Biochemical and Biological Studies on the Growth of an RNA Containing Bacteriophage

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BIOCHEMICAL AND BIOLOGICAL STUDIES ON THE GROWTH
OF AN RNA CONTAINING BACTERIOPHAGE

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ABSTRACT

The growth of the RNA-containing bacteriophage f2 has been studied.

DNA synthesis appears normal after phage infection. It is possible to dissociate the synthesis of viral RNA from the synthesis of DNA using thymine-less bacteria with fluorinated pyrimidines and Mitomycin C as inhibitors of DNA synthesis. It is concluded that the growth of f2 is independent of DNA synthesis.

Since the phage RNA has no special characteristics, its synthesis has been followed by using P³² to label RNA which is eventually incorporated into virus particles. Using this method of measuring phage RNA synthesis, it has been shown that the material for the synthesis of the virus nucleic acid (Phosphorus) comes almost entirely from the components of the medium, and not from the host cell. Also, only 10% of the virus particles released from an infected cell can form plaques in the phage assay system.

Chloramphenicol (CA) has been used to elucidate the relationship of protein synthesis to the synthesis of phage f2 RNA. Phage RNA synthesis is inhibited if CA is added within two minutes after infection. If CA is added at later times, some phage RNA is synthesized. Addition of CA 15 minutes after infection allows phage RNA to be synthesized in amounts similar to the amount synthesized in uninhibited cells.

The protein coat of f2 does not contain the amino acid histidine. By using as host bacteria a strain which requires histidine and methionine, it was shown that the synthesis of f2 becomes independent of the presence of histidine halfway through the latent period, but remains dependent on the continued presence of methionine. These results suggest that a protein, other than the coat, is synthesized early after phage f2 infection, which is essential for the synthesis of virus RNA.

An enzyme activity which catalyzes the incorporation of ribonucleoside triphosphates into an acid precipitable material appears after infection. It has a number of characteristics which indicate that it is a new phage induced enzyme.
PREFACE

This thesis is concerned with various aspects of the growth of the recently discovered RNA-containing bacteriophage f2. As it was desired to make this thesis concise, only those implications directly related to RNA phage growth will be discussed. No attempt has been made to present a complete review of the field of virology, and the introduction reviews only those areas which are necessary for an understanding of the experiments reported in this thesis.

I wish to thank Mrs. Georgia Fisher for her cooperation in the typing of this thesis; Miss Muriel Fabrizio for her excellent illustrations; Mrs. Doris Degan Grano for her aid in the laboratory; Mr. Henry Smilowitz for his assistance in the performance of some of these experiments; Mr. Flinn for his kindness during my stay at the Institute; Dr. Brink and Dr. Bronk for making this experience at the Rockefeller Institute possible; the members of the Institute library, purchasing office, and illustration service for their help during the past four years; and my wife, Alexandra, to whom I dedicate this thesis, for her aid both in the laboratory and in the preparation of this thesis.

Above all, I wish to thank my advisor and teacher, Professor Norton D. Zinder, for his guidance and counsel during the course of this work.
TABLE OF CONTENTS

ABSTRACT ......................................................... ii.
PREFACE ........................................................... iii.
TABLE OF CONTENTS .............................................. iv.

I. INTRODUCTION .................................................. 1
   A. Phage f2 ......................................................... 1
      1. Isolation of Phage f2 ........................................ 1
      2. Relationship of Phage f2 to Bacterial Sexuality .......... 1
      3. Physical and Chemical Characteristics of Bacteriophage f2 1
      4. Growth and Biological Characteristics of Bacteriophage f2 2
      5. Effect of f2 on Host Metabolism ............................ 2
      6. Infectious Ribonucleic Acid from f2 ......................... 2
      7. Appearance of Bacteriophage f2 in the Electron Microscope 2
   B. Special Aspects of Virus Growth ............................. 3
      1. The Growth of the T-even Bacteriophages ................. 3
      2. Viability of Virus Particles ............................... 3
      3. Inhibition of Host Biosyntheses ......................... 4
      4. Complementary Nucleic Acid Synthesis .................... 4
      5. Early Protein -- Biological Results ....................... 5
      6. Early Protein -- Enzyme Studies .......................... 6

II. MATERIALS AND METHODS ...................................... 7
   A. Materials ..................................................... 7
      1. Chemicals ................................................... 7
      2. Media ........................................................ 7
      3. Bacterial and Phage Strains ............................... 8
   B. Methods ...................................................... 8
      1. Preparation of Phage stocks ............................... 8
      2. Phage Growth Experiments ................................. 8
      3. Chemical and Radiochemical Determinations ............... 9
      5. Assay of Ribonucleoside-triphosphate Incorporating Ability 10

III. RESULTS ...................................................... 11
   A. Relationship of DNA Synthesis to the Growth of f2 ........ 11
      1. Nucleic Acid Synthesis in Phage Infected Cells .......... 11
      2. Inhibition of f2 by Fluorinated Pyrimidines ............. 11
      3. Inhibition of DNA Synthesis Measured by Uptake of
         Adenine-C14 .................................................. 12
      4. Additional Experiments on the Inhibition of DNA Synthesis 13
      5. Mitomycin C and the Growth of f2 .......................... 13
      6. Summary .................................................... 14
TABLE OF CONTENTS

III. RESULTS (continued)

<table>
<thead>
<tr>
<th>Section Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. Experiments on Synthesis of f2 RNA</td>
<td>15</td>
</tr>
<tr>
<td>1. Source of Phage RNA</td>
<td>15</td>
</tr>
<tr>
<td>2. Viability of Progeny Virus Particles</td>
<td>16</td>
</tr>
<tr>
<td>3. Time Course of Phage RNA Synthesis</td>
<td>16</td>
</tr>
<tr>
<td>4. Summary</td>
<td>17</td>
</tr>
<tr>
<td>C. Relationship of Protein Synthesis to the Synthesis of Phage RNA</td>
<td>17</td>
</tr>
<tr>
<td>1. Synthesis of Phage RNA in the Presence of Chloramphenicol</td>
<td>18</td>
</tr>
<tr>
<td>2. Inhibition of Phage RNA Synthesis by Chloramphenicol</td>
<td>18</td>
</tr>
<tr>
<td>3. Escape from Chloramphenicol Inhibition</td>
<td>19</td>
</tr>
<tr>
<td>4. Growth of f2 in the Absence of Histidine</td>
<td>20</td>
</tr>
<tr>
<td>5. Summary</td>
<td>21</td>
</tr>
<tr>
<td>D. Appearance of a New Enzyme Activity after Phage Infection</td>
<td>22</td>
</tr>
<tr>
<td>1. Appearance of a New Enzyme Activity</td>
<td>22</td>
</tr>
<tr>
<td>2. Characteristics of the New Enzyme Activity</td>
<td>23</td>
</tr>
<tr>
<td>3. Summary</td>
<td>24</td>
</tr>
<tr>
<td>IV. DISCUSSION</td>
<td>25</td>
</tr>
<tr>
<td>V. BIBLIOGRAPHY</td>
<td>27</td>
</tr>
</tbody>
</table>
INTRODUCTION

The first part of the introduction is a short summary of the discovery and pertinent physical, chemical, and biological characteristics of bacteriophage f2. The second part is a brief review of some of the concepts of virus growth and biosynthesis which have formed the background of the experiments presented in this thesis.

A. Phage f2

1. Isolation of Phage f2: Bacteriophage f2 was isolated from New York sewage by Loeb (1960) as one of a group of seven phages (f1 - f7) which would form plaques only on F+ and Hfr strains of Escherichia coli. These phages fall into three serological groups with the prototypes f1, f2, and f4. Other bacteriophages similar to f2 have been isolated (Paranchych and Graham, 1962; and Marvin and Hoffman-Berling, 1963). They are similar to f2 in host specificity and physical characteristics. Feary et al. (1963) have isolated a small RNA containing bacteriophage which grows on Pseudomonas aeruginosa.

2. Relationship of Phage f2 to Bacterial Sexuality: Bacterial sexuality and the heterothallic nature of bacterial mating have been reviewed by Jacob and Wollman (1962) and Loeb (1962). "Male" strains of E. coli (F+ and Hfr) can associate with "female" strains (F-) and inject some genetic material into the female strains. Phage f2 cannot adsorb to female strains of E. coli. Therefore, the resistance of female strains to f2 is due to the inability of the virus to penetrate the cell, rather than to any internal inhibition of virus growth. This was confirmed by Loeb (1962) when he showed the RNA isolated from the virus can lead to the growth of mature phage when added to protoplasts of a female bacterium. Preliminary results by the author indicate that f2 does not have much effect on the mating of bacteria. It may be concluded that the growth of the phage f2 does not have any necessary dependence upon the sexual type of the host cell other than the inheritance of some specific viral receptors which will allow the virus to attach to and penetrate the cell.

3. Physical and Chemical Characteristics of Bacteriophage f2: The physical and chemical characteristics of Phage f2 are listed in Table I. Since the molecular weight of the nucleic acid (as determined by light scattering)
**TABLE I**

Physical and Chemical Properties of Phage f2

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter of phage particle</td>
<td>200 Å</td>
</tr>
<tr>
<td>Molecular weight of f2*</td>
<td>3,000,000</td>
</tr>
<tr>
<td>Molecular weight of f2 RNA*</td>
<td>700,000</td>
</tr>
<tr>
<td>Per cent RNA</td>
<td>23-25</td>
</tr>
<tr>
<td>Per cent DNA</td>
<td>0</td>
</tr>
<tr>
<td>No Histidine in protein coat</td>
<td></td>
</tr>
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</table>

**Nucleic Acid Composition**

- Adenine: 22
- Guanine: 26
- Cytosine: 27
- Uracil: 25

*Light scattering determination by Dr. Muriel Roger*
is approximately one quarter of the molecular weight of the phage particle, and the amount of nucleic acid in the phage is one quarter of the mass of the virus particle, the ribonucleic acid of the virus is probably in a single molecule. Chemical analysis of the phage reveals that there is no histidine in the protein coat. Analysis of the RNA has shown that there are no particular abnormal bases or unusual base ratios which will allow direct chemical identification of the phage in the presence of the host RNA.

4. **Growth and Biological Characteristics of Bacteriophage f2:** The growth of phage f2 as reported by Loeb and Zinder (1961) is presented in Figure 1. The burst size is large, approximately 5,000 to 10,000 particles from each infected cell, and the entire period of growth is completed by about 60 minutes after infection. Calcium is required for infection, although it is not required for adsorption (Zinder, unpublished results). There is a 15 minute eclipse period during which no mature phage particles can be recovered from the infected cells. Phage f2 is lysis inhibited; high multiplicities of infected phage, presumably leading to continuous infection of the cells, prolong lysis, leading to high burst sizes. Phage f2 makes smaller plaques than would be expected from a phage of its size, and this is presumably due to the difficulty of infecting physiologically old cells on the assay plate and lysis inhibition during plaque formation. The best infection occurs when the cells are at a relatively low density -- in the range of 1-2 x 10^8 cells per milliliter.

5. **Effect of f2 on Host Metabolism:** Infection of E. coli does not seem to cause any major changes in host metabolism. Enzyme synthesis and enzyme induction after infection are not very different from uninfected cells (Zinder, 1963), and protein synthesis (Paranchych and Graham, 1962) and nucleic acid synthesis appear to be quite normal (III, A, 1).

6. **Infectious Ribonucleic Acid from f2:** Infectious ribonucleic acid can be extracted from phage f2 by treatment of the phage with phenol and precipitation of the nucleic acid with alcohol. Protoplasts placed in a hypertonic medium expand when the RNA in a hypotonic solution is added. This leads to the incorporation of the viral nucleic acid by the protoplasts, and to the subsequent formation of mature progeny phage.

7. **Appearance of Bacteriophage f2 in the Electron Microscope:** An electron micrograph of phage f2 is shown in Figure 2. The virus is small and spherical, similar in appearance to the small RNA containing animal viruses
FIGURE 1

Single Cycle Growth of Phage f2

From Loeb and Zinder, (1961)
The phage were negatively stained by embedding in neutral phosphotungstate. The micrograph was taken by Dr. W. Stoeckenius.
(such as poliovirus) or plant viruses. It is approximately 22 mµ in diameter and has no apparent tail.

B. Special Aspects of Virus Growth

The DNA containing T-even phages have been well studied and may be considered to be the best understood viruses. The concepts and methods derived from the study of the T-even viruses have provided a stimulus for the study of similar phenomena in other virus systems. It would therefore be of value to review briefly the growth of the T-even bacteriophages with emphasis on those results and concepts which will be most relevant to the understanding of the growth of phage f2. The descriptions of the various aspects of virus growth will be brief, as the growth of the T-even phages has been discussed in many reviews (Kellenberger, 1961; Mahler and Fraser, 1961; Stent, 1959; Adams, 1959; Sinsheimer, 1960; and Luria, 1962).

1. The Growth of the T-even Bacteriophages: Figure 3 and its legend provide a brief description of the main aspects of the synthesis of the T-even bacteriophages. The main purpose for presenting this schematic outline is to provide a framework for the discussion of the results to be described on studies of f2. The main points to note in the outline presented in Figure 3 are the synthesis of RNA from the phage DNA "template", and the synthesis of enzymes which are needed for the synthesis of the phage DNA. As described below the existence of the enzymes was first suggested by studies using inhibitors of protein synthesis (I, B, 6), and only later were the enzymes actually identified with enzyme assays (I, B, 7).

2. Viability of Virus Particles: The ability of virus particles to form plaques (or cause infection, lesions, or death) can vary widely depending upon the virus and the assay system. Reviews of the reported abilities of viruses to initiate infection have been presented by Isaacs (1957), Schwerdt (1959), and Wildy and Watson (1962). The main point to note is that with few exceptions the animal and plant viruses have a very low plaque forming ability (expressed as Plaque Forming Units (PFU) particle). With the bacteriophages, however, Luria, Williams, and Backus (1951) have reported that every T2 bacteriophage particle is able to initiate infection. This difference between bacterial and other viruses is usually ascribed to some inefficiency in the assay systems of the latter and not to any intrinsic inviability of the particles. The plaque forming ability of f2 is described in section III, B, 2.
A phage attaches to a bacterium by its tail (A) and in some manner injects its contents, a large molecule of DNA into the cell (B). The nucleic acid then directs the inhibition of the host cell metabolism, and also the breakdown of the host cell DNA (B). Ribonucleic acid molecules are then synthesized using the DNA as a directing template (C). These molecules, denoted by m, have been termed "messenger" RNA. These "messenger" molecules then attach to ribosomes (D). The ribosome-messenger RNA complex then directs the synthesis of proteins which have been collectively termed "early protein". This "early protein" has been interpreted to be phage induced enzymes synthesized after infection (E). These enzymes direct the synthesis of various precursors of DNA and also the DNA of the phage progeny, using the DNA of the infecting phage as a template (F). These progeny DNA molecules the synthesis of more messenger molecules (F), which lead to the synthesis of the protein components of the phage coat. A lysozyme is also produced which acts on the cell wall of the bacteria leading to lysis of the cell (G). In some manner the nucleic acid produced by the enzymes (F) is incorporated into protein coats (H), and these mature virus particles are then released where they can again initiate infection.
FIGURE 3

Growth of the T-even Bacteriophages
3. **Inhibition of Host Biosyntheses:** After infection by the T-even viruses the ability of the host cell to synthesize new host enzymes is inhibited. Net synthesis of RNA ceases, and there is a delay of approximately 6 minutes before DNA synthesis resumes after infection. The mechanism of inhibition of host cell biosyntheses appears to be distinct from the breakdown of the host nucleic acid. Other virus-cell systems exhibit an inhibition of host cell syntheses. Host cell DNA, RNA, and protein synthesis are inhibited after infection with mengovirus (Baltimore and Franklin, 1962) and Newcastle disease virus (Wheelock, 1961). Reovirus inhibits only DNA synthesis after infection (Dr. P. Comatos, unpublished observations).

4. **Complementary Nucleic Acid Synthesis:** The work of Volkin and Astrachan (1956) and of Hall and Spiegelman (1961) has demonstrated that the synthesis of the DNA-containing bacteriophage T2 requires the synthesis of specific RNA molecules. This RNA was initially recognized by the similarity of its base composition to the DNA of the infecting virus. The discovery of this kind of RNA led to the development of current ideas regarding the transfer of genetic information from the "primary" genetic material (DNA in the case of the T2 phage) through a "messenger" (RNA) to the synthesis of phage-specific protein (I, B, 8). There is evidence that DNA-containing animal viruses also require RNA synthesis for their development (Tamm et al., 1960; Tamm and Bablanian, 1960).

Studies with RNA viruses have indicated that their synthesis is not dependent upon DNA synthesis. The work of Simon (1961) indicated that poliovirus and Newcastle disease virus can replicate when DNA synthesis is inhibited. Also, Mitomycin C, an inhibitor of DNA synthesis has no effect on mengovirus or poliovirus synthesis (Reich and Franklin, 1961). Staehelin and Gordon (1960) have suggested that the synthesis of tobacco mosaic virus is independent of DNA synthesis, although their conclusion was limited by technical factors. Gierer (1962) reports that Davern and Bonner have confirmed that the growth of TMV is not dependent upon DNA synthesis.

The main conclusion to be derived from these studies is that DNA viruses are dependent upon the synthesis of RNA for virus growth, while the RNA viruses investigated are independent of DNA synthesis. The question may therefore be asked, is the synthesis of phage \( f_2 \) dependent upon the synthesis of DNA?
5. Early Protein--Biological Results: The studies of Burton (1955), Tomizawa and Sunakawa (1956), and Hershey and Melechen (1957), indicated that after T-even phage infection there was some synthesis of protein necessary for the initiation of phage DNA synthesis. It was found that if protein synthesis was inhibited at the start of infection, no phage nucleic acid would be synthesized. If a period of synthesis was allowed before protein synthesis was inhibited, phage nucleic acid could be synthesized. The protein necessary for the synthesis of virus nucleic acid after infection will be called "early protein" and will be defined by a strict operational definition. Early protein is that protein synthesized after virus infection which will allow virus nucleic acid to be synthesized even after protein synthesis is subsequently inhibited. It is defined experimentally by adding an inhibitor of protein synthesis (chloramphenicol or 5-methyltryptophan to bacteria and p-fluorophenylalanine or puromycin to animal cells) at the start of infection, and comparing the synthesis of virus nucleic acid with the synthesis of virus nucleic acid when protein synthesis is inhibited some time after the start of virus infection. If viral nucleic acid is synthesized only when protein synthesis is inhibited at some period after infection, then the conclusion is made that some "early protein" synthesis is necessary for virus nucleic acid synthesis.

There are three points to note regarding this definition. First, it is assumed that the penetration of the virus and release of nucleic acid is not inhibited when protein synthesis is inhibited. Second, "early protein" will only be found when the synthesis of virus nucleic acid is not "coupled" to the synthesis of proteins, (the synthesis of virus nucleic acid is not dependent upon the continuation of protein synthesis). And third, one must be able to recognize virus nucleic acid.

Evidence for some "early protein" synthesis after virus infection has been presented for a number of other viruses including poliovirus (Levintow, et al., 1962), mengovirus (Franklin and Baltimore, 1962) and T5 (Kellenberger, 1961), although in the last case the criterion of "early protein" synthesis was the initiation of nuclear breakdown after phage infection. It should be noted, however, that in some cases of animal virus synthesis, evidence has been presented which suggests that concomitant protein synthesis is needed for the continuing synthesis of viral nucleic acid (Wecker and Richter, 1962; Scharff, et al., 1963).
6. Early Protein--Enzyme Studies: The biochemical basis for the necessary protein synthesis (as discussed in section I, B, 5) was implicit in the finding of Wyatt and Cohen (1953) of an unusual pyrimidine base in the DNA of the T-even bacteriophages. One could have postulated the need for a new enzyme in order to produce the 5-hydroxymethylcytosine that is a component of the bacteriophage DNA. Flaks, Lichtenstein, and Cohen (1959) showed that after infection a new enzyme activity did appear--a deoxycytidylate hydroxymethylase. Pizer and Cohen (1962) have shown that there is no detectable synthesis of this enzyme before infection. Further work by a number of groups has led to the discovery of a number of new enzymes synthesized after phage infection (deoxycytidylate deaminase, Keck, et al., 1960; hydroxymethyldeoxycytidylate kinase, deoxycytidine triphosphatase, hydroxymethyl deoxycytidylate kinase, and hydroxymethyl deoxycytidylate glycosylases, Kornberg, et al., 1959). The evidence for the appearance of phage-induced enzymes have been reviewed by Cohen (1961) and Kornberg (1961).

Besides the synthesis of new enzymes (not present in the uninfected cell) there is an increase in the activity of enzymes similar to those found in the host (Kornberg, et al., 1959). It is probable that the increase is due to the appearance of new enzymes with the same functional specificity as the host enzymes. Aposhian and Kornberg (1962) have shown that the DNA polymerase synthesized after phage infection is different from the E. coli DNA polymerase, and the two enzymes can be separated by chromatographic techniques. Other phage induced enzymes have been shown to be functionally similar but chemically different from host cell enzymes (thymidylate synthetase, Greenberg, et al., 1962; thymidylate kinase, Bello, et al., 1961a; deoxyguanylate kinase, Bello, et al., 1961b).

The appearance of new enzymes after virus infection has also been reported for phage T5 (Crawford, 1959) and certain animal viruses. A virus-induced thymidine kinase is synthesized after vaccinia virus infection (Kit, Dubbs, and Piekarski, 1963). Evidence has also been presented for the appearance after mengovirus infection, of a new activity which incorporates ribonucleoside triphosphates into an acid insoluble product (Baltimore and Franklin, 1962).
II

MATERIALS AND METHODS

A. Materials

1. Chemicals: 5-Fluorodeoxyuridine (FUDR) was obtained from Dr. Duchinsky of Hoffmann-LaRoche, Inc. Fluorouracil was kindly supplied by Dr. A. Tomasz. Mitomycin C was obtained from the Kyowa Hakko Kogyo Co., Ltd. Radioactive phosphate (P32) was purchased from the Oak Ridge National Laboratory, and adenine-8-C14 was obtained from the California Biochemical Company. Chloramphenicol (CA) was purchased from Park-Davis, Inc.

2. Media: The tryptone broth used for general growth and assay of the phage contained per liter: 10.0 g Bacto-tryptone; 1.0 g yeast extract; 1.0 g glucose; 8.0 g NaCl; and 0.22 g CaCl2. Agar plates were prepared by supplementing the tryptone broth with 15.0 g of agar per liter. Top agar contained 7.0 g of agar per liter.

The peptone medium used as a low-phosphate broth contained per liter: 10.0 g peptone (Difco); 1.0 g glucose; 5.0 g NaCl; and 0.22 g CaCl2.

Tris-glucose (TG) medium was used when a low phosphate minimal medium was needed. It contained per liter: 1.48 g KCl; 4.60 g NaCl; 1.06 g NH4Cl; 13.0 g Sigma 121 (Tris); 10.0 g glucose; 0.25 g CaCl2; and 0.12 g MgSO4. Ten milliliters of casein hydrolysate from Nutritional Biochemicals was added per liter to make the TG-HC medium. Phosphate was added in the form of KH2PO4 to give a desired phosphate concentration. The medium was adjusted to pH 7.2 with HCl before using.

The Vogel-Bonner (VB) medium was used as a minimal medium when low phosphate concentration was not needed. It contained per liter: 0.2 g MgSO4·7 H2O; 3.5 g NaNH4HP04·4 H2O; 2.0 g citric acid ·1 H2O; 10.0 g K2HP04; and 2.0 g glucose. After sterilization 0.22 g of CaCl2 was added.

The Eosin-Methylene Blue (EMB) contained per liter: 8.0 g N-Z Case; 1.0 g yeast extract; 5.0 g NaCl; 2.0 g K2HP04 (anhydrous); 0.3 g eosin (Hilton-Davis); 0.05 g meth blue (chloride); 16.0 g agar; and 1.0 g dextrose. Cultures were checked for contamination with phage by drawing a loopful of a culture repeatedly across the surface of an EMB plate. After incubation of the agar plate, any contamination of the culture can be noted by the presence of reddened,
mottled colonies. Physiological saline used for dilutions of the phage and bacteria was supplemented with 0.22 g of CaCl₂ per liter.

3. **Bacterial and Phage Strains:** The following bacterial and phage strains were used in this study:

- *Escherichia coli* K13, a prototroph derived from Hfr Reeves, used as an indicator and host for phage f₂;
- E. coli K20, a derivative of K13 requiring uracil (or thymine) for growth;
- E. coli K24, from Dr. F. Ryan, F⁺, requiring thymine for growth;
- E. coli K35, from Dr. E. Borek, is a histidine and methionine requiring strain relaxed for the control of RNA synthesis (Borek, et al. 1955); it can synthesize RNA when some amino acids are absent.
- E. coli K36, is an f₂ resistant derivative of K35; does not adsorb f₂.
- λv, a virulent mutant of phage λ.

**B. Methods**

1. **Preparation of Phage Stocks:** Phage stocks were made in tryptone medium by one cycle of growth. Because the growth of f₂ is lysis inhibited, lysozyme and chloroform were added to liberate the phage from the cells. Cellular debris was removed by centrifugation and the stocks were kept frozen until used. Assays for the phage and general phage methods are described by Adams (1959).

2. **Phage Growth Experiments:** Calcium is not a cofactor for adsorption, but is required for the successful completion of infection. Even in the presence of calcium, however, the killing of bacteria is not very efficient. There is usually a small proportion of bacteria (10-30%) that remains viable after infection with a high multiplicity of phage. This resistance is not genetically determined, for subsequent testing of the descendants of the viable survivors showed that they were still sensitive. The measurement of infective centers was complicated by the desorption of phage from the bacteria; this desorption led to relatively high titers of free phage particles. These two effects were larger in minimal media than in broth. In order to assure accurate assays of infected bacteria, free phage were removed by two washings of the cells in saline-calcium, and treatment with phage antiserum. Even with
these precautions, about 10% of the adsorbed phage were viable after treatment of the infected cells with chloroform.

For growth experiments, overnight cultures of bacteria were inoculated into fresh medium and aerated by shaking until a density of 1 to 2 x 10^8/ml (as determined in a Petroff-Hausser Counter) was obtained. Bacteria in greater densities were sometimes refractory to infection. Phage was added to a concentrated and washed culture of the bacteria, and after time for adsorption (5-10 minutes) the infected cells were freed from unadsorbed phage (as described above) and diluted into media for growth or chemical studies.

3. Chemical and Radiochemical Determinations: Deoxyribose was determined by the diphenylamine reaction (Dische, Z, 1955). Phosphate was determined by the method of Fiske and SubbaRow (1925). Radioactive nucleic acids were fractionated by means of the modified Schmidt-Thannhauser method of Hershey (1953). For this fractionation, an aliquot of the cells was precipitated with cold 5% TCA (bovine serum albumin was added as a carrier) and then hydrolyzed in 1 N NaOH for 18 hours at 37°. The TCA precipitable fraction after the hydrolysis was called DNA. The use of P^32 incorporation as a measure of nucleic acid synthesis, probably gives values which are somewhat high for the synthesis of DNA owing to contamination of the DNA fraction with phosphoproteins, phospholipids, and undigested RNA. Radioactive phosphate and carbon were counted on a thin-window gas-flow counter and the counts were corrected for self-adsorption.

4. Measurement of Phage RNA Synthesis: Because f2 RNA does not contain any unique component, and infection with f2 does not appear to arrest host RNA synthesis, a relatively indirect method was used to measure the synthesis of phage RNA, defined as RNA synthesized after infection and eventually incorporated into virus particles. This definition excludes any pool of phage RNA which by the time of lysis has not been encased in a protein coat.

The general method used in measuring the amount of phage RNA synthesized during any interval after infection is summarized below:

E. coli K13 is grown to a density of 1-2 x 10^8/ml in peptone medium, the culture is centrifuged and the cells are resuspended in fresh, prewarmed medium, infected with a multiplicity of approximately 10 plaque forming units per bacterium, and aliquots distributed as indicated in the various experiments. Radioactive label (P^32) and chloramphenicol are added for various times during
the growth cycle. When CA is added during the growth cycle a period of growth must be allowed after removing the inhibitor in order to allow any phage RNA formed to be encased in a protein coat. The CA and P\textsuperscript{32} are removed by centrifuging the cells, washing once with saline calcium, and resuspending in fresh media. After the growth cycle is completed, lysozyme and chloroform are added to insure complete lysis of the cells, and the lysate is incubated with DNase and RNase. A known amount of pure phage is added to the lysates as carrier and the phage is isolated by the procedures described in Figure 4. The specific activity of the purified phage fraction is measured. From the amount of carrier phage added and the specific activity of the labeled phosphate added, one can calculate the amount of phage RNA that had been synthesized during the labelling period (see legend to Table VI).

5. **Assay of Ribonucleoside-triphosphate Incorporating Ability**: The assay is similar to the assay for DNA-dependent RNA polymerase, with the exception that DNase is present at a high concentration. The assay mixture contained, in one milliliter: Tris (pH 8.5), 20 μmoles; MgCl\textsubscript{2} μmoles; 2-mercaptoethanol, 20 μmoles; Phosphoenolpyruvate, 4 μmoles; Phosphoenolpyruvate kinase, 4 μg; GTP\textsuperscript{32} (approximately 20,000 CPM/μmole) 60 μmole; ATP, CTP, UTP, 100 μmole each; f\textsubscript{2} RNA, 180 μg; and DNase, 40 μg. Infected or uninfected cells were ground with 2 cell volumes of alumina and extracted with 8 cell volumes of buffer (0.02 M Tris (pH 7.5); 0.01 M 2-mercaptoethanol, and 0.0015 EDTA). The extract was centrifuged for 10 minutes at 30,000 x g and the supernate was added to the assay mixture. The assay was incubated at 37°C for 20 minutes. The product was precipitated with 5% TCA and 0.01 M pyrophosphate, and collected and washed on a millipore filter. The radioactivity on the filter was determined with a windowless gas-flow Geiger-Muller counter.

Infected cells were prepared in two ways. For purification and general study, 40 liter batches of bacteria were grown to a density of 2 x 10\textsuperscript{8} ml in a Biogen (American Sterilizer Company) and infected with approximately 10 viable particles per bacterium. After 30 minutes incubation, the infected cells were removed from the biogen and added to ice cubes to prevent further growth. The cells were then collected by centrifugation in a Sharples continuous flow centrifuge. For smaller experiments, cells were grown in 250 ml aliquots in liter flasks in a shaker. At various times after infected the cells were cooled, and centrifuged in bottles in a Lourdes centrifuge. Cells were usually stored frozen until extracted, as the activity was stable in frozen cells.
FIGURE 4

Purification of Phage f2

Lysate
Add 0.3 gm (NH₄)₂SO₄ per ml; add 2.0 mg purified carrier phage; store in cold overnight; centrifuge at 20,000 g for 15 minutes

Pellet
Supernate
discard

Wash with 28% (NH₄)₂SO₄; centrifuge at 20,000 x g for 15 minutes; dissolve pellet in water; centrifuge at 20,000 x g for 15 minutes

Pellet
discard

Centrifuge at 100,000 x g for 2 hours

Pellet
(purified phage—may be purified further)
Supernate
discard

Cesium Chloride Centrifugation
Centrifugation or Filtration

Take up pellet in CaCl₂ (0.65 gm per ml); Centrifuge at 115,000 x g for 15 hours; collect drops and save visible phage band; dilute phage fraction and centrifuge at 100,000 x g for 2 hours.

Supernate
discard

Purified phage

Pellet
discard

Remove any visible particles with a low speed centrifugation or passage through an HA Millipore filter.
III
RESULTS

A. Relationship of DNA Synthesis to the Growth of f2

The need for complementary nucleic acid synthesis in DNA virus growth has been noted (I, B, 5). An investigation was made into the relationship of DNA synthesis to the growth of the RNA-containing f2. This was done by adding various inhibitors of DNA synthesis to f2 infected cells, and seeing if f2 could grow in the absence of DNA synthesis.

1. Nucleic Acid Synthesis in Phage Infected Cells: Radioactive phosphate was used to follow the synthesis of nucleic acid in phage infected cells. Figure 5 shows the incorporation of P\(^{32}\) into the nucleic acids of f2 infected E. coli. The synthesis of DNA parallels the synthesis of RNA quite closely and continues until virus synthesis ceases, approximately 40 minutes after infection. This result is similar to the results of Loeb and Zinder (1961) who used chemical methods to follow the synthesis of nucleic acids after infection.

Loeb and Zinder (1961) reported a slight lag in the synthesis of DNA and RNA after infection with f2. Measurement of the synthesis of nucleic acid using radioactive phosphate confirmed the existence of this lag period. The results are shown in Figure 6. Radioactive label was added prior to infection in order to eliminate a lag in uptake of phosphate caused by a pool of unlabeled nucleic acid precursors.

2. Inhibition of f2 by Fluorinated Pyrimidines: Infective centers were prepared and distributed to tubes containing VB medium and additions of 5-fluorouracil (FU) and 5-fluorodeoxyuridine (FUDR). Uracil and thymidine were added as noted. The synthesis of f2 in three different hosts in the presence of various additions is described in Table II. It may be seen that f2 synthesis is inhibited by FU and FUDR, and that this inhibition is reversed only by uracil, thymidine has no effect.

There are numerous reports in the literature indicating that FU and FUDR inhibit DNA synthesis by inhibiting the synthesis of thymine. Horowitz, et al. (1960) studied the effect of fluorinated pyrimidines on E. coli. They found that fluorouracil inhibits DNA synthesis unless the medium is supplemented with thymine. Their results indicate that the inhibition of DNA synthesis is
K13 was grown in peptone medium, centrifuged, and resuspended in saline calcium, and infected with a high multiplicity of f2. After adsorption of phage, the cells were centrifuged and resuspended in peptone medium with $^{32}P$. Aliquots were removed and fractionated for RNA and DNA as indicated in the Methods (II,B,3).
Figure 6

Nucleic Acid Synthesis After f2 Infection (II)

$^{32}$P uptake

$\text{C.P.M. (x10^{-3})}$

Time (minutes)

$^{32}$P was added to a growing culture of K13 in peptone medium 30 minutes prior to infection. Phage were then added as indicated by the arrow and aliquots were removed and assayed for total radioactivity. Further fractionation as described in the Methods (not shown) indicated that both DNA and RNA synthesis exhibit lags after infection.
TABLE II

Inhibition of f2 by Fluorinated Pyrimidines

<table>
<thead>
<tr>
<th>Additions</th>
<th>Burst Size in</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K13</td>
<td>K20(U⁻ or T⁻)</td>
<td>K24(T⁻)</td>
</tr>
<tr>
<td>No additions</td>
<td>226</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>U</td>
<td>359</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>T</td>
<td>77</td>
<td>40</td>
<td>120</td>
</tr>
<tr>
<td>U, T</td>
<td>664</td>
<td>200</td>
<td>75</td>
</tr>
<tr>
<td>FU</td>
<td>8</td>
<td>&lt;1</td>
<td>5</td>
</tr>
<tr>
<td>FU, U</td>
<td>665</td>
<td>90</td>
<td>200</td>
</tr>
<tr>
<td>FU, T</td>
<td>8</td>
<td>1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>FU, U, T</td>
<td>382</td>
<td>100</td>
<td>200</td>
</tr>
<tr>
<td>FUDR</td>
<td>5</td>
<td>&lt;1</td>
<td>8</td>
</tr>
<tr>
<td>FUDR, U</td>
<td>310</td>
<td>100</td>
<td>120</td>
</tr>
<tr>
<td>FUDR, T</td>
<td>5</td>
<td>3</td>
<td>&lt;1</td>
</tr>
<tr>
<td>FUDR, U, T</td>
<td>502</td>
<td>58</td>
<td>200</td>
</tr>
</tbody>
</table>

Cells were grown in VB medium, washed, and then grown for 30 minutes in medium without any additions. The cells were then washed again, suspended in saline-calcium, and infected with 0.25 particles per bacterium. Infective centers were then prepared as described in the Methods, and then the bacterial-phage complexes were distributed to the various media. After 90 minutes' incubation, lysozyme and chloroform were added and the yields were assayed. The thymidine (T), uracil (U), FU, and FUDR were present in concentrations of 50 μg/ml.
quite complete, while RNA synthesis continues at a normal level. In a uracil-requiring strain, in the presence of uracil, fluorouracil inhibited DNA synthesis as measured by chemical analysis or by the uptake of labeled adenine.

FUDR exhibits the same inhibition spectrum as FU (Table II). Cohen, et al. (1958) showed that this deoxyribonucleoside is converted to the ribotide in E. coli, and that this compound is an irreversible inhibitor of thymidylate synthetase. The inhibition of thymine synthesis then leads to an inhibition of DNA synthesis.

The inability of f2 to grow in the thymine-requiring K24, in the absence of added uracil, cannot be explained. It is possible that this strain is unable to synthesize uracil in the absence of thymidine.

Thus, the results in Table II are consistent with one main conclusion. Since the FU and FUDR in the presence of uracil lead to an inhibition of DNA synthesis, the unrestricted growth of a phage containing RNA under these conditions indicates that DNA synthesis is not needed for phage replication. Further chemical analysis reported below supports this conclusion.

FU alone prevents the production of any viable phage. This is probably due to the incorporation of FU into the phage RNA leading to a non-viable phage RNA. The result may be contrasted to the results of Salzman, et al. (1962) who showed that infectious poliovirus could be produced in a medium containing three times the concentration of FU used in the experiments described in Table II, and that 35% of the uracil in the virus produced was replaced by fluorouracil. The reasons for the difference in sensitivity to FU of poliovirus and f2 are not understood at the moment.

3. Inhibition of DNA Synthesis Measured by Uptake of Adenine-C\textsuperscript{14}: In order to demonstrate that DNA synthesis was stopped under the conditions described in the previous section, radioactive adenine was used as a label for nucleic acids. Horowitz et al. (1960) and Roberts et al. (1955) have shown that virtually all the adenine is incorporated into nucleic acids, practically none being detectable in any other fraction. The uptake of adenine was therefore followed with a Schmidt-Thannhauser fractionation. The results are presented in Table III.

It can be seen that the burst size is independent of the amount of DNA synthesized, and that normal growth can be maintained in the presence of a very
### TABLE III

**Synthesis of Nucleic Acids in Thymineless Bacteria Measured by the Uptake of Adenine-Cl4**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Yield/Bact.</th>
<th>RNA (cpm/ml)</th>
<th>DNA (cpm/ml)</th>
<th>RNA (μg/ml)</th>
<th>DNA (μg/ml)</th>
<th>% DNA synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>A, U</td>
<td>846</td>
<td>51,000</td>
<td>350</td>
<td>14.0</td>
<td>0.10</td>
<td>8.1</td>
</tr>
<tr>
<td>A, U, T</td>
<td>520</td>
<td>71,000</td>
<td>4350</td>
<td>19.7</td>
<td>1.20</td>
<td>100.0</td>
</tr>
<tr>
<td>A, U, FU</td>
<td>780</td>
<td>57,000</td>
<td>130</td>
<td>15.8</td>
<td>0.036</td>
<td>3.0</td>
</tr>
<tr>
<td>A, U, FUDR</td>
<td>806</td>
<td>80,000</td>
<td>730</td>
<td>22.2</td>
<td>0.20</td>
<td>16.8</td>
</tr>
</tbody>
</table>

Adenine was present in a concentration of 6.11 μg/ml. The pyrimidine additions, uracil (U), thymidine (T), 5-fluorouracil (FU), and 5-fluorodeoxyuridine (FUDR), were present at a concentration of 50 μg/ml. There were 220,000 cpm/ml, a specific activity of adenine of 36,000 cpm/μg. Per cent DNA synthesis is based on the synthesis of DNA in the presence of thymidine as 100%. For calculation, adenine is taken as 10% of RNA and DNA. Thymineless K24 was grown up in VB medium with adenine and thymidine present. The cells were washed once and then suspended in media with adenine along. After 30 minutes' incubation in the VB medium without thymine the cells were concentrated, infected (multiplicity of 10), and distributed to flasks containing VB medium with radioactive adenine and the various additions listed. The yields are calculated on the basis of a 100% infection of the 1.5 x 10^8 cells/ml. After growth for 90 minutes an aliquot was removed for assay of the yield, and the remaining solution was fractionated by precipitation with TCA and treatment with NaOH as described in the Methods. The counts reported are corrected for self-absorption.
low level of DNA synthesis. The limit of detection of DNA was probably reached in the FU-containing sample. The material in the DNA fraction could be attributed to a small amount of incomplete hydrolysis of RNA, or incomplete washing of the alkali insoluble precipitate. If the hydrolysis of RNA was only 99.7% complete, the contamination could completely account for the residual counts in the DNA fraction.

The data eliminates one possible role of DNA in the growth of f2. The amount of DNA synthesized is less than the amount of RNA actually incorporated into mature phage. A sample with FU had at least 0.1 μg of phage RNA synthesized per milliliter, as calculated from the phage yield and the RNA content of a phage particle (about 10^{-12} μg), and only one-third this amount of DNA was synthesized. Thus, a stoichiometric relationship between the amount of phage synthesized and the amount of DNA synthesized is excluded. An alternative hypothesis might be that a few molecules of DNA are synthesized and then stamp out phage replicas. It is difficult to eliminate the possibility of a few molecules of DNA being synthesized in each bacterium, but it may be seen in Table III that the phage yield is constant even though there are large variations in the amount of DNA synthesized. If some DNA molecules played a role in phage synthesis, one might expect that there would be some fluctuation in phage synthesis; this however, is not found.

4. Additional Experiments on the Inhibition of DNA Synthesis: In order to corroborate the results obtained with radioactive adenine, other experiments were done using chemical analysis, and P^{32} incorporation, to measure the synthesis of nucleic acids after infection.

The use of radioactive phosphate to follow nucleic acid synthesis gave results almost identical with those in the experiment with C^{14}-labeled adenine. The procedure used was as described in Table III except that P^{32} was used as a tracer in TG-HC medium. One experiment, with K24 (thymine requiring) as a host, compared two infected cultures—one with uracil and thymidine additions, the second with uracil and FUDR. After growth, aliquots were removed for phage yield assay and the cultures were then subjected to a series of chloroform and phenol extractions to remove phosphate-containing proteins and phospholipids that could contaminate the DNA fraction. Then a Schmidt-Thannhauser fractionation was applied to the remaining TCA-precipitable material, and the relative amounts of P^{32} labeled DNA and RNA were measured. The amount of DNA synthesized
in the FuDR sample was less than 1\% of the RNA synthesized, or less than 10\% of normal DNA synthesis. (The residual radioactivity in the DNA fraction is possibly contamination that was not removed by deproteinizations or alkaline hydrolysis.) The yields of virus were identical in the two cultures.

When chemical analysis (the diphenylamine reaction) was used to measure DNA synthesis in an experiment similar to the one using radioactive phosphate, there was a slight decrease in the amount of DNA in the FuDR sample during the period of virus growth. There was normal DNA synthesis in the control supplemented with thymidine, and again, the virus yields were the same in both cultures. Thus, with three different measures—chemical analysis, incorporation of \(p^{32}\), and incorporation of adenine-C\(^{14}\)—it can be shown that phage synthesis continues when DNA synthesis is inhibited.

5. Mitomycin C and the Growth of f2: Sekiguchi and Takagi (1960) have shown that Mitomycin C inhibits the synthesis of DNA. The mechanism of inhibition appears to be the breakdown of the "template" DNA in the cell (Reich, et al. 1960). Sekiguchi and Takagi (1960) have shown that the injection of phage DNA into a cell restores some of the DNA synthesis, probably by replacing a template for the synthesis of DNA. The effect of Mitomycin C on the growth of f2 and \(\lambda_V\) (a DNA-containing phage) is shown in Table IV. It can be seen that the growth of f2 is almost normal in the presence of Mitomycin C whereas the synthesis of \(\lambda_V\) was inhibited. In experiments with Mitomycin C a slight loss of infective centers in cells killed by Mitomycin was observed. Experiments have shown that this loss of infective centers is much less rapid than the loss of bacterial viability, and that the growth of f2 in cells killed by Mitomycin is the same whether the antibiotic is present or absent after infection. Reich (personal communication) has noted that bacteria killed by Mitomycin are relatively fragile and are killed in pipetting and handling. This could account for the losses of infective centers. On the other hand, the effect of Mitomycin might be less specific than reported, and this could lead to a loss of infective centers on Mitomycin-killed cells. Thus, the results with Mitomycin are consistent with the conclusion that DNA synthesis is not needed for RNA virus production. The results are not as conclusive as the chemical results reported above.

6. Summary: The conclusion of the experiments described above is clear: there is no participation of DNA synthesis in the growth of the bacteriophage f2. The growth of f2 in the absence of DNA synthesis is similar to results reported with other RNA-containing viruses (I, B, 5). It is quite probable that this property—indeedence of DNA synthesis—is common to all the RNA-viruses; and furthermore f2 may be a good model for the study of RNA virus growth.
TABLE IV

The Effect of Mitomycin C on Growth of RNA and DNA Bacteriophages

<table>
<thead>
<tr>
<th>Phage</th>
<th>Addition</th>
<th>Yield/infective center</th>
</tr>
</thead>
<tbody>
<tr>
<td>f2</td>
<td>M</td>
<td>200</td>
</tr>
<tr>
<td>f2</td>
<td>S</td>
<td>500</td>
</tr>
<tr>
<td>λV</td>
<td>M</td>
<td>1</td>
</tr>
<tr>
<td>λV</td>
<td>S</td>
<td>100</td>
</tr>
</tbody>
</table>

Tryptone broth was used. Strain K13 was grown to give a density of 10^8 cells/ml. Mitomycin (M) was added at a concentration of 40 μg/ml (20 μg/ml gives 10^{-3} viability after 30 minutes incubation) and the cells were incubated for 30 minutes. Another aliquot of the cells was incubated with a saline (S) addition as a control. Phage were added at a multiplicity of 0.05 to aliquots of the incubated cultures. Infective centers were checked after 10 minutes and the burst after 75 minutes.
It has also been noted (III, A, 1) that there is no drastic change in net nucleic acid synthesis after f2 infection. Paranchych and Graham (1962) have reported that protein synthesis does not appear to be affected by RNA phage infection. Zinder (I, A, 5) has noted that there is only a slight effect of f2 infection on the synthesis of a bacterial enzyme, β-galactosidase. One can therefore conclude, that f2 infection does not appear to have any serious effect on host cell biosyntheses (see I, B, 4).

B. Experiments on the Synthesis of f2 RNA

A method of measuring of virus nucleic acid synthesis is described in the Methods (II, B, 4). The method is simply to add radioactive label to the infected culture for a given period during the growth cycle and then to measure the label in the virus produced. Since $\text{P}^{32}$ is used as the label the radioactivity is then a measure of the amount of virus nucleic acid, defined as RNA which is eventually incorporated into virus particles, synthesized during the labeling period. The addition of purified phage in large amounts to the progeny virus allows one to obtain quantitative results (see legend to Table VI).

The method has two major theoretical limitations. First, this method does not measure the synthesis of viral nucleic acid which is not incorporated into stable virus particles before lysis. Thus, this method probably leads to a minimum estimate of virus nucleic acid synthesis; any viral nucleic acid formed but not incorporated into a phage coat at the time of lysis will be lost into the medium. A second theoretical limitation is that extraneous RNA which does not have the genetic potentiality of phage nucleic acid may be incorporated accidentally into phage coat and thus lead to a high estimate of viral nucleic acid synthesis. These limitations are not critical for the experiments to be presented in this and the following section.

The experiments in this section will not only provide some information on the synthesis of virus RNA and virus particles, but will also serve to illustrate the method of measuring virus RNA.

1. Source of Phage RNA: The source of phage RNA phosphorus was determined (Table V). It can be seen that the medium supplies almost all of the phosphorus of the bacteriophage RNA. It is therefore probable that all of the components of the phage RNA are derived from the medium. Since the bacterial contribution to phage RNA is negligible, the amount of incorporation of label
### TABLE V

**Source of Phage RNA (Phosphorus)**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>P&lt;sup&gt;32&lt;/sup&gt; Source</th>
<th>CPM in Phage</th>
<th>% Bacterial Label in Phage</th>
<th>% Phage Label from Bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bacteria and Medium*</td>
<td>640</td>
<td>0-0.12%</td>
<td>0-0.7%</td>
</tr>
<tr>
<td></td>
<td>Bacteria</td>
<td>659</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>94,780</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Bacteria</td>
<td>1,200</td>
<td>0.16%</td>
<td>1.2%</td>
</tr>
<tr>
<td></td>
<td>Bacteria and Medium</td>
<td>99,400</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Uninfected

Cells (K13) were grown in peptone medium with or without added P<sup>32</sup>. Pre-labeled cells were incubated in the absence of label for 20 minutes before infection, in order to remove any pool of P<sup>32</sup> labeled phosphorylated precursors of nucleic acid. The cells were then infected as indicated, incubated and lysed. The label in the phage was determined as described in the Methods (I,B,4).
16.

from the medium is a simple measure of the synthesis of phage RNA. The results in Table V also indicate the limit of sensitivity of the experimental technique. It can be seen that if adequate controls are done, the procedure is sensitive enough to measure less than 1% synthesis. Since all of the label comes from the medium or nucleic acid precursors in the cell, one can perform experiments without the need for compensating for the contributions from the host nucleic acid.

2. Viability of Progeny Virus Particles: During the course of these experiments it became clear that only a fraction of the particles released from infected cells could initiate plaques. The results of a few experiments are presented in Table VI. It can be seen that only 10% of the particles formed after infection are able to initiate the formation of a plaque. Growth in the presence of CA for a period of time after infection did not lead to any significant change in the ratio of viable titer to total number of particles synthesized.

3. Time Course of Phage RNA Synthesis: The kinetics of phage RNA synthesis was measured by the "subtractive" method of Darnell and Levintow, (1960). P$^{32}$ is added at various times after infection to aliquots of the culture and kept present until the growth cycle is over. The number of counts in phage obtained with the P$^{32}$ present at the start of infection is taken as a measure of the total phage RNA synthesized. The difference between this number and that obtained when P$^{32}$ is added at later times is taken as a measure of the amount of phage RNA synthesized prior to the addition of label.

This method has two main limitations. First, it is very insensitive during the important early period, where the difference between two relatively large numbers is indicative of the amount of phage RNA synthesized. Thus, it is difficult to determine exactly when the first phage RNA molecules are formed. Second, the existence of a large pool of phosphate (Carnegie Institute of Washington Yearbook, 1961) does not allow the accurate determination of the actual time of synthesis of phage RNA macromolecules. The time actually measured is the time of formation of organic-phosphate material (not exchangeable with the phosphate of the medium) which is the precursor of phage RNA. For the experiments to be reported below, these limitations are not critical.

The results of two experiments which measure the time course of phage RNA synthesis are shown in Figure 7. It can be seen that RNA is synthesized
TABLE VI

Plaque Forming Ability of f2 Particles

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Particles/ml</th>
<th>FFU/ml</th>
<th>Particles/FFU</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$5.9 \times 10^{12}$</td>
<td>$3.4 \times 10^{11}$</td>
<td>17</td>
</tr>
<tr>
<td>2</td>
<td>$6.9 \times 10^{12}$</td>
<td>$5.2 \times 10^{11}$</td>
<td>13</td>
</tr>
<tr>
<td>3</td>
<td>$1.0 \times 10^{13}$</td>
<td>$4.2 \times 10^{11}$</td>
<td>24</td>
</tr>
<tr>
<td>4</td>
<td>$1.9 \times 10^{12}$</td>
<td>$2.4 \times 10^{11}$</td>
<td>8</td>
</tr>
<tr>
<td>5</td>
<td>$6.7 \times 10^{12}$</td>
<td>$1.7 \times 10^{11}$</td>
<td>39</td>
</tr>
<tr>
<td>6*</td>
<td>$2.6 \times 10^{11}$</td>
<td>$3.5 \times 10^{10}$</td>
<td>7</td>
</tr>
</tbody>
</table>

* Tris-minimal medium

Each result was obtained from an independent experiment. Peptone medium was used except in #6 where tris minimal medium was substituted. The particle concentration was determined by use of the formula:

\[
\text{Particles/ml} = \frac{\text{CPM in pure phage/OD} \times \text{OD added carrier/ml lysate} \times \text{ug P/ml medium} \times (10^{13} \text{ particles/ug P})}{\text{CPM/ml medium}}
\]

Radioactive phosphate was added at the time of infection, and was present until lysis. The progeny phage and added carrier were purified as described in the Methods.
Cells were prepared as described in the Methods. $^{32}P$ phosphate was added at the indicated times after infection ( • or ○) and the various aliquots incubated until lysis. The results are from two different experiments. The dashed line (---) indicates the formation of mature virus. The method of calculation is described in the text.
during a large fraction of the growth cycle. The time when 50% of phage RNA has been synthesized is about 25 minutes after infection. This is approximately 15 minutes before the synthesis of 50% of the phage particles. As mentioned above, the actual time of phage RNA synthesis is somewhat later than indicated. There appears to be a pool of phage RNA molecules with an estimated average lifetime of 5-10 minutes prior to their incorporation into mature phage particles.

4. **Summary**: Three experiments on the synthesis of virus RNA have been described in this section. It was shown that the components of the virus nucleic acid are derived almost entirely from the medium, with a negligible contribution from the host cell material. Therefore viral RNA synthesis can be measured by following the uptake of radioactive label from the medium. If there were any substantial incorporation of material from the host, the measurement of viral RNA synthesis by the uptake of label from the medium would lead to an underestimate of viral RNA synthesis.

It was also shown that only a small fraction of the virus particles released from the cell are infectious. Zinder (unpublished observations) had noted that a loss in viable titer occurs when a virus preparation is stored without being frozen, and during purification (particularly when the virus is sedimented at high centrifugal forces). Since the measurements of infectivity and particle number (III, B, 2) were made on fresh lysates, inviable phage are produced directly by infected cells. Whether the cause of the inviability measured in this section is the same as the cause of the loss in inviability noted by Zinder remains to be determined.

The determination of the time course of viral RNA synthesis, used P$^{32}$, is made uncertain by the presence of a large pool of phosphorylated precursors of nucleic acid. The existence of this pool of precursors makes the measured RNA synthesis appear earlier than the actual synthesis of RNA macromolecules. This limitation is not critical for the experiments to be reported in the next experimental section.

C. **Relationship of Protein Synthesis to the Synthesis of Phage RNA**

The relationship of "early protein" synthesis to virus growth has been discussed in the Introduction (I, B, 5 and I, B, 6). The experiments in this section indicate that some protein synthesis after virus infection is necessary for the initiation of viral RNA synthesis. That is, after f2 infection there is some "early protein" synthesis.
1. **Synthesis of Phage RNA in the Presence of Chloramphenicol:** Chloramphenicol inhibits bacterial protein synthesis while RNA synthesis continues unimpaired. The synthesis of phage particles is reversibly inhibited by chloramphenicol. It was of interest to see whether phage RNA could be synthesized when protein synthesis was inhibited by chloramphenicol. Since the only measure of phage RNA available was the amount of RNA which appears in phage particles, if we are to recognize the phage RNA synthesized in the presence of chloramphenicol, the RNA would have to be incorporated into particles synthesized after the removal of the CA. The details and results of an experiment to test whether phage RNA can be synthesized in the presence of chloramphenicol are presented in Table VII. The labeling period was chosen to be after the start of phage particle synthesis, and during the period when a major portion of phage RNA is synthesized. By having label present both when CA is present and after CA is removed, we have a measure of the amount of phage RNA synthesized during these two intervals. It can be seen that the amount of phage RNA synthesized in the presence of chloramphenicol is similar to the amount synthesized in its absence.

The synthesis of phage coat does not occur until relatively late in the growth cycle (Cooper, preliminary results), and thus there is a negligible amount of phage coat synthesized at the time of addition of chloramphenicol. Thus the phage RNA synthesized in the presence of chloramphenicol could not be incorporated into phage coats which had been synthesized prior to the inhibition of protein synthesis. Control experiments have also indicated that there is no major turnover of RNA synthesized after infection, thus breakdown and reutilization of p\(^{32}\) for the synthesis of phage particles after removing the CA is eliminated.

The results show that phage RNA can be synthesized in the presence of chloramphenicol, and further, since the phage RNA is recognized by its incorporation into protein coats, the RNA synthesized in the presence of CA can be incorporated into protein coats.

2. **Inhibition of Phage RNA synthesis by Chloramphenicol:** Since phage RNA synthesis can proceed if protein synthesis is inhibited late in infection, a second experiment was performed to see if phage RNA synthesis is inhibited if protein synthesis is inhibited at the start of infection. The results are given in Table VIII. The correction applied to the first and fourth lines in Table VIII is to subtract the counts obtained from the cells which had label incorporated but were not infected until the label was removed. This
TABLE VII

**Synthesis of Phage RNA**

*in the Presence of Chloramphenicol*

<table>
<thead>
<tr>
<th>CA Present</th>
<th>$^32$ P Present</th>
<th>CPM in Phage RNA</th>
<th>% Total, Incorporated in the Presence of CA</th>
</tr>
</thead>
<tbody>
<tr>
<td>24-45</td>
<td>24-45</td>
<td>12,600</td>
<td>64</td>
</tr>
<tr>
<td>24-45</td>
<td>24-90</td>
<td>19,350</td>
<td></td>
</tr>
<tr>
<td>24-45</td>
<td>45-90</td>
<td>7,090</td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>24-45</td>
<td>20,300</td>
<td>83</td>
</tr>
<tr>
<td>---</td>
<td>24-90</td>
<td>26,600</td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>45-90</td>
<td>4,030</td>
<td></td>
</tr>
</tbody>
</table>

Cells were grown in peptone medium, centrifuged, resuspended in fresh prewarmed medium, and infected. The infected cells were divided into six portions and at the indicated times radioactive phosphate and chloramphenicol (50 µg/ml) were added to the appropriate cultures. The CA and label were in the separate cultures for the times indicated. (The times are the minutes after infection). After incubation all aliquots were centrifuged, washed, and resuspended in fresh medium with radioactive phosphate as indicated. In this manner all cultures were treated exactly the same except for the additions as noted. After lysis, the phage fraction was purified and assayed as described in the Methods (I,B,4).
TABLE VIII

Effect of Chloramphenicol

on the Synthesis of Phage RNA

<table>
<thead>
<tr>
<th>CA Present</th>
<th>P32 Present</th>
<th>CPM in Phage RNA</th>
<th>% Total, Incorporated in the Presence of CA (corrected)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0---23</td>
<td>0---23</td>
<td>79</td>
<td>19</td>
</tr>
<tr>
<td>0---23</td>
<td>0---23-----90</td>
<td>418</td>
<td></td>
</tr>
<tr>
<td>0---23</td>
<td>23---90</td>
<td>337</td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>0---23</td>
<td>1,168</td>
<td>82</td>
</tr>
<tr>
<td>---</td>
<td>0---23-----90</td>
<td>1,413</td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>23---90</td>
<td>369</td>
<td></td>
</tr>
<tr>
<td>---*</td>
<td>0---23</td>
<td>122</td>
<td></td>
</tr>
<tr>
<td>0---23*</td>
<td>0---23</td>
<td>51</td>
<td></td>
</tr>
</tbody>
</table>

* Uninfected during labeling period; infected after removing label

Bacteria were grown, infected, and handled in the same manner as described in Table VIII. The correction applied subtracts the counts found in the phage fraction when labeled cells were infected in unlabeled medium (see text).
is a measure of the label entering phage from the pool of phosphorylated precursors present in the cells after removing the label. Since phage RNA synthesis begins soon after removing the chloramphenicol, some phage RNA would be labeled with the radioactive organic-phosphate precursors. The correction is probably a minimal estimate of the incorporation of precursor material into phage RNA as the control cells (lines 7 and 8) must first be infected before the events leading to the synthesis of virus RNA can occur, while in the experimental aliquots (lines 1 and 4) the infection has already taken place. Since the experimental cells therefore probably start synthesis of virus RNA more quickly than the control cells, they might be expected to incorporate a larger fraction of soluble precursor material into the virus RNA. When the results are corrected for this contaminating incorporation, it can be seen that no phage RNA synthesis can be observed.

Two conclusions may be derived from the experiments described in this and the preceding section. First, the synthesis of phage RNA is not coupled to the synthesis of protein. Second, there does appear to be a need for some protein synthesis early in the infectious cycle in order to allow phage RNA synthesis to proceed.

3. Escape from Chloramphenicol Inhibition: An experiment was performed to determine more precisely the time when the necessary "early protein" has been synthesized. Chloramphenicol was added to aliquots of the same culture at different times after infection, and P³² was added to the cultures for a constant label period from 17 to 45 minutes after infection. The chloramphenicol and label were then removed and, after incubation and lysis, the progeny phage were isolated by the method previously described. The results are given in Figure 8.

The RNA synthesis during the labeling period was used as a measure of the rate of phage RNA for the various cultures. It can be seen that if chloramphenicol is given within the first two minutes after infection no phage RNA is synthesized. If the chloramphenicol is added at later times, the ability to synthesize phage RNA increases, until by 15 minutes the rate of RNA synthesis is similar to the rate without added chloramphenicol. (Fifteen minutes is the time of appearance of the first mature intracellular phage.)
Chloramphenicol (50 µg/ml) was added to aliquots of the infected cultures at different times after infection as noted. Phosphate label was present in all cultures between 17 and 45 minutes. At 45 minutes after infection label and chloramphenicol were removed from each flask. After incubation and lysis the released phage were purified as described in the Methods. All points are corrected for incorporation due to pool of organic phosphate precursors in the cell which was not washed out before removing the chloramphenicol.
The increasing ability of the infected cell to escape chloramphenicol inhibition may be an indication that there is a continuing synthesis of "early protein" during the first few minutes after infection, and any "early protein" synthesized before addition of chloramphenicol is subsequently able to participate in the synthesis of phage RNA.

4. Growth of f2 in the Absence of Histidine: The protein coat of f2 does not contain the amino acid histidine (I, A, 3). If the conclusions of the preceding sections are correct, and there is a protein produced early in phage infection which is necessary for the synthesis of phage nucleic acid, then one criterion for distinguishing coat protein from the "early protein" could be based on the probable presence of histidine in the latter. Accordingly, one would expect that the phage would not be synthesized in a histidineless bacterium in the absence of histidine. However, if histidine is supplied for some period after infection, its removal after the synthesis of the early protein, might not prevent the further synthesis of f2. On the other hand, removal of an amino acid such as methionine, although it represents less than 1% of the coat protein, might prevent further synthesis of f2.

The bacterium K35 is an Hfr strain which requires methionine and histidine and is derepressed for the synthesis of RNA (Borek et al., 1955). It can synthesize RNA even in the absence of these amino acids. In order to insure the rapid removal of amino acids at different times after infection, infected cells were diluted into flasks which were missing the required amino acids, and which contained an excess of an f2 resistant histidine and methionine requiring bacterium (K36) which would quickly remove any amino acids carried over during the dilution. This procedure, however, did not insure the rapid removal of any pool of intracellular amino acids that may be present in the infected cells.

The results of an experiment to see whether f2 can be synthesized in the absence of histidine are illustrated in Figure 9, and may be summarized as follows:

1. In the absence of histidine or methionine f2 cannot grow.
2. If histidine is removed 10-15 minutes after infection the phage yield is approximately 50% of that which is obtained when histidine is not removed.
3. If methionine is removed the yield is reduced to the level of that which obtains upon removing both amino acids, approximately 10% of normal.
K35 (histidine and methionine requiring) cells were grown in VB medium with Histidine (H) and Methionine (M). Amino acids, when present were at a concentration of 5 µg/ml. When the cell density reached $2 \times 10^8$/ml the cells were centrifuged and resuspended in VB medium with no additions for 30 minutes in order to eliminate any residual pool of amino acids. The cells were resuspended in saline and infected with 0.1 phage per bacterium. After 10 minutes for adsorption, the cells were washed to remove free phage and then distributed (this point to be taken as 0 minutes) into four flasks—A with no additions, B with histidine added, C with methionine added, and D with both histidine and methionine added. At the indicated times after infection (measured from the time of addition of the infected bacteria to the 0 minute flasks) a 1/100 dilution was made from the 0 minute flask D (marked *) into four flasks, A, B, C, and D with additions as noted. At 90 minutes the yield was assayed after lysis of the cells by lysozyme and chloroform. In this medium the latent period of the phage is delayed about 10 minutes as compared to that in broth. K36, an f2 resistant derivative of K35, was present in all flasks at a concentration of $10^8$/ml. Fφ indicates the mature phage which had been formed in the D* flask at the time of transfer.
Figure 9

Synthesis of f2 in the Absence of Histidine

- A - his, - meth
- B + his, - meth
- C - his, + meth
- D + his, + meth

Phage per infective center vs. Minutes after infection removal of amino acid.
Thus, there is a definite quantitative difference in the phage yield after the removal of histidine when compared with the removal of methionine. The slight growth of phage in the flasks without methionine is probably due to the utilization of the pools of this amino acid that accumulated in the bacteria during the incubation in the presence of histidine and methionine. The results are consistent with and support the concept of a necessary "early protein" synthesis after f2 infection. Further, the results indicate that the "early protein" probably contains histidine in contrast to the phage coat which does not contain histidine.

5. Summary: The data presented in this section suggest the existence of a protein, produced soon after phage f2 infection, which is necessary for the synthesis of phage RNA. As the postulated "early protein" appears to contain histidine, it is different from the phage coat protein which does not contain histidine. Once the "early protein" is synthesized, continued protein synthesis is not necessary for continued RNA production.

The fact that the "early protein" appears to contain histidine leads to an interesting correlation. Nathans, Notani, Schwartz, and Zinder (1962) have shown that f2 RNA added to the cell free amino acid incorporating system of Nirenberg and Matthaei (1961), can lead to the synthesis of a product which appears to be similar to phage coat protein. Nathans, et al. (1962) noted that the RNA stimulated the incorporation of histidine into an acid insoluble product, which indicates that other proteins were made besides the phage coat. One may speculate that the histidine containing product synthesized in vitro may be the same protein, the existence of which has been implied by the results reported above. It is not known how many proteins comprise the operationally defined "early protein" (see I, B, 5) or how many proteins, other than the coat, are synthesized in the cell free system. There may be theoretical limitations on the number of proteins that could be synthesized by the relatively small f2 RNA molecule.

In the next section, experiments will describe the appearance, after phage infection, of a new enzyme activity. The new enzyme activity may be the biochemical basis for the necessary "early protein", as the enzymes synthesized after T-even infection have been interpreted as the biochemical basis for "early protein" synthesis.
D. Appearance of a New Enzyme Activity after Phage Infection:

In the introduction (I, B, 5, and I, B, 6) it was noted that the necessary "early protein" appearing after T-even phage infection was interpreted as the synthesis of various enzymes necessary for the synthesis of the phage DNA. The possibility was therefore explored that a similar explanation may hold for the "early protein" synthesized after f2 infection.

There is no direct participation of DNA in the synthesis of f2 (III, A). Since the RNA is the genetic material of f2 (f2 RNA alone can infect bacteria) there should be some enzyme mechanism in the infected cell for the synthesis of progeny RNA molecules similar to the infecting RNA. Since an enzyme with the ability to direct the synthesis of RNA using an RNA primer has not yet been found in E. coli, it is likely that such an enzyme is synthesized after infection.

Therefore, a search for a phage induced, RNA dependent, RNA polymerase was initiated in collaboration with Dr. J. T. August of the Department of Microbiology of the New York University Medical School. The results presented below indicate that such an enzyme is synthesized after infection with phage f2.

1. Appearance of a New Enzyme Activity: The assay used, as finally developed, is described in the methods (II, B, 5). It is similar to the assays for DNA polymerase and DNA-dependent RNA polymerase, except for the presence of a large amount (40 μg/ml) of DNase. At various times after infection cells were collected by centrifugation and extracted as described in the Methods (II, B, 5). The extracts were tested for enzyme activity. The results of two separate experiments are illustrated in Figure 10. It can be seen that the ability of cell extracts to catalyze the incorporation of GTP$^{32}$ into an acid insoluble form increases after infection. Uninfected extracts have a uniformly low incorporating ability. The increase in activity appears between 10 and 20 minutes after infection, reaching a maximum at approximately 30 to 40 minutes after infection. In other experiments no detectable increase, and even some decrease in activity was noted after 40 minutes. There is an uncertainty of about 3-5 minutes in the time course as there is some time needed to cool the cultures and spin out the infected cells. It is quite probable that the enzyme may actually appear at slightly later times than indicated in Figure 10.
Cells were grown and infected in peptone medium and harvested by centrifugation at various times after infection. The incorporating ability of the cells was assayed as described in the Methods (II,B,5).
2. Characteristics of the New Enzyme Activity: A study of some of the characteristics of the new enzyme activity described above has been carried out on a relatively crude extract of infected cells. The results are listed in Table IX.

The fact that the new enzyme activity appears only after phage infection (1) (numbers refer to Table IX) not being found in uninfected cells, and that the appearance of this activity is inhibited by chloramphenicol (2) indicate that the new activity is synthesized after phage infection and that this is not an activation of some host enzyme by the infecting RNA.

The insensitivity of the new enzyme activity to DNase and Antinomycin (3) and phosphate (4) indicate that the incorporation of GTP is not due to a DNA-dependent RNA-polymerase (which is sensitive to Actinomycin and DNase, Hurwitz et al., 1962) nor to polynucleotide phosphorylase (which is inhibited by phosphate, Ochoa, et al., 1961). Also, the amounts of pyruvate kinase and phosphoenolpyruvate in the assay are such that one could not assay polynucleotide phosphorylase in their presence.

The ability of the new enzymatic activity to incorporate CTP as well as GTP (5) and the sensitivity of the GTP product to ribonuclease (6) suggests that the acid precipitable product formed is not merely a homopolymer, but has some CTP (or UTP) incorporated along with GTP. In addition the necessity for added triphosphates (7) (in more purified preparations this dependence upon added triphosphates is almost complete) indicates that the enzyme reaction is probably the synthesis of RNA on some template. In those cases where a template mechanism has been shown to be operative (as in DNA synthesis by DNA-polymerase, and RNA synthesis by DNA-dependent RNA-polymerase) there has been a requirement for the presence of all triphosphates in the reaction mixture. The activity is sensitive to ribonuclease (8) but it is difficult to distinguish this effect of RNase from the sensitivity of the product to RNase. The activity is magnesium dependent (9), sediments with the ribosomal fraction (that is, it is associated with a particulate element) (10), and is very unstable (11). The activity can be stored in frozen cells, but upon extraction there is almost complete loss of activity after a day at 0°.

It should be noted that the activity shows no dependence upon added RNA (12). One would expect such a dependence if this were a mechanism for replicating RNA. The crude extracts have a large amount of RNA, and thus may be saturated with enough RNA so that added RNA has no effect.
**TABLE IX**

**Characteristics of the New Enzyme Activity***

1. Present only in infected cells
2. Synthesis of activity inhibited by chloramphenicol
3. Enzyme activity resistant to DNase and Actinomycin
4. Activity insensitive to presence of phosphate
5. Incorporates GTP and CTP to similar levels
6. Product with GTP<sup>32</sup> sensitive to RNase
7. Maximum incorporation dependent upon added triphosphates
8. Activity sensitive to RNase.
9. Sediments with ribosomal fraction
11. Activity is unstable
12. No dependance upon added RNA in crude extracts

*Characteristics studied on the 30,000 x g supernate (II,B,5)*
3. **Summary:** The new enzyme activity which has been described in this section may be necessary for the replication of virus nucleic acid. The enzyme results strongly suggest (but not conclusively) that the replication of RNA is the function of the enzyme. The activity is insensitive to the presence of DNase, and yet shows some characteristics which would be expected of an enzyme which synthesized RNA from a template. The enzyme fits all the necessary criteria for an RNA-dependent RNA-polymerase, except the most important one, that the incorporation of triphosphates into an acid insoluble product be dependent upon added RNA. The crude extracts and even the partially purified extracts have large amounts of RNA present, presumably in saturating amounts. Therefore it may be impossible to see the effect of any added RNA. It is possible that when the enzyme is purified the activity will be dependent upon added RNA.

The search for the enzyme was begun after the experiments in the preceding section showed that there was some protein synthesis required for the initiation of virus nucleic acid synthesis. The "early protein" begins to appear at approximately 4 minutes after infection (Figure 8) while the enzyme activity is not measurable until 15-20 minutes after infection (Figure 10). If the enzyme activity was the "early protein" one would presumably expect them to appear at the same time. One explanation for this discrepancy may be suggested. The synthesis of enzyme may start very soon after infection, but the activity cannot be measured above the background counts until much later. The *in vivo* activity of the enzyme may be enough, however, to synthesize large amounts of virus RNA. If this were the case then the "early protein" as measured by the ability of the cells to synthesize phage RNA would appear before the enzyme activity as measured by an enzyme assay.

The results reported in this section are of a preliminary nature. It is expected that when the enzyme is purified a study of its specificity and requirements will lead to an understanding of its function in the infected cell.
IV. DISCUSSION

In this thesis an investigation was made into the biochemical events occurring after phage f2 infection. In particular, the relation of macromolecular synthesis to the production of viable phage particles was studied.

The first question asked was whether DNA synthesis was necessary for the synthesis of f2. From the results reported in the experimental section, it is concluded that DNA synthesis is probably not involved in production of f2 particles. Experimental limitations on the complete elimination of DNA synthesis preclude a stronger conclusion.

An investigation of the role of protein synthesis in the biosynthesis of phage f2 indicated that some protein synthesis soon after infection was necessary for the synthesis of phage RNA. These two results—the non-participation of DNA synthesis, and the need for "early protein" synthesis—suggested that the "early protein" was probably an enzyme which was necessary for the synthesis of phage RNA. Further, it was hypothesized that the enzyme used RNA as a template for RNA synthesis.

Preliminary results are presented indicating that an enzyme is synthesized after phage infection. The enzyme has a number of properties which suggest that it probably is an RNA-dependent RNA-polymerase.

From the results reported in this thesis and summarized above, and from the results obtained by other workers with bacterial viruses, it is possible to present a tentative outline of the main events in the growth and biosynthesis of phage f2. After adsorption of the virus to a susceptible host, the RNA of the virus enters the cell. It then proceeds to direct the synthesis of an enzyme (presumably an RNA-dependent RNA-polymerase) which can synthesize progeny RNA. There is probably no participation of bacterial DNA in the synthesis of the virus. The viral RNA synthesized by the enzyme can then participate in the synthesis of phage coat molecules. Eventually RNA molecules of the virus and the coat molecules combine, in some unknown manner, to form mature virus particles.

One item not mentioned concerning virus growth is "control". The problem of "control" may be illustrated by the following considerations. Each RNA molecule must be enclosed by approximately 100 protein coat molecules. Each of these coat molecules is synthesized under the direction of the phage RNA. If there are approximately 100 molecules of coat protein synthesized for each phage RNA synthesized, it would be a waste of biosynthetic potential if the same
number of enzyme molecules were synthesized. As the enzyme can be presumably direct the synthesis of many RNA molecules, it is likely that there would be fewer molecules of enzyme synthesized than RNA molecules made. In Figure 10 it can be seen that the rate of enzyme synthesis appears to decrease when the rate of phage RNA synthesis becomes maximal (Figure 7). This may be the result of some specific control mechanism which shuts off enzyme synthesis when enough enzyme molecules have been produced to allow the synthesis of all the phage RNA molecules.

Two possible models of control may be noted. One may imagine a control mechanism acting through a specific set of "repressors" as has been postulated for the control of bacterial enzyme synthesis (Jacob and Monod, 1961). This would imply that at some time after infection the phage infected cell synthesizes a specific "repressor" which inhibits enzyme synthesis, but allows phage coat synthesis. In the same manner one may imagine a specific "repressor" inhibiting phage coat synthesis until enough enzyme has been produced. It is difficult to conceive how such a mechanism would enable a switchover to occur, and the amount of information needed for such a mechanism may be too much for the small RNA molecule. An alternative model (analogous to one suggested by Luria (1962) for T-even phages) postulates that there is a difference between the synthesis directed by the infecting molecules and that of the progeny RNA molecules. The early functions (such as enzyme synthesis) would be performed only by the infecting RNA, while the late functions (such as synthesis of phage coat) would be performed only by the progeny phage RNA. This would allow the synthesis of many more coat molecules than enzyme molecules.

The mechanism by which f2 penetrates a susceptible host is also unknown. Phage f2 does not have a tail as do other virulent phages, and appears to be devoid of the unique method of injection of virus nucleic acid through such an appendage. One of the first points to be studied is whether the virus RNA alone penetrates the cell (as the RNA alone is enough to initiate infection) or whether the coat of the virus also penetrates the cell. One experimental approach to the problem may be the results of Loeb (1962) and Brock (1962) that RNase and streptomycin respectively prevent infection--but only if they are present at the instant of infection. If RNase or streptomycin are present only before, or only after the addition of the virus to the bacterial cells, they have no affect. It is possible that the virus nucleic acid is exposed momentarily to the action of these two substances when it attaches to the cell.
In section III, C, 5 it was conjectured that the "early protein" synthesized after phage f2 infection may be identical to the histidine product formed in the cell free amino acid incorporating system of E. coli. In section III, D, 3 it was suggested that the new enzyme activity appearing after phage infection may be identical to the "early protein." Further work should be concentrated on proving (or disproving) the identity of these three products of phage infection. When this is accomplished a major step in the understanding of phage f2 biosynthesis will have been taken.
Bibliography


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