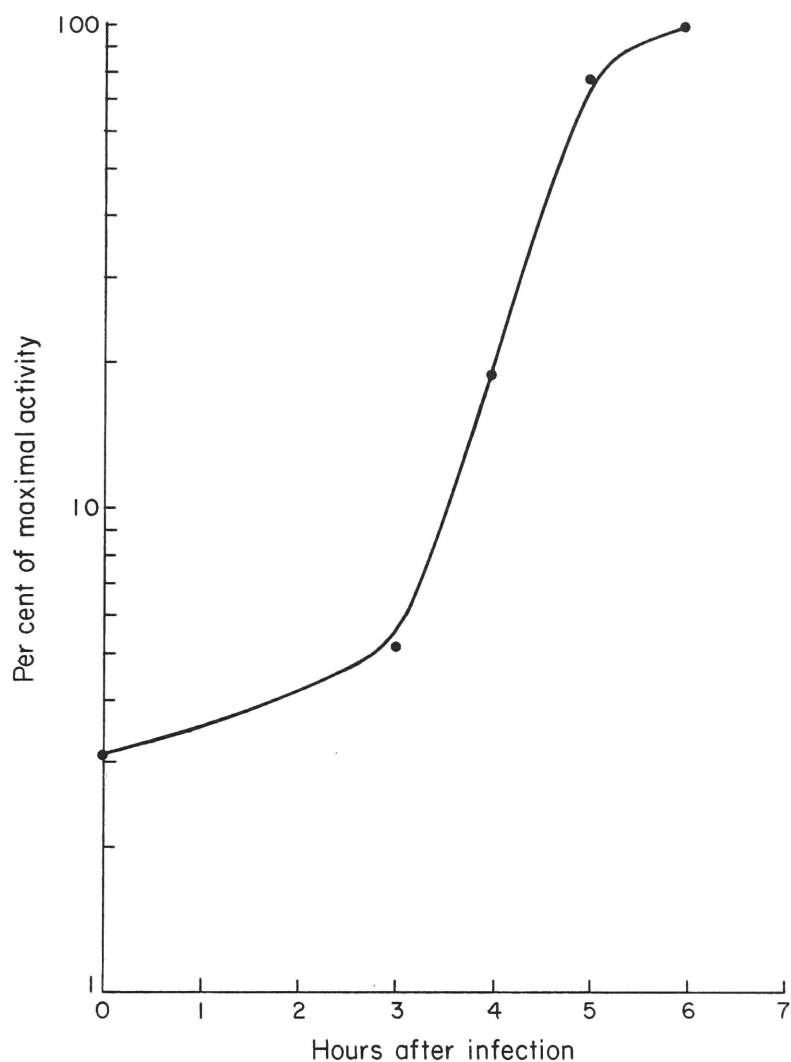


Fig. 13. Kinetics of enzyme appearance. At various times after infection, cells were harvested and enzyme preparations made. After 10 minutes' incubation in the assay mixture described in Table 6, acid precipitable radioactivity was measured. Protein concentrations were determined (Lowry et al., 1951), results were expressed as cpm/mg protein, and converted to per cent of maximum. Enzyme preparation was by sucrose homogenization.

A number of lines of evidence indicate that this enzyme is not present in uninfected cells. Study of actinomycin-inhibited cells indicates that the only DNA-independent reactions occurring in L-cells involve terminal and sub-terminal addition to preformed soluble RNA molecules (Merits, 1963; Franklin, 1963). Also, as demonstrated here, extracts from uninfected L-cells have no incorporating activity for one nucleotide which is dependent on the addition of the others. When  $\alpha$ - $P^{32}$ -ATP was used as substrate, the incorporation in enzyme preparations which occurred in the absence of the other nucleotides involved mainly polyadenylic acid (poly A) formation, as shown by the nearest neighbor studies, and some incorporation next to CMP. The poly A formation probably results from the activity of the ribosomal enzyme originally isolated by August et al. (1962), while the incorporation next to CMP is probably terminal addition to transfer RNA species (Preiss et al., 1961).

The DNA-dependent RNA polymerase from bacteria also uses RNA as a primer (Nakamoto and Weiss, 1962; Krakow and Ochoa, 1963). Since much of the DNA-dependent polymerase activity disappears after Mengovirus infection (see Section II), it might be imagined that this enzyme migrates into the cytoplasm after infection. Several arguments against this hypothesis can be raised. The nuclear enzyme is stimulated by manganese ions (see Section IIC), but the virus polymerase is inhibited, even at very low concentrations of the ion. Furthermore the decrease of nuclear activity occurs much sooner after infection than the increase of cytoplasmic incorporation (Franklin and Baltimore, 1962a). Also, in poliovirus-infected HeLa cells a similar viral polymerase appears three hours after infection (Baltimore et al., 1963), before any decrease of nuclear RNA synthesis is evident (Zimmerman et al., 1963). Under two conditions, nuclear RNA synthesis decreases without the appearance of virus RNA synthesis: when poliovirus infected HeLa cells are treated with guanidine at 2 hours after infection (Holland, 1963), and when infected cells are kept at a supra-optimal temperature (Fenwick, 1963). Lastly, there is evidence that the viral polymerase activity is markedly unstable and that the enzyme must be constantly renewed by a process requiring protein synthesis (Scharff et al., 1963; Baltimore and Franklin, 1963a; Eggers et al., 1963). The latter two points are probably the most compelling evidence for believing that the viral RNA polymerase represents a protein which is not in the

cell prior to infection and which is made under the control of the viral genome.

A word of caution must be said here with regard to the interpretation of certain of these results. In these studies, when a measurement of enzyme activity is performed, the concentration of enzyme plus template is the limiting factor. Therefore, a decrease or increase in enzyme activity could reflect simply a loss of template. For this reason, the effects which have been interpreted above as resulting from an instability of the polymerase could, in fact, be a reflection of a decreased availability of template.

It should be noted that an enzymatic activity similar to the one reported here has been demonstrated in extracts of bacteria infected with RNA bacteriophages (Weissman et al., 1963; Kaye et al., 1963). Also, it has been claimed that RNA-dependent RNA polymerase is found in both normal and EMC virus-infected Krebs II ascites cells (Burdon and Smellie, 1962; Cline, et al., 1963), but in this system terminal addition reactions have not been ruled out.

#### IV. GENERAL DISCUSSION

The foregoing data and discussions have shown that soon after attaching to the surface of its host cell, and penetrating the cell's surface, Mengovirus initiates the synthesis of a protein which causes the inhibition of the cellular DNA-dependent RNA polymerase. The virus later initiates the appearance of a cytoplasmic RNA polymerase which functions independently of DNA: the viral RNA polymerase

##### A. Related Literature and Some Assumptions

In order to provide a unified picture of the process of Mengovirus infection, and presumably of infection by poliovirus and other picornaviruses, a number of different aspects of the question must be considered. Looking first at protein synthesis in the infected cell, a fairly comprehensive picture has recently become available. Mengovirus induces a rapid inhibition of cellular protein synthesis within the first 1.5 hours after infection (Franklin and Baltimore, 1962) by a process which apparently involves viral protein synthesis (Baltimore and Franklin, 1963a). Poliovirus has a comparable effect on its host cell (Zimmerman et al., 1963), which has been shown to be due to the breakdown of polysomes to inactive, single ribosomes (Penman et al., 1963). At the time of Mengovirus production in the cell, there is an increased rate of protein synthesis (Franklin and Baltimore, 1962) which parallels the synthesis of viral protein (Krug and Franklin, 1963). Penman et al. (1963) have shown that in poliovirus-infected cells a specific species of very large, functioning polysomes appears at the time of viral protein synthesis.

A second aspect of the synthesis of picornaviruses which has become clear in the last few years is that of the intracellular site of synthesis. In 1959, Horne and Nagington described large cytoplasmic vesicles which were replete with entrapped poliovirus particles, and they suggested that this was the site of maturation of the virus. Becker et al. (1963) found that short pulses of uridine or amino acids given to actinomycin-pretreated cells (which were thus synthesizing mainly virus-related materials) were taken up into large, deoxycholate-soluble structures. The polymerized amino acids were found largely in virus-specific polysomes which were bound to these membranous structures. Uninfected cells, conversely, showed

uptake of amino acids into free polysomes and of uridine into the nucleus. Thus, biochemical evidence points to large membranous structures in the cytoplasm as being involved in the synthesis of viral protein and RNA. Some electron microscopic evidence on EMC-infected Krebs ascites cells has also indicated the existence of virus-specific cytoplasmic membranes, which were, in this work, seen in situ (Sanders and McGee-Russell, 1962). A further indication of the existence of virus-specific centers of protein synthesis comes from the work of Baltimore et al. (1963b), who demonstrated that a heavy cytoplasmic fraction from infected cells is much more active than its counterpart from uninfected cells in the in vitro incorporation of labeled amino acids into protein. Since guanidine, a specific inhibitor of poliovirus precursor synthesis, prevents the appearance of this enhanced incorporating activity, it is taken to represent virus-specific synthesis which is occurring on polysomes in large vesicles.

Thus, evidence is accumulating that picornavirus infection causes the manufacture of a new system of cytoplasmic vesicles on or inside of which are attached the polysomes involved in the synthesis of viral proteins. Virus particles may mature in association with these structures. The viral RNA polymerase also appears to be attached to these heavy particulates (Baltimore, in press).

The problem of the attachment of picornaviruses to their host cells and the release of their nucleic acid from its protein shell is still unsettled, but a certain amount of relevant data are available (Holland and Hoyer, 1962; Mandel, 1962). There are receptors at the surface of cells which can reversibly bind specific viruses; cells which are not infectable by a given virus lack these receptors. At 37°C, the bound virus undergoes a reaction whereby it is "irreversibly eclipsed". This procedure does not involve release of the nucleic acid from its protein coat, but apparently represents a change in organization of the subunits of the coat protein; a second reaction must occur which frees the nucleic acid. Joklik and Darnell (1961) found that 50% of the incoming viral RNA becomes sensitive to RNase within 30 minutes after infection, indicating that the real "uncoating" of the viral RNA is a rapid process.

Before proceeding to a statement of a model picornavirus biosynthesis, two assumptions must be stated. The first is that the viral RNA polymerase



discussed in Section III is actually the enzyme responsible for the biosynthesis of Mengovirus RNA. This assumption is reasonable: the enzyme appears at the time of virus RNA synthesis and has the properties of an enzyme synthesizing an RNA of predetermined base sequence. Studies with poliovirus have demonstrated in many ways that the ability of the cell to produce viral RNA and the presence of polymerase go hand in hand (Baltimore et al., 1963a; Eggers et al., 1963). This assumption, however, can only be considered proven when the product of the reaction can be demonstrated to be viral RNA.

The second assumption is that there are at least four separate virus-directed proteins which carry out the four viral functions which have been delineated: an inhibitor of cellular RNA synthesis, an inhibitor of cellular protein synthesis, the viral RNA polymerase, and the viral coat protein. This assumption is not critical to the model, but does facilitate discussion. There is no evidence for or against this proposal except for the difference between the times of appearance of the functions.

#### B. The Model

The conclusions from the experimental work reported here, along with the material discussed in Section IVA, lead to the following general model. It is concerned with the events occurring after a single Mengovirus particle encounters (or interacts with) a single L-cell. Although almost all data on viral infections have been obtained in experiments with mass cultures of cells, it is easier to present a model of the infective process in one cell, which can be envisaged as the prototype cell of the culture. In fact, the time relations given below probably vary greatly from cell to cell in a culture and the timing which is specified is thus only an average, and probably has a large variance.

When a virus particle comes in contact with a cell it apparently interacts with receptors on the cell surface. The virus nucleic acid must then penetrate the cell surface and be released from its protein coat. The mechanism of neither of these processes can be specified, but it is clear that half of the virus particles in a mass culture have released nucleic acid into the cell cytoplasm within 0.5 hours after adsorption, so that this time can be taken as representative for the prototype cell.

The viral nucleic acid must now find an appropriate number of free ribosomes in the cell in order to form a polysome. The polysome then provides the structure on which the viral nucleic acid can act as a template for the synthesis of at least three proteins: an inhibitor of DNA-dependent RNA synthesis, a protein which can break down cellular polysomes, and an RNA dependent RNA polymerase. The two inhibitors begin to work immediately and cellular RNA synthesis falls to about 10% of normal within 1.5 hours, while protein synthesis falls to 40% within an hour.

Sometime during the first three hours of infection an RNA-dependent RNA polymerase is formed under the direction of the viral genome. During this time the incoming strand of viral RNA cannot have been degraded since it represents the only copy of the viral information which the cell possesses. By analogy with the synthesis of DNA (Kornberg, 1961), it is plausible to assume that RNA is reduplicated by the process of complementary base pairing elucidated by Watson and Crick (1953). The first action of the new RNA polymerase must be to polymerize the complementary strand to the viral RNA, thus initiating the replication of viral RNA. The new strand can now select the nucleotides necessary to remake the viral RNA.

After the initial copy of viral RNA is made, a sequence of replication starts whereby a large number of strands of viral RNA are synthesized. Base ratio determinations (Darnell, 1962) and hybridization studies (Baltimore et al., 1964) indicate that in the cell, viral RNA is greatly in excess of complementary RNA. Therefore, either the reaction by which viral RNA is made is regulated such that only the viral strand is made or there exists a method for the preferential degradation of complementary RNA. The replication of viral RNA may involve a double strand composed of viral RNA plus complementary RNA. If this is so, the polymerase might only copy one strand of the duplex (the complementary strand) and thus produce only viral RNA.

The newly made viral RNA now has to initiate the formation of more polysomes in order that more viral proteins can be made. The progeny RNA molecules will find this a much easier process than did the initially infecting molecule. Before infection two-thirds of the cell's ribosomes are in polysomes; at 3.5 hours of infection, almost all ribosomes are free due to the virus-induced degradation of the polysomes (Penman et al., 1963).

Also, the cessation of nuclear RNA synthesis means that the viral RNA does not have to compete with cellular messenger for ribosomes.

The new polysomes now start making more viral RNA polymerase and coat protein. Whether more inhibitor proteins are made and whether the initial polysome synthesized any coat protein can not be specified. The new polymerase may combine with some of the viral or complementary RNA, or with the double-stranded structure, and thus mediate the synthesis of more viral RNA. This cycle would then continue and a large pool of coat protein and viral RNA would build up from which whole virus could crystallize.

One important element of virus synthesis has not been discussed. The viral polysomes and the viral polymerase are found associated with large membranous structures. Furthermore, large concentrations of virus have been seen in large membranous structures in the cytoplasm of infected cells. We therefore can consider that most or all of the processes specified in the model occur in or on these vesicles. The value of this would be that the viral precursors could be retained in a concentrated state instead of diffusing throughout the cytoplasm where the protein and RNA might find difficulties in aggregating together to form virus particles.

The supporting evidence for much of this model has been detailed above. A viral RNA polymerase has been shown to exist, as have virus-specific polysomes. Very recently Montagnier and Sanders (1963b) have reported the existence of a double-stranded infectious form of EMC virus RNA in infected Krebs ascites cells. This is presumed to be the replicating form of the viral RNA, analogous to the replicating form of ØX174 bacteriophage, a virus with single stranded DNA (Sinsheimer et al., 1962). The high, sharp melting point of the double-stranded RNA is consistent with its having a Watson-Crick base-paired structure. Reovirus RNA, which is double-stranded (Gomatos and Tamm, 1963), also has a high melting point (Gomatos and Tamm, 1963). Reovirus RNA has a DNA-like secondary structure (Langridge and Gomatos, 1963) and equal quantities of adenine and uracil, guanine and cytosine, which indicates a Watson-Crick configuration. The base composition of EMC RNA is too near to equimolar amounts of the four bases (Faulkner et al., 1961) to allow determination of the base-pairing mode in the double-stranded EMC RNA simply by base composition analysis. The virtual equality of the melting points of this RNA and Reovirus RNA

argues strongly that the replicating form has the same structure as native DNA. The existence of a double-stranded viral RNA in poliovirus-infected cells has been confirmed (Baltimore et al., 1964) and we are at present attempting to determine if it is an intermediate in viral RNA synthesis.

Other aspects of the model have yet to receive experimental support, although work is in progress to elucidate them. This includes the association of the incoming viral RNA with a polysome and the demonstration that the virus-specific polysomes are made from ribosomes which were depolymerized by the viral inhibitor of protein synthesis.

One important aspect of virus synthesis has not been covered in this model because no relevant data are available. This is the matter of control of viral protein synthesis. Are the inhibitors and the polymerase made at the same rate as coat protein throughout infection? A priori it appears wasteful for a large excess of these other proteins to be made, but there may be no choice, and since the cell is due to die anyway, the waste is not very significant. If a cell makes  $10^5$  virus particles, this represents  $1.3 \times 10^{-12}$  gms of protein. Since a cell contains about  $440 \times 10^{-12}$  gms of protein ((Salzman, 1959)), even an amount of protein 10 times greater than that of viral protein would not represent more than 5% of the cellular protein. On the other hand, cellular protein synthesis falls markedly after infection and is still low during the time of virus synthesis. Because of this, it is apparent that between 3 and 5 hours post-infection, when virus protein is made, the total amount of protein made is not equal to 5% of the normal cellular protein. One hour of maximal protein synthesis is necessary for a cell with a 20 hour generation to make 5% of its protein.

Certain models for control of viral protein synthesis are interesting to consider. Although most models of control in bacteria assume that it involves regulation of messenger RNA synthesis (Jacob and Monod, 1961), there are indications of control at the level of messenger function (Ames, 1963) which would be more analogous to the present system. Three models for the control of viral protein synthesis are considered below.

Model 1. All proteins are made at the same rate, but the excess protein is rapidly degraded or inactivated. This model, which is clearly inefficient and of limited value, has one feature to recommend it. If the

polymerase were made as rapidly as the coat protein, an imbalance between viral RNA synthesis and virus maturation would occur since the polymerase is used catalytically, while the coat protein is used stoichiometrically. An unstable polymerase would rectify this disparity. In fact, there is much evidence for the instability of the polymerase (Baltimore et al., 1963a; Baltimore and Franklin, 1963b, c; Eggers et al., 1963) which makes this proposal plausible. This model can be incorporated into either of the other two models, especially as it relates to the instability of the polymerase. No evidence about the stability of the inhibitory proteins is available.

Model 2. The problem of why complementary RNA is not made in large quantities has not been settled, although the data of Holland (1962a) and Darnell (1962) clearly require either preferential synthesis of viral RNA or rapid degradation of the complementary RNA. If one were to suppose that both RNA's are made but that the complementary RNA is used preferentially for polysome synthesis, while the viral RNA goes mainly into virus particles, this difficulty would be overcome and a control mechanism would emerge. The complementary RNA is thus assumed to have the information for coat protein synthesis, while the viral RNA encodes the polymerase and other proteins. Since some viral RNA does get into polysomes (the incoming strand must do this, so progeny molecules probably also can) polymerase synthesis continues throughout infection (as it is known to do; Eggers et al., 1963; Baltimore and Franklin, 1963b). However, on this model polysomes are mainly formed using the complementary RNA so the bulk of viral protein synthesis is of coat protein. The model, then, postulates that polysome RNA is degraded with reasonable rapidity, and that viral RNA in virus and virus precursor stages is insusceptible to degradation.

Model 3. On this model viral RNA is the messenger for the polysome, but a reason for the lack of accumulation of complementary RNA cannot be specified. This model was suggested by the results of Ames (1963) and would be applicable to the bacterial system he has studied. The virus RNA is assumed to have a unique, linear arrangement of cistrons with the information for the coat protein monomer at one end. This end is then thought to be the initiation point at which the ribosome begins to read out the RNA into protein. After rapid completion of the coat protein monomer, the ribosome is prevented from continuing on to the next cistron, and often

is released from the viral RNA. A certain percentage of the time the ribosome can continue on and read out the next protein, but again at the end it has a probability of not continuing. Thus, there would be more coat protein made than any other protein and the rate of ribosome release at the end of one cistron would determine how often the following cistron is read out. The model could be changed such that the ribosomes initiate the reading out of each cistron separately and control would then be exerted at this initiation point, but this would be at variance with Ames' (1963) results which indicate sequential reading of proteins from a multicistronic message.

Various combinations of these models can also be envisaged, but in the absence of relevant data, such speculation is unlikely to be profitable.

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