EFFECTS OF p-FLUOROPHENYLALANINE ON MS2 REPLICATION

By

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CHAPTER I

INTRODUCTION

The order of the flow of information during protein synthesis is DNA* ⟶ RNA ⟶ Protein, therefore, a specific nucleotide sequence in DNA gives a unique amino acid sequence in the polypeptide chain of a protein. The RNA participates in this process as a messenger between the DNA and the protein. Since DNA is commonly composed of only four nitrogenous bases, by necessity the information of protein synthesis is contained in a coded form. This code is designated the genetic or amino acid code, and is defined as the means by which the sequence of bases found in nucleic acids may be translated into a given amino acid sequence in a functional protein.

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*The following abbreviations are used: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; sRNA, soluble or acceptor ribonucleic acid; Phe, phenylalanine; F-Phe, p-fluorophenylalanine; poly U, poly uridylic acid; U, G, A and C, the nucleosides of uracil, guanine, adenine and cytosine respectively; PFU, plaque forming units; ATP, adenosine triphosphate; mRNA, messenger ribonucleic acid; TPG, Tris pyruvate glucose medium; MS Broth, normal growth medium; A_{260}, absorbance at 260 mμ; Tris, Tris (hydroxymethyl) amino methane; EDTA, Ethylenediaminotetraacetate; RF, Replicative form; Tm, melting temperature; T, time; RNase, ribonuclease; and TK^-, thymidine kinaseless.

Combinations utilizing different numbers of the bases for code words might be a doublet code 4^2 or 16, a triplet code 4^3 or 64, or a quadruplate code 4^4 or 256. Since there are more natural amino acids than could be coded for by a doublet code and the quadruplate code yields too many possible words, the triplet code is the most probable. The code is degenerate since an individual amino acid responds to more than one code word and has more than one sRNA species. Most code words do code for amino acids and are designated as sense words. There are a few, however, which do not code for amino acids, or have as yet not been shown to code, and are called nonsense words. Never-the-less these may play an integral part in protein synthesis as punctuation marks giving the command to stop or start reading.

Evidence for degeneracy has been demonstrated by a number of workers (1-7). Chapeville, Lipmann, von Ehrenstein, Weisblum, Ray and Benzer (8) proved that sRNA functions as an adaptor in specifying the fit of an amino acid on a template. Reducing cysteine-sRNA to alanine-sRNA using Rainey nickel, they found the coding properties remained unchanged as shown by the absence of incorporation obtained with poly UG. Since an amino acid, once attached to sRNA, no longer participates in selection, it follows that the correspondence between nucleotide sequence in template RNA and amino acid sequence in proteins is embodied in the precise structure and interrelationships of the set of sRNA adaptors and activating enzymes (8). In E. coli, leucine responds to poly UC, poly UG, poly UA or poly U for incorporation into polypeptide chains (1, 7). Two leucine sRNA's have been separated by the counter current distribution technique (9) making possible examination of degeneracy in cell-free systems using natural messenger RNA. Benzer found no

apparent discrimination in the incorporation of leucine into particular peptides of rabbit hemoglobin from leucyl-sRNA I or leucyl-sRNA II (10). Yamane and Sueoka(11) presented evidence that loose specificity is due to exchange of leucine between leucyl-sRNA I and II catalyzed by the leucyl-sRNA synthetase. von Ehrenstein, Weisblum and Benzer (12) then confirmed the earlier results obtained with a synthetic polynucleotide by proving the incorporation of alanine in the place of cysteine, after reduction with Rainey nickel, into a peptide of the α chain of rabbit hemoglobin.

If two sRNA's correspond to different codons, they should transfer amino acids into different positions in polypeptide chains (13). One of the leucyl sRNA's from Escherichia coli which is separable by counter current distribution was found to introduce leucine only into a single position in the α-chain of rabbit hemoglobin, thus indicating at least two distinct codons for leucine in the amino acid code, and that degeneracy of the code is reflected in a multicomponent of sRNA for each amino acid. The translation of mRNA, therefore, should be affected if a change in the adaptor is introduced. Sueoka and Kano-Sueoka (14) investigated the metabolic transition of E. coli infected with bacteriophage T4 from the early to the late phases of gene expression and found a major shift in the pattern of protein synthesis. To account for control during virus infection the adaptor modification hypothesis was developed (14) and it complements the modulation hypothesis proposed by Ames and Hartman (15). The adaptor modification hypothesis explains a change of the spectrum of functioning genes during development by modifying sRNA without provoking mutation of genes and is thus one means by which a phage can take over control of the host cell's metabolic

machinery. This modification consists of changing one leu sRNA type into another leu sRNA which is used in transcribing phage information.

Nirenberg and co-workers (16) have established patterns of degeneracy in code words such as XpYpU and XpYpC for the same amino acid. Also, XpYpA and XpYpG were found to serve for the same amino acid. Other degeneracies may exist for the 5'-terminal, 3'-terminal and internal codons.

Since Woods showed in 1940 (17) that p-aminobenzoic acid competed with the sulfonamide drugs for essential enzymatic sites, many structural analogues of natural amino acids as well as other types of compounds have been tested to see if they compete with the natural metabolite for incorporation. Sometimes the incorporation of amino acid analogues into protein leads to complete growth inhibition (18).

The use of isostere of phenylalanine, p-fluorophenylalanine, as an antagonist has been reviewed by Richmond (18). In chemically defined media containing exponentially growing cultures of E. coli or yeast the analogue reduced the rate of growth and the inhibition is reversed by L-Phe.

Fluorophenylalanine is assimilated in the intracellular amino acid pool and then incorporated into protein (18). Using E. coli, Cohen and Rickenberg (19) found that L-Phe ^{14}C could be replaced by F-Phe by using a 20 fold greater concentration of the analogue. However, when equal concentrations were present the natural amino acid was incorporated preferentially into the protein.

Westhead and Boyer (20) demonstrated the incorporation of F-Phe into the rabbit proteins aldolase and glyceraldehyde 3-phosphate dehydrogenase with replacement of up to 25% of the Phe residues with no

modification of enzyme activity. Richmond has reported random replacement of the eight Phe's in E. coli alkaline phosphatase (21) and also incorporation into penicillinase (22).

p-Fluorophenylalanine has been used as a protein synthesis inhibitor in many viral systems. Ackermann, Rabson and Kurtz (23) reported that F-Phe produced a marked inhibition of the replication of poliovirus, but did not interfere with the cytopathology in HeLa cells. The analysis of the effects of F-Phe in a virus - host system may be divided into the effect upon the host cell's metabolism and the effect upon the virus and viral constituents.

Baltimore, Franklin and Callender (24) observed that Mengovirus infection of L cells induces a rapid decline in the rate of synthesis of RNA and protein but F-Phe can supress this inhibition of host cell RNA synthesis and can prevent inhibition of host protein synthesis, possibly by inhibiting proteins synthesized under control of viral RNA. Verwoerd and Hansen (25) demonstrated that along with suppressed formation of viral particles, there was inhibition of virus-specific products in ME virus infected L cells. Viral infection depends upon the synthesis of several types of "early proteins" responsible for inhibition of normal RNA and protein synthesis, and one or more necessary for the production of virus specific material; but all of which are coded for by the viral genome.

Brown, Planterose and Steward (26) found that addition of F-Phe reduced the yield of infective RNA and resulted in a corresponding decrease in foot and mouth disease virus titer. With poliovirus Scharff, Thoren, McElvain and Levintow established that early addition of F-Phe prevented initiation of viral RNA synthesis and if added later in the

infectious cycle, the synthesis of viral RNA was interrupted showing continual protein synthesis was required for viral RNA synthesis. Baltimore and Franklin (28) observed that F-Phe will halt Mengovirus RNA synthesis even after its initiation, but low concentrations of the analogue allows viral precursor synthesis with inhibition only of the infectious virus formation. Scholtissek and Rott (29) also found that in the fowl plaque virus system F-Phe if given immediately after infection inhibits the formation of any viral material while with post infection addition all viral material appeared as normal, but no infective particles were produced. Flake (30) discovered that in the presence of F-Phe formation of infectious herpes-simplex virus was inhibited.

Tanami and Pollard (31) found that extracellular psittacosis virus particles were intact, but that F-Phe interfered with the maturation of intracellular virus. If the antagonist was added early in the infectious cycle, maturation was suppressed; but if added later, infectious virus were produced.

Townsley (32) reports that in TMV F-Phe is incorporated at a slower rate than Phe; and several workers (27, 33-35) have demonstrated F-Phe reversal by Phe in fowl plague virus, poliovirus and vaccinia virus.

The formation of thymidine kinase in vaccinia infected L-M (TK^-) cells is inhibited by F-Phe (36). L-M (TK^-) cells are mutants which lack the ability to synthesize thymidine kinase and thus F-Phe is inhibiting the synthesis of the enzyme as directed by the viral RNA.

Poliovirus has been extensively studied by several laboratories (37-39). Dainell, Levintow, Thoren and Hooper (37) established that the synthesis of viral RNA and maturation were very closely coordinated in time. In other experiments (38) F-Phe reduced viral yields to less than

2% of control without affecting the formation of infectious RNA during early synthesis and during later synthesis the rate decreased only slightly giving 80% of the control. The inhibition was reversed by the addition of Phe but the course of maturation was displaced by 2 hours. Hummeler and Wecker (39) have observed that particles formed in the presence of low concentrations of F-Phe are structurally altered. Since appreciable amounts of viral protein and infectious RNA are synthesized with production of few infectious viral particles, the presence of F-Phe may prevent assembly of physical particles, or, if formed, they are not infective.

To differentiate between these two possibilities sensitivity to RNase was determined and electron micrographs were made. Treatment with RNase reduced the titer of F-Phe - grown virus by 99%. Electron photo micrographs of virus synthesized in the presence of F-Phe showed an obvious disarrangement of the subunits and appeared less dense due to the penetration of phosphotungstic acid. Structurally defective poliovirus particles are formed in the presence of F-Phe.

The availability of RNA containing bacteriophages which attack E. coli provides a natural messenger RNA that can be used in the cell-free protein synthesizing system of its host. The first bacteriophage, f2, containing RNA as its nucleic acid was isolated by Loeb (40) and characterized by Loeb and Zinder (41). Several other RNA coliphages which lyse only the male strains of Escherichia coli and produce a very high average yield of progeny have been described (42-44). Since DNA is not involved in the process of replication, all of the information for phage protein synthesis and RNA replication must be in the bacteriophage RNA.

The phages f2 and MS2 have been extensively studied and characterized (45,46) while the other RNA phages have been examined to a lesser degree. Phage f2 is a small particle 200 Å in diameter and contains approximately 2,500 nucleotides in its nucleic acid and has some 100 molecules of a molecular weight 20,000 in its protein coat. The fingerprint pattern for the tryptic digested peptides is known (46,47) and some sequences have been determined (48). Bacteriophage MS2 has a particle weight of 3.6×10^6 and is estimated to be 32% RNA by weight. The particle weight of the isolated RNA is $1.05 \pm 0.1 \times 10^6$, indicating that there is one molecule of RNA per virus particle contained in its 240 Å diameter. Light scattering studies and temperature denaturation studies both suggest considerable hydrogen bonding in the viral RNA molecule and a compact structure which sediments abnormally in the ultracentrifuge (45). Several other antigenically different RNA phages have been isolated and partially characterized by Bishop and Bradley (42).

Studies on the synthesis of the coat protein and the structure of the coat protein of phage f2 have been conducted (49,50). Nathans, Notani, Schwartz and Zinder (49) have shown that the RNA from bacteriophage f2 can act as a natural messenger in the cell-free E. coli synthesizing system and Schwartz, Eisenstadt, Brawerman and Zinder (50) have obtained synthesis of f2 coat protein in extracts of Euglena gracilis. The products of these reactions have the same tryptic digest peptide pattern as isolated coat protein and also contain a certain amount of unidentified material.

Certain bacterial strains carry a suppressor gene affecting mutants in many different cistrons. Using nitrous acid treatment of f2, Zinder has obtained mutants which will grow only on the strain

carrying the suppressor. In vivo complementation occurs defining at least two phage cistrons (51). Pfeifer, Davis and Sinsheimer (52) have also obtained complementation with a series of temperature mutants of MS2 and have been able to determine whether the mutants are blocked in an early or late stage of virus synthesis.

Davis and Sinsheimer (53) have found no evidence for any type of conservative or semiconservative transfer from parental nucleic acid to progeny phage. They also demonstrated that dispervive transfer was limited to 3%. A number of workers (54-57) have also shown that DNA is not involved in the replication of RNA viruses.

Although the RNA of these bacteriophage is normally single stranded, during the process of replication a negative strand is formed and the double-stranded product is known as the replicative form (RF). Weissman, Simon and Ochoa (58) described the properties of an enzyme induced on viral infection which catalyzes the incorporation of the ribonucleotide triphosphages in RNA but does not depend on added RNA primer. The characterization of a product made a few minutes after viral infection from the parental RNA described by Weissman, Borst, Burdon, Billeter and Ochoa (59) is the RNase resistant RF. This RF is found attached to the RNA synthetase during purification.

This research examines the effect of p-fluorophenylalanine on MS2 replication and on protein synthesis in the E. coli cell-free system using MS2 RNA as messenger. It was undertaken to develop a system for the study of phenylalanine degeneracy using phenylalanine and p-fluorophenylalanine.

CHAPTER II

EXPERIMENTAL PROCEDURE

Materials

The sodium salt of GTP was obtained from Schwarz Bio Research, Inc. The sodium salt of ATP was obtained from P-L Biochemicals, Inc. The silver-barium salt of phosphoenolpyruvate, pyruvate kinase (2.7.1.40), p-fluorophenylalanine, and p-fluorophenylalanine-1'^{14}C Sp. Act. 3.5 mC per mmole, were obtained from California Corporation for Biochemical Research. L-Phenylalanine-UL-^{14}C (Sp. Act. 367 mC per mmole) was obtained from New England Nuclear Corp. RNase (2.7.7.16) was obtained from Mann Research Laboratories. Bacto-tryptone, Bacto-yeast extract and Bacto-agar were purchased from Difco Laboratories. Magnesium chloride and ammonium sulfate came from Fisher Scientific Company and 99% pyruvic acid was obtained from Matheson, Coleman and Bell, Inc. Freon 11 ($CFCl_3$) was obtained from Union Carbide Chemical Company and cesium chloride came from Alfa Inorganics, Inc. Ammonium chloride and calcium chloride came from J. T. Baker Chemical Company. Sodium perchlorate was purchased from G. Frederick Smith Chemical Company, and Thiamine hydrochloride was obtained from Merck and Company, Inc. The bacteriophage MS2 and its host bacterium, Escherichia coli HFr C 3000, was very kindly supplied by Dr. Alvin J. Clark, University of California, Berkeley.

Methods

Media

MS broth, the normal growth medium for the host bacterium, contains per liter: 10 g Bacto-tryptone, 8 g NaCl and one g Bacto-yeast extract. After autoclaving, 10 ml of sterile 10% glucose solution, 2 ml sterile 1 M $CaCl_2$ solution, and 1 ml of a sterile solution containing 10 mg/ml of thiamine hydrochloride are added per liter.

Top agar for plating contains the same ingredients as the broth, plus 0.8% Bacto-agar. Bottom agar also contains the same ingredients as broth plus 1.0% Bacto-agar.

TPG medium contains per liter: 0.5 g NaCl, 8.0 g KCl, 1.1 g NH_4Cl, 0.2 g $MgCl_2 \cdot 6H_2O$, 0.64 ml 99% Pyruvic acid, 12.1 g Tris, 0.046 g KH_2PO_4, and 1.0 ml 0.16 M Na_2SO_4. These salts are adjusted to pH 7.4 with HCl and then the following sterile solutions are added: 1.0 ml of $FeCl_3 \cdot 6H_2O$, 20 ml 0.1 g/ml glucose, 2 ml of 1 M $CaCl_2$, and 1 ml of a solution containing 10 mg/ml thiamine hydrochloride (60,63).

Growth and Assay

The bacteriophage was added to an exponentially growing culture of host cells in MS broth at 37° and up to 90% of the input titer was adsorbed within 5 minutes. In any culture there was a variable fraction (usually around 10% of the *E. coli*) which retained colony-forming ability. Burst sizes have usually been between 10^3 and 10^4 phage per bacterium and lysate titers may exceed 10^{13} phage/ml.

For mass cultures, 20 liter carboys containing 15 liters of MS broth were used. The culture was aerated vigorously at 37° and when the concentration reached 10^8/ml, the virus were added at a multi-

plicity of 10. Foaming at lysis was controlled by the addition of Dow-Corning anti-foam B. Aeration was continued for 6 hours after addition of phage then 0.5 ml of chloroform was added per each 10 - 20 ml to lyse the remainder of the bacteria. The chloroform was then removed by aeration.

Assay of the virus was performed by the agar layer method (61). Plating bacteria were E. coli Hfr C 3000 cultures grown overnight diluted one to 10, then grown for two additional hours. To each tube containing 3 ml melted top agar at 48° was added 0.3 ml of a solution of bacteria and 0.1 ml of the bacteriophage-containing solution. Plates were incubated at 37° until plaques appeared.

Purification of the Virus

The method used is a modification of the ϕX174 purification procedure of Sinsheimer (62) which was also adapted for the MS2 purification by Strauss and Sinsheimer (45).

To the lysate was added 25 ml 0.1 M EDTA, pH 7/liter followed by 280 g ammonium sulfate/liter with vigorous stirring. After 12 hours in the cold, the precipitate was centrifuged in the Sharples Super Centrifuge and the supernatant solution was discarded.

The precipitate was thoroughly mixed with 10 ml of a buffer containing 0.1 M NaCl, 0.05 M tris, 0.01 M EDTA, pH 7.6 per liter of lysate. To this suspension was added 10 ml of Freon-11 per liter of lysate. The resulting mixture was shaken in a separatory funnel for 3 minutes, and the emulsion then broken by centrifugation for 10 minutes at 12,000 X g. The aqueous layer which contained the phage was decanted and saved.

To the Freon layer was added 4.3 ml of the MS2 buffer per liter of lysate, and the mixture treated as before. The aqueous layers were then pooled.

By the process of three cycles of differential centrifugations at 16,000 x g for 30 minutes followed by 40,000 x g for 6 hours, the phage was greatly purified and the volume greatly condensed. If further purification was necessary, CsCl was added until the density of the solution was 1.38 ± 0.01 (approximately 0.550 g CsCl/ml). The solution was placed in lusteroid tubes and centrifuged in the SW rotor of the Spinco Model L-2 Ultracentrifuge for 40 hours at 35,000 rpm at 4°.

Preparation of MS2 RNA and Coat Protein

A modification of the method of Freifelder (63,64) was used to release the bacteriophage RNA. The phage (8 mg/ml) is added to neutralized 10 M $NaClO_4$ containing 5 x 10^{-3} M EDTA 1:1 by volume and incubated at room temperature for 30 minutes. The protein which precipitated out was collected in a clinical centrifuge.

Other Determinations

The method of Katz and Comb (65) was used to determine the base composition of the RNA. Preparation of the E. coli 30,000 x g supernatant solution was as described by Nirenberg (66).

Radioactive determinations were made using a Tri-Carb Liquid Scintillation Counter having an operating efficiency of 65-70% for ^{14}C and 4-6% for ^{3}H. For the incorporation into MS2 precipitate the counting solution and procedure of Kihara, Ikawa and Snell (67) was used and for all other radioactivity measurement Bray's liquid scintillation solution (68) was used.

CHAPTER III

RESULTS

Characterization of MS-2

Effect of MS2 Infection on Absorbance of an E. coli Culture

The course of MS2 infection can be determined by measuring absorbance at 630 mμ of an infected E. coli culture as is shown in Figure 1. For 40 minutes after infection the absorbance values of both cultures are the same. With the MS2 infected culture the expected absorbance increase does not occur at the 60 minute interval. Then there is a rapid decline of absorbance to approximately the original absorbance of the culture. The absorbance of the control E. coli culture increases exponentially as would be expected for normal logarithmic growth. If measurements are continued on an infected culture, increasing absorbance occurs due to the growth of MS2-resistant E. coli. In order to follow MS2 infection by this technique time intervals must be chosen so that the overgrowth by resistant bacteria does not interfere with the measurments.

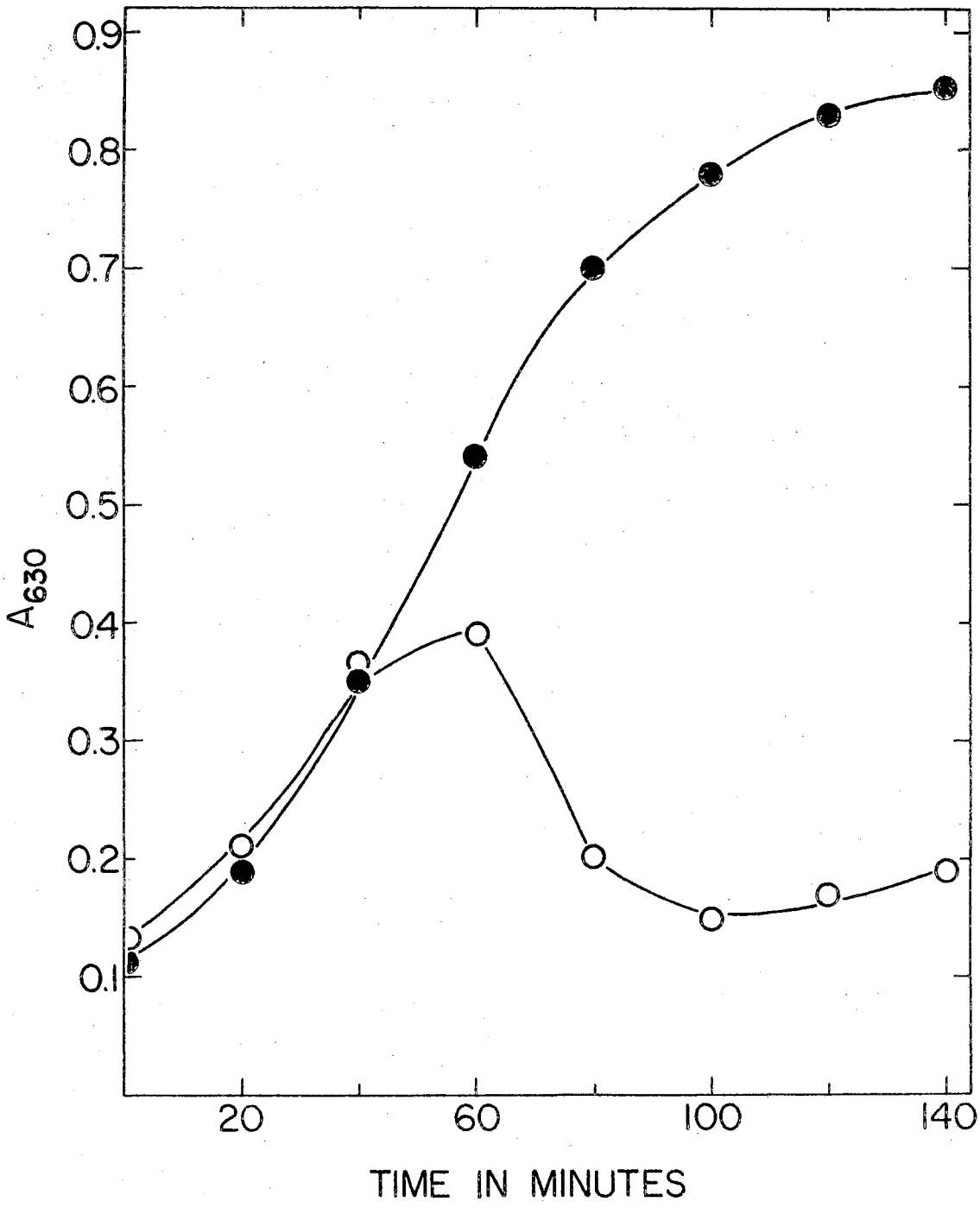
One Step Growth Curve in MS Broth

One of the best techniques for following progress of bacteriophage infection is the one step growth curve (69), an example of which can be

Figure 1

Effect of MS2 Infection on the Absorbance of an E. coli Culture

One sample of E. coli cells, O, growing in MS broth were infected with MS2 at a multiplicity of one. The absorbance was determined at 630 mμ at the indicated time intervals. Absorbance measurements for a control culture are also shown ●.


0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
A_{630}
20
60
100
140
TIME IN MINUTES

seen in Figure 2. Because of the unavailability of MS2 antiserum, the normal procedure for one step growth curves was modified by omitting this step and correcting for the number of unadsorbed MS2 virus by determining the titer shortly after adsorption occurs and before progeny are produced.

Plaque Types

Photographs of typical MS2 plaques developed on infection of E. coli Hfr C3000 are shown in Figures 3A and 3B. No published data is available on the plaque types of MS2; however, Dr. Edward Carusi communicated that plaque type heterogenicity such as Figures 3A and 3B illustrates has also been obtained in Dr. R.L. Sinsheimer's laboratory. Several homogeneous plaque type cultures were obtained by cloning five times. These plaque type mutants were not further characterized.

Ultraviolet Absorption Spectrum of MS2

Figure 4 shows an ultraviolet spectrum of purified MS2 virus with a maximum at 257 mμ and a minimum at 241 mμ. This is very similar to the ultraviolet spectrum published by Strauss and Sinsheimer (45).

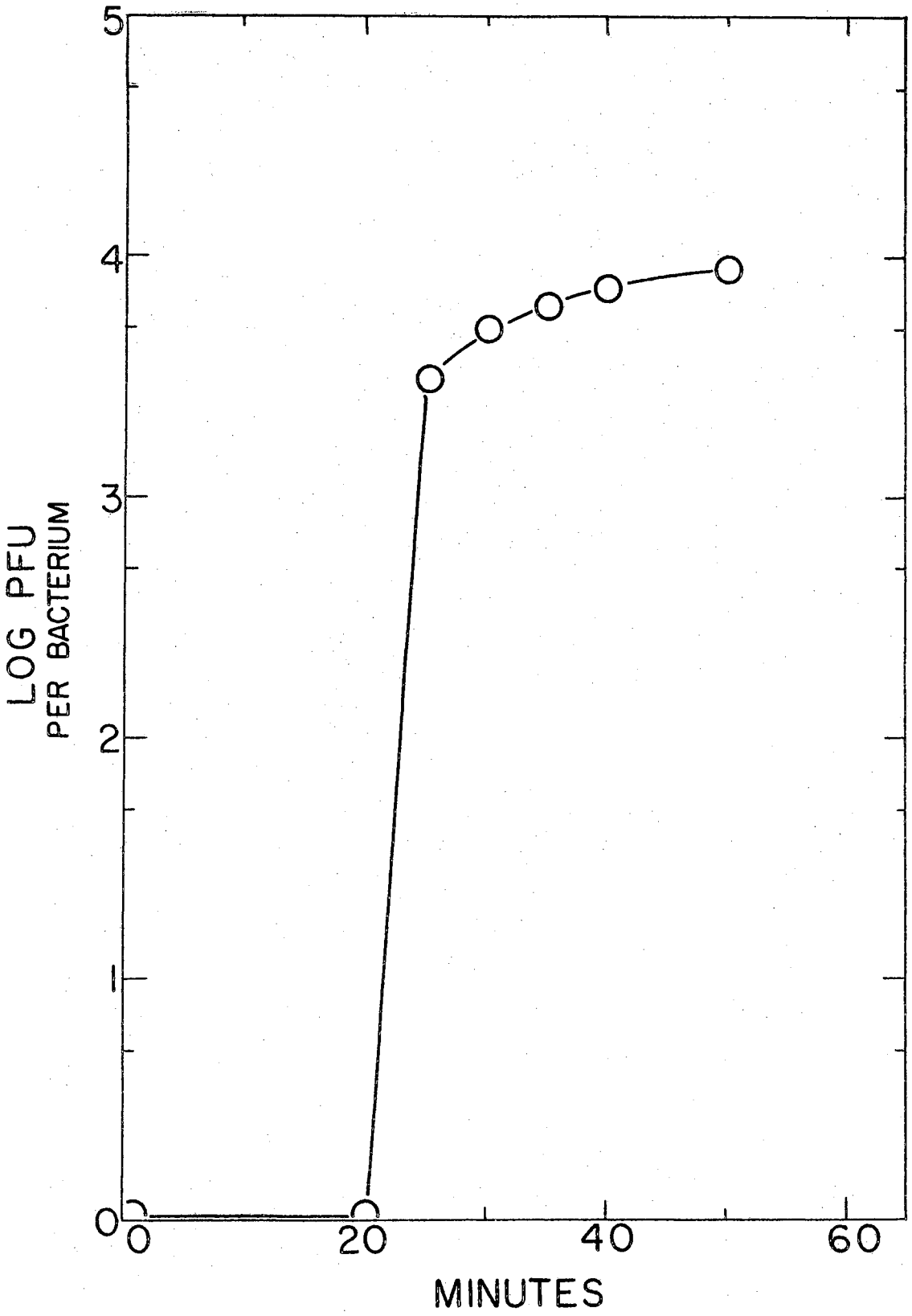
Cesium Chloride Density Gradient Analysis of MS2

For characterization of MS2 and correlation of radioisotope incorporation the cesium chloride density gradient pattern was obtained. There was a bluish white opalescent band about one third the distance up the tube which gave a very distinct absorbance peak when measured at 260 mμ as seen in Figure 5.

Figure 2

One Step Growth Curve without Use of Antiserum

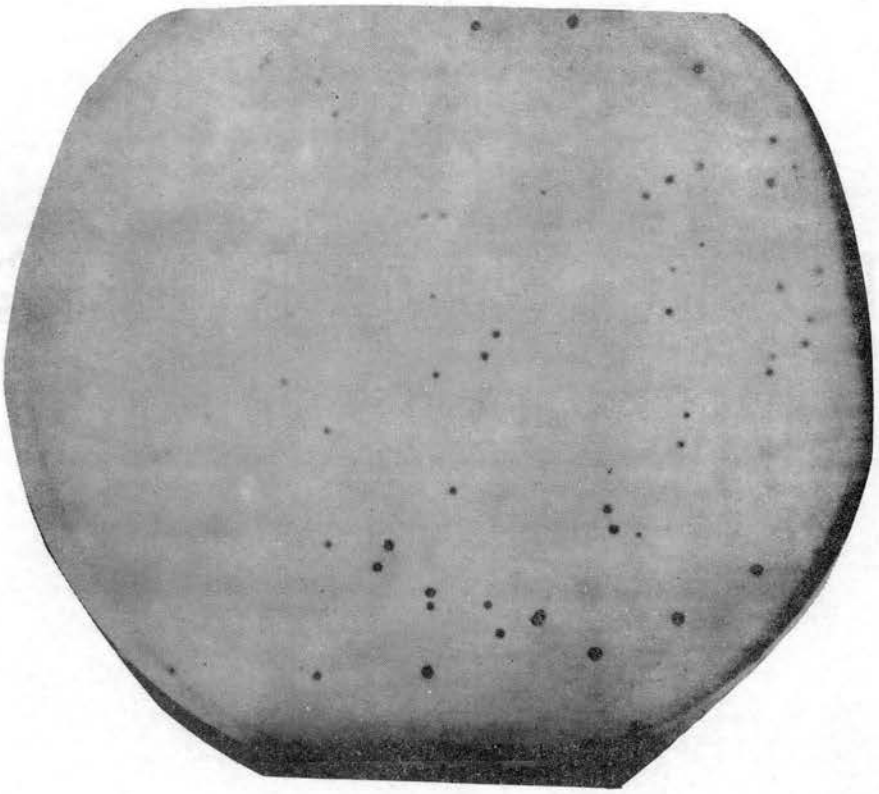
A culture of E. coli growing in MS broth was infected with MS2 at a multiplicity of one. Samples were taken at the indicated time and treated with chloroform. The titer was determined as described in the text. The results are presented on the basis of PFU per bacterium.


LOG PFU
PER BACTERIUM
5
4
3
2
1
0
0
20
40
60
MINUTES

Figures 3A and 3B

Common Plaque Types of MS2

After incubation at 37° for 6 hours, photographs were made using a Polaroid camera, of an E. coli lawn containing common type plaques obtained. They were illuminated from the side by florescent lights on a Polaroid copy stand.



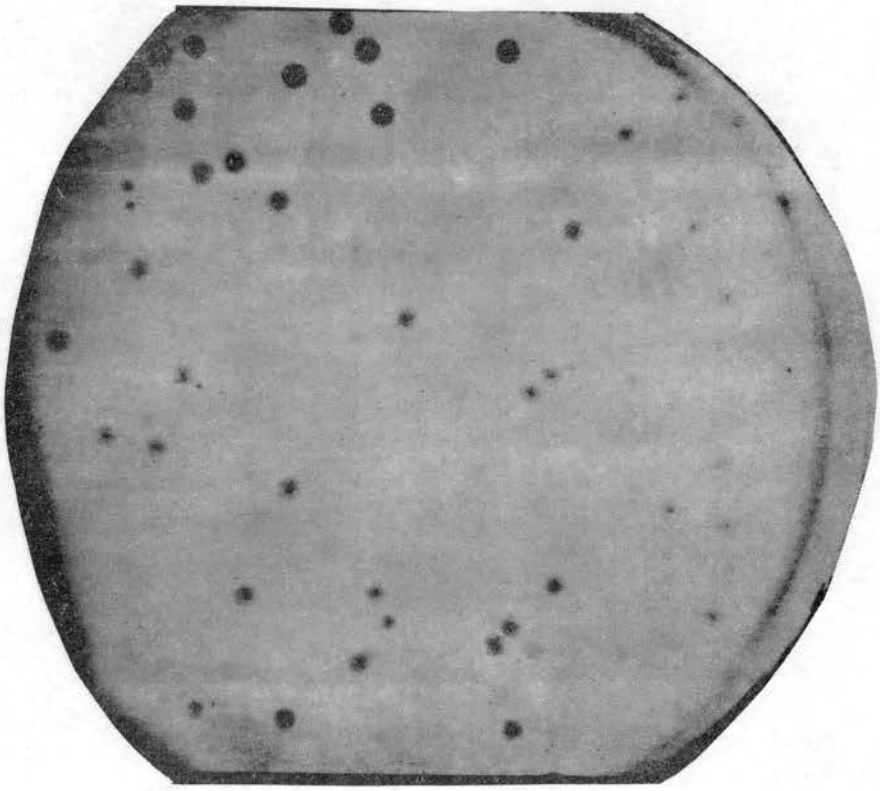


Figure 4

Ultraviolet Absorption Spectrum of Bacteriophage MS2

The spectrum of a sample of MS2 in 0.1 M NaCl, 0.05 M Tris and 0.0. M EDTA, pH 7.6 was determined using a Beckman DB recording spectrophotometer. Concentration of the phage was 90 μg/ml.

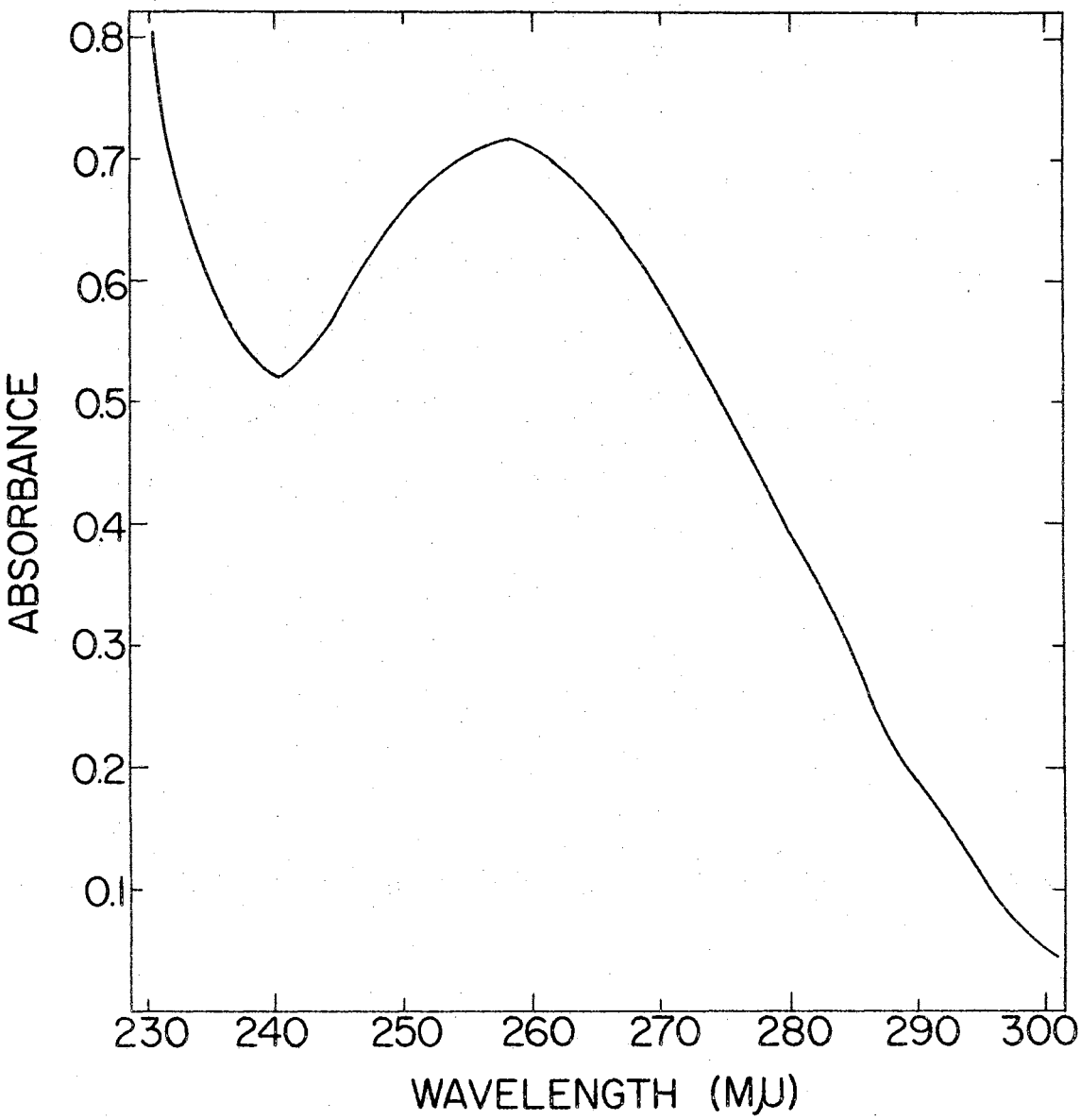
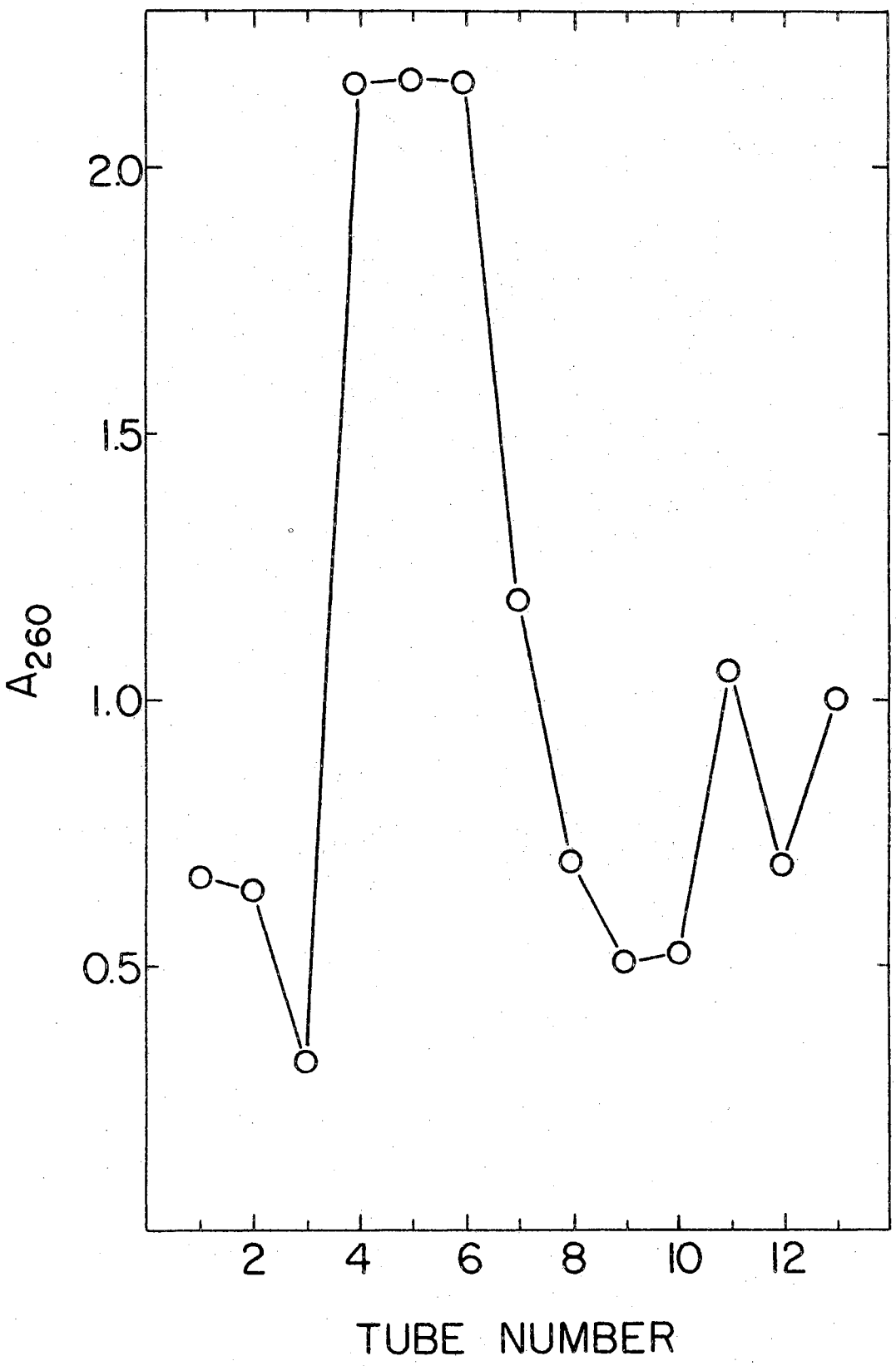

ABSORBANCE
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
230
240
250
260
270
280
290
300
WAVELENGTH (MJU)

Figure 5

Cesium Chloride Density Gradient Centrifugation of MS2 Virus

A 3.2 ml sample of differentially centrifuged MS2 virus in P=1.4. was centifuged for 60 hours in the SW 39 rotor of the Spinco L-2, centrifuged at 35,000 rpm and 4°. Four drop fractions were collected, diluted to 1 ml and the absorbance at 260 mμ was determined.


2.0
1.5
1.0
0.5
A_{260}
2
4
6
8
10
12
TUBE NUMBER

Sucrose Density Gradient Analysis of MS2

To prepare further for correlation of radioisotope incorporation data and to examine the relative efficiency of a sucrose density gradient, MS2 virus was centrifuged on a 5-20% w/v buffered sucrose gradient. The pattern shown in Figure 6 indicated a sharply defined absorbance peak when analyzed at 260 mμ and suggested that banding of a small amount of radioactive phage would be feasible.

Effect of F-Phe on MS2 Replication

Experiments Using Enriched Medium

In Figure 7, F-Phe was added to MS broth in a sufficient amount to successfully compete with the Phe present. When F-Phe was added at the time of infection the virus had an increased latent period, a decreased rate of replication, and a significantly reduced viable phage titer. If F-Phe was added at the time of dilution of E. coli and infection occurred 60 minutes later, the rate of replication compared favorably with the control, the period of latency was again increased, and only a slight reduction in viable phage titer was observed.

Growth Curve with F-Phe Added 60 Minutes Before Infection

The results of an experiment with F-Phe being added at the time of dilution into fresh medium when infection was made 60 minutes later indicated that the virus had an increased ability to absorb the E. coli followed by a slower rate of release over a period of 10 minutes than control infected cultures. The control in Figure 8 indicates a yield of 2×10^9 PFU at 25 minutes in the absence of F-Phe.

Figure 6

Sucrose Density Gradient Centrifugation of MS2 Virus

A 0.3 ml sample of MS2 virus was layered on a 3 ml linear density gradient of 5-20% w/v sucrose buffered with 0.1 M NaCl, 0.05 M Tris, and 0.01 M EDTA, pH 7.6, and centrifuged 3 hours at 35,000 rpm and 4°. Four drop fractions were collected, diluted to 1 ml and the absorbance at 260 mμ was determined.

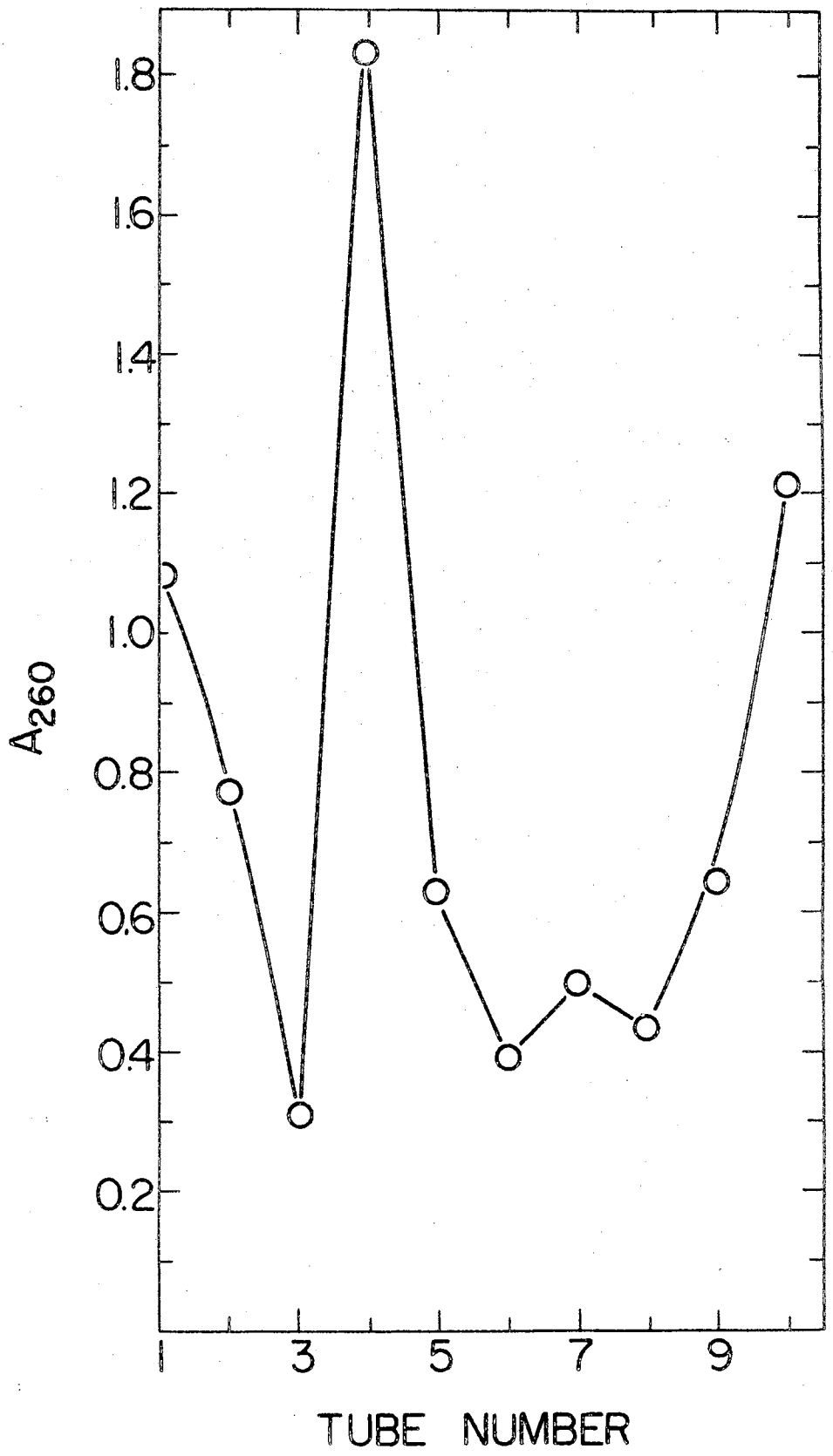

1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
A_{260}
1
3
5
7
9
TUBE NUMBER

Figure 7

Effect of F-Phe Addition on MS2 Replication

To a culture of E. coli in MS broth F-Phe (2 mg/ml) was added 60 minutes prior to infection O, and concomitant with infection ● with 3×10^4 PFU/ml of MS2 virus all cultures were treated with chloroform to lyse resistant bacteria and the titer was determined as described in the text. No F-Phe was present in sample ●.

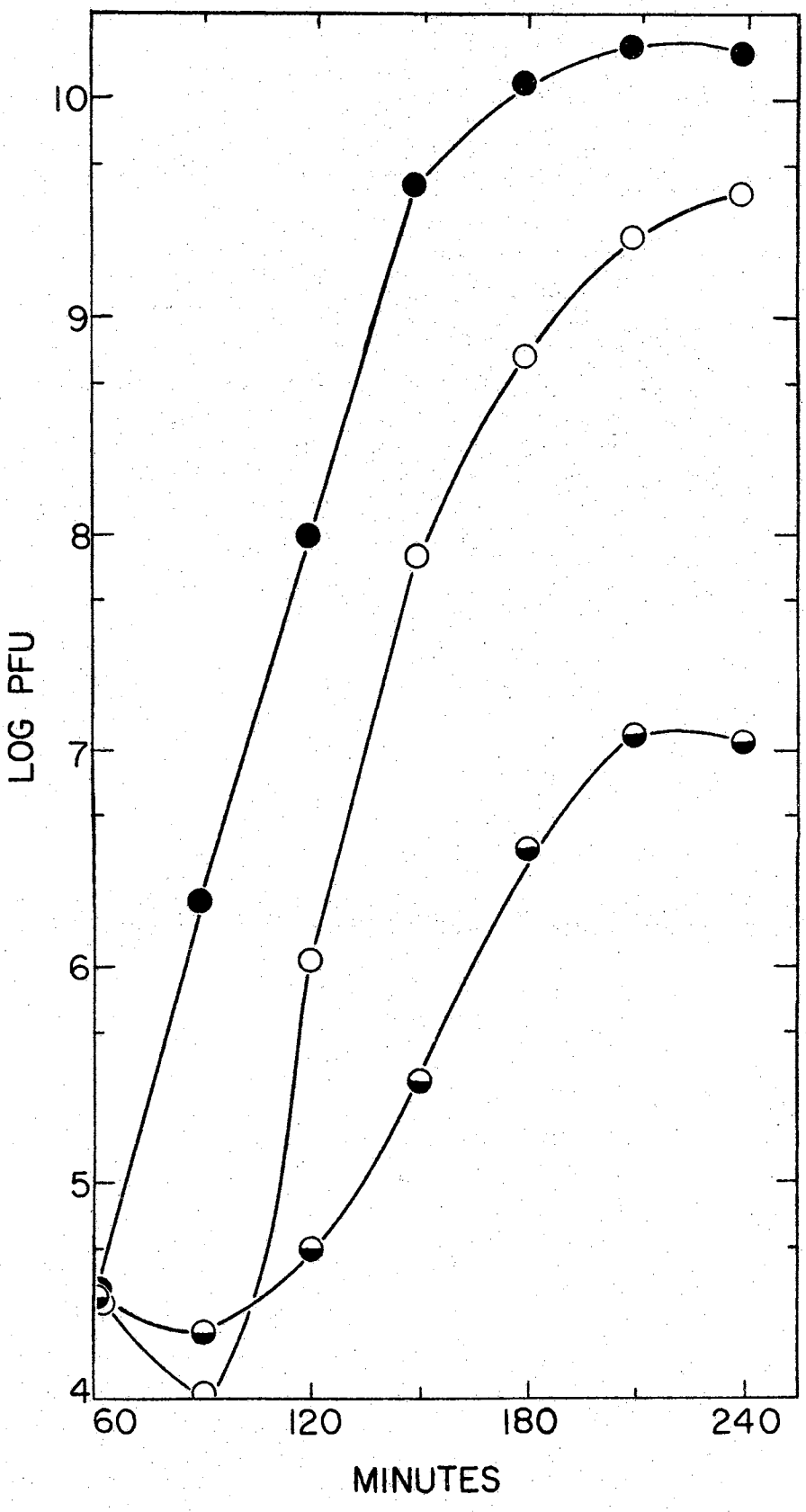
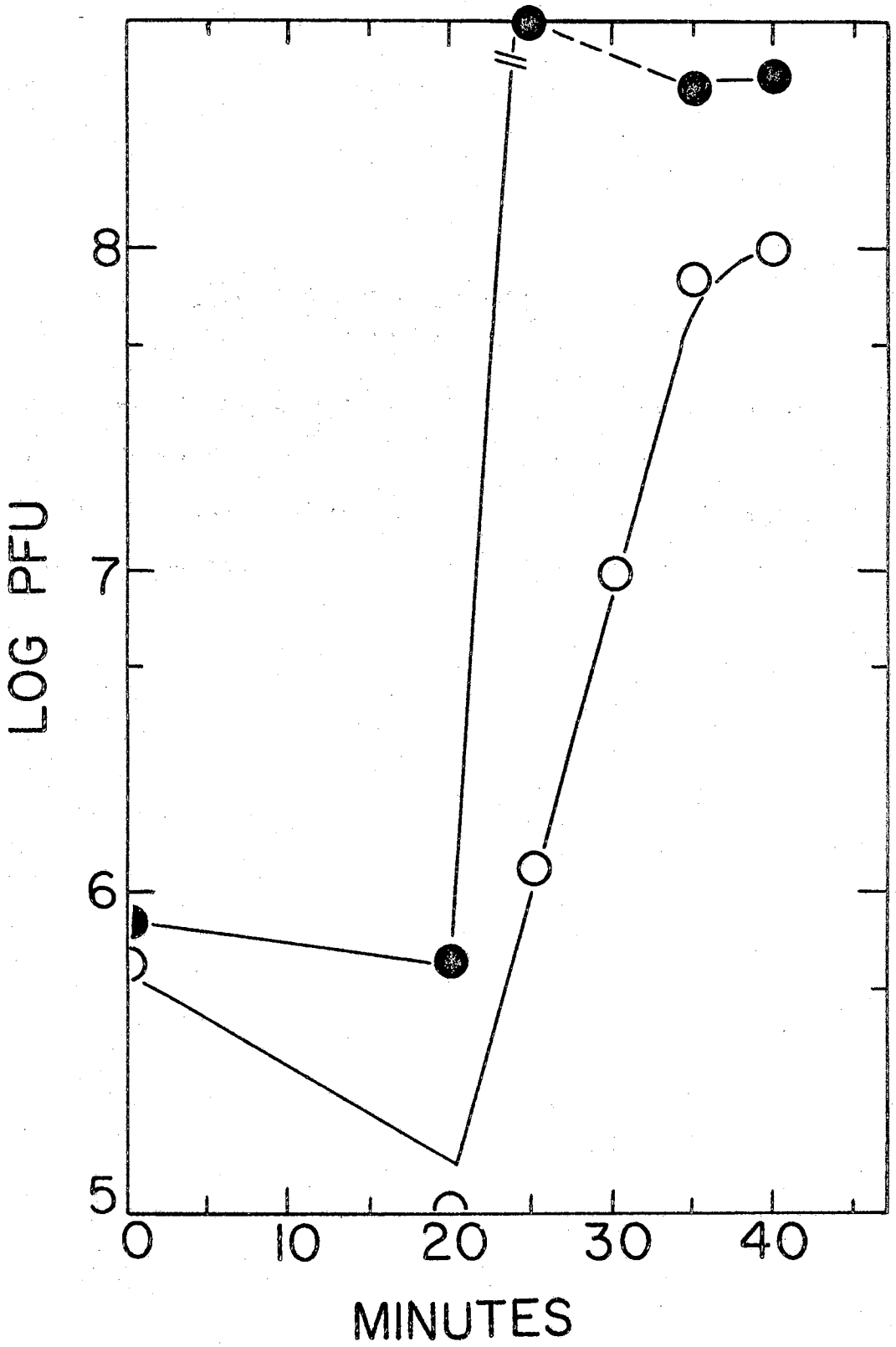

LOG PFU
10
9
8
7
6
5
4
60
120
180
240
MINUTES

Figure 8

Replication of MS2 with F-Phe Added 60 Minutes Before the Time of Infection

To a culture of E. coli growing in MS media, 2 mg/ml F-Phe was added at 60 minutes before infection with 8 x 10^5 PFU/ml. Samples were removed at the indicated times, chloroformed, and titer determined as described in the text for control ● and F-Phe containing sample O.


LOG PFU
8
7
6
5
0
10
20
30
40
MINUTES

Subsequent absorption of part of the viral population left a titer of 3×10^8 PFU/ml.

One Step Growth Curve with F-Phe Added at Time of Infection

Greater inhibition resulted when the analogue in added concomitantly with infection as can be seen in Figure 9. The latent period was increased by 10 minutes and there was a 30-fold reduction of final phage titer as compared to control infection.

Determination of the Amount of Phe in MS Broth

Due to the extremely high concentration (2 mg/ml) of F-Phe needed to produce a 90% inhibition the amount of Phe in MS broth was analyzed by a microbiological assay. Using a modification of the techniques described by Henderson and Snell (70) with Lactobacillus arabinosus, the amount of Phe is MS was found to be 572 μg per ml. Because of the difficulties of competing radioactive F-Phe against such an excess of Phe, further experiments were made in a modified TPG medium (60). The growth rates of E. coli and MS2 were reduced in this medium by a factor of about 2.

Experiment Using Synthetic Medium

Single Burst Experiment with F-Phe Added 60 Minutes Before Infection.

Figure 10 shows a one step growth curve in chemically defined TPG medium. When F-Phe was present at a concentration of 10 μg/ml, the latent period was increased by 10 minutes and a reduction in final titer was observed.

Figure 9

Single Burst Experiment with F-Phe Added at Time of Infection

To a culture of E. coli growing in MS broth 2.0 mg/ml F-Phe was added at the time of infection with 4×10^6 PFU/ml. Samples were removed at the indicated time intervals and treated with chloroform to lyse residual bacteria. The titer was determined as described in the text for control infection ● and F-Phe containing sample O.

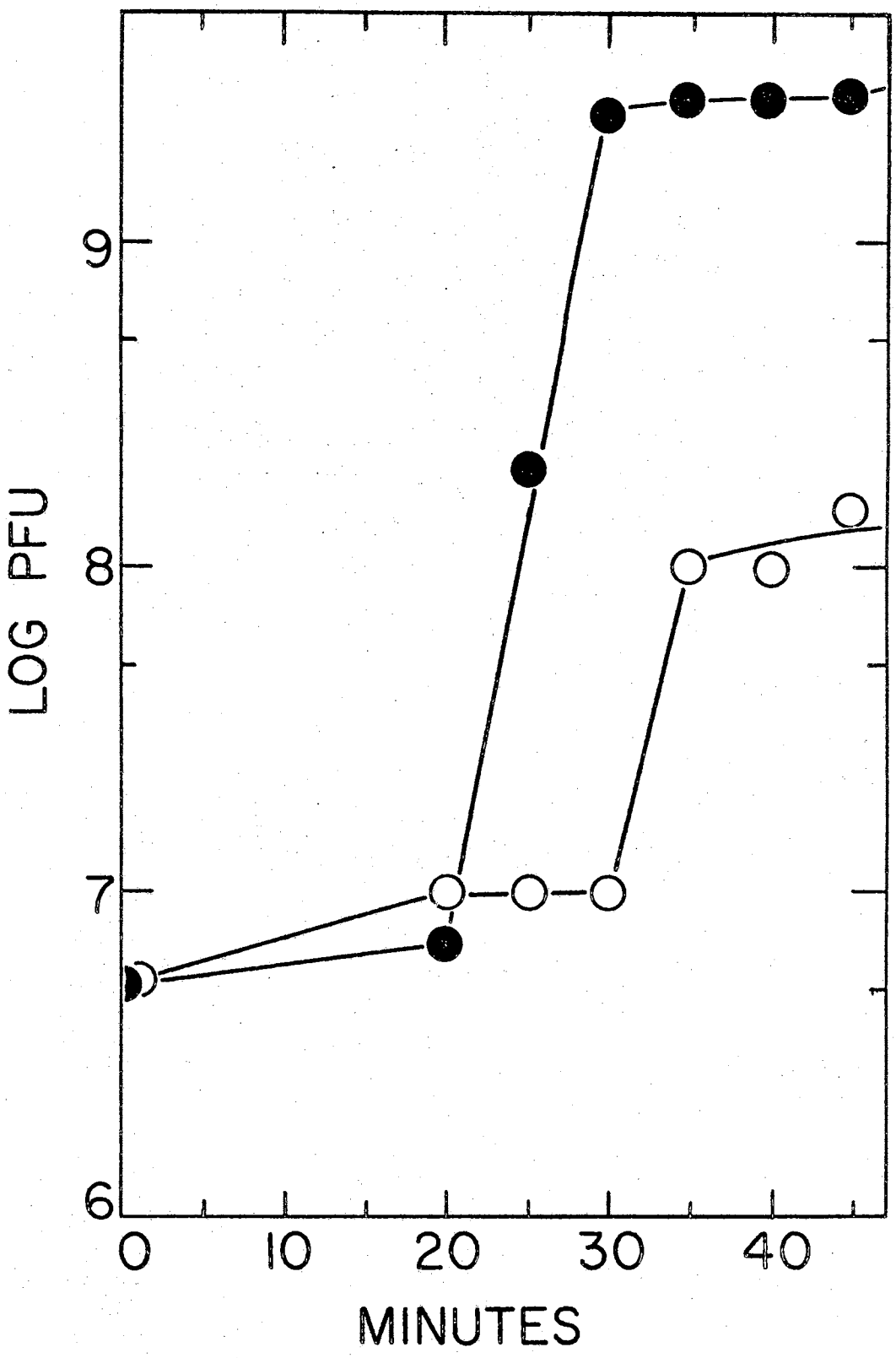
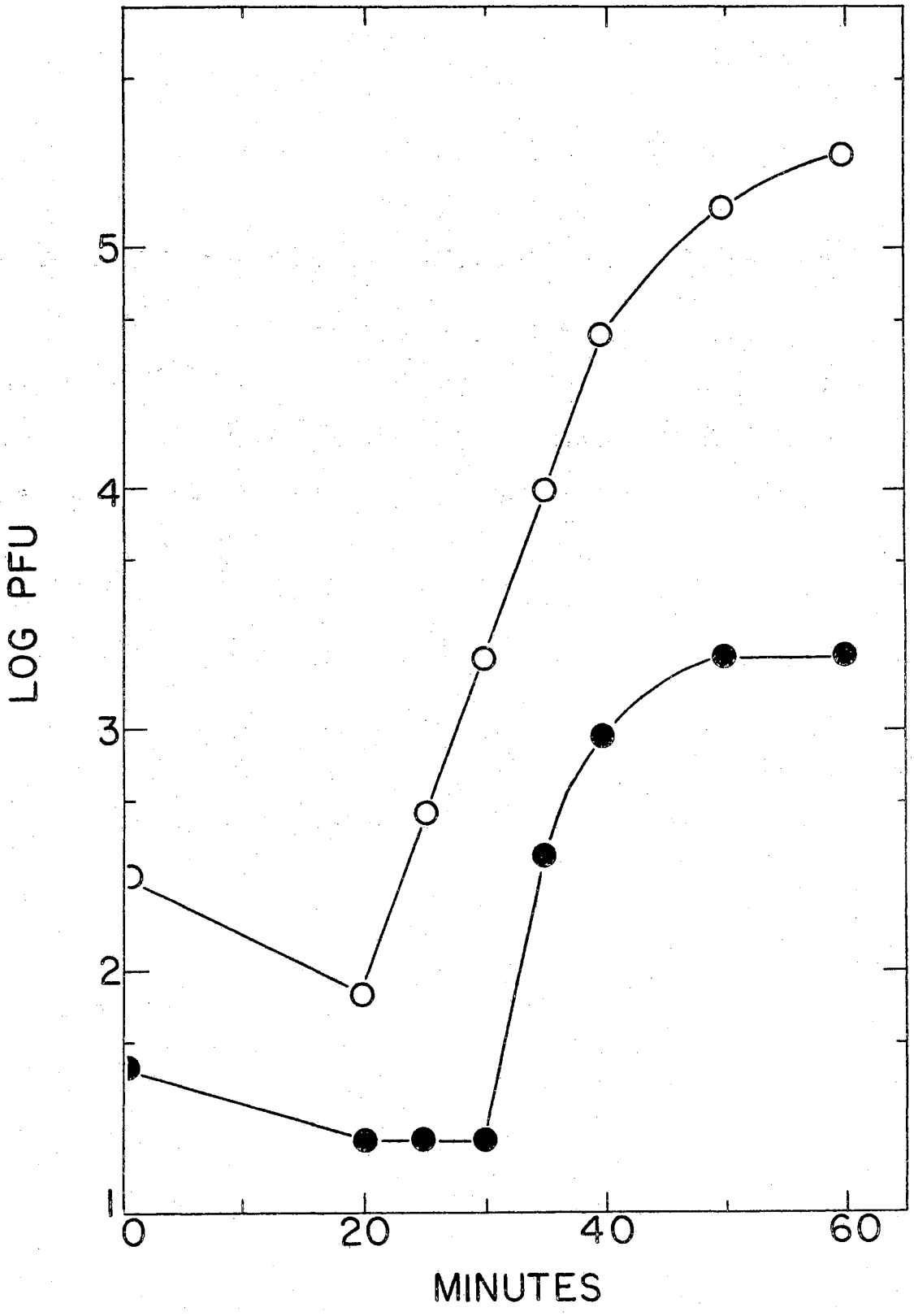

LOG PFU
9
8
7
6
0
10
20
30
40
MINUTES

Figure 10

Single Burst Experiment with F-Phe Added 60 Minutes Prior to Infection

To a culture of E. coli growing in TPG media 10 μg/ml F-Phe was added 60 minutes before infection with 3 x 10^5 PFU/ml. Samples were removed at the times indicated, chloroformed, and titer determined as described in the text for control O and F-Phe containing sample ●.


LOG PFU
5
4
3
2
1
0
20
40
60
MINUTES

Effect of Time of Addition of F-Phe on Burst Size and Time

The programming of viral replication is under temporal control and by adding F-Phe at various time intervals relative to infection it should be possible to determine a specific time period during replication when there is a marked inhibition. The results from such an experiment are shown in Figure 11. Addition of F-Phe at the same time as infection produced the greatest inhibition suggesting that the synthesis of an early enzyme was the site of F-Phe effect. However, the inhibitory effects observed with the addition of F-Phe at 20 and 30 minutes show that a later protein synthesizing step is also inhibited. From this experiment it is not possible to determine which processes are being inhibited without measuring other parameters of viral infection beside latent period and burst size.

Effect of Time of Addition of Chloramphenicol and F-Phe on Progeny Production

In Table I the time effects of the addition of chloramphenicol are compared with those of F-Phe. The temporal and quantitative effects of the two protein inhibitors were quite different. The F-Phe inhibition is similar to that shown in Figure 11.

The early protein synthesis was greatly inhibited by chloramphenicol because addition of the inhibitor between 60 prior to and 10 minutes after infection prevented formation of infective progeny. The steps in viral titer observed with chloramphenicol addition at 15 and 60 minutes suggests that the synthesis of at least two other proteins is being inhibited.

Figure 11

Effect of Time of Addition of F-Phe on Burst Size and Time

Replicate flasks containing E. coli growing in TPG medium were infected at 0 time with 1.2×10^6 PFU/ml MS2 virus. F-Phe (10 μg/ml) was added at the times indicated by the arrows and samples were taken and treated with chloroform at the indicated time intervals. The progeny titer was determined as described in the text. No F-Phe was present in sample △ while F-Phe was added at -60, ◐; 0, ●; 10, ◐; 20, ◐; 30, ◐; and 60,0 minutes relative to time of infection.

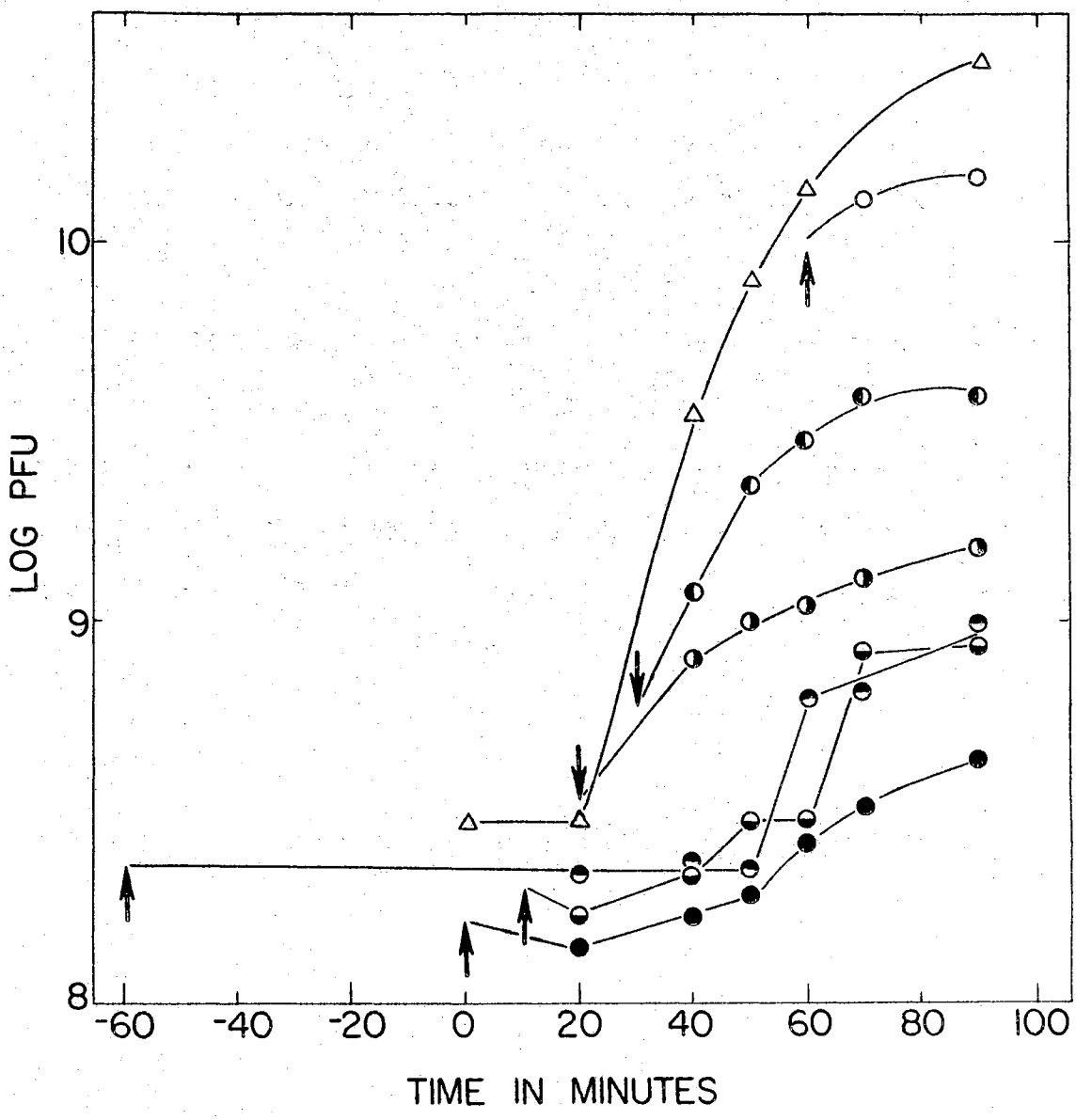

LOG PFU
10
9
8
-60
-40
-20
0
20
40
60
80
100
TIME IN MINUTES

TABLE I

COMPARISON OF THE EFFECT OF TIME OF ADDITION OF CHLORAMPHENICOL AND FLUOROPHENYLALANINE ON PROGENY PRODUCTION

	PFU per ml	
	Inhibitor	
Time of inhibitor addition	Chloramphenicol	F-Phe
minutes		
-60	2.0×10^4	4.0×10^4
-30	2.5×10^4	2.3×10^4
-10	1.8×10^4	1.7×10^4
0, infection	2.2×10^4	9.0×10^3
5	2.0×10^4	1.2×10^4
10	2.4×10^4	1.4×10^4
15	1.2×10^5	1.4×10^4
30	1.2×10^5	6.0×10^4
60	6.0×10^5	2.0×10^5
no inhibitor	2.1×10^6	

At the indicated time intervals 10 μg per ml of chloramphenicol and 10 μg per ml of F-Phe were added to replicate cultures of E. coli growing in TPG medium. Infection was made with 6×10^3 MS2 virus at 0 time and after 60 minutes the cultures were treated with chloroform. Virus titer was determined as described in the text.

Specific Infectivity

Table II shows that a higher specific infectivity (PFU/A_{257}) with normal MS2 than with MS2 grown in the presence of F-Phe. This result is consistent with the possibility that some non-infectious particles are produced. These particles are purified along with the infectious virus.

Effect of Ribonuclease on MS2

If the incorporation of F-Phe occurs into the coat protein of MS2, the structure of the particles might be changed so that their resistance to RNase would be decreased. In vacccina the incorporation of F-Phe produces particles more permeable to RNase and phosphotungstate stain. There was no reduction in the infectivity of F-Phe grown MS2 when incubated in the presence of RNase for up to 8 hours (Table III).

Incorporation of Radioactivity from ^{14}C-F-Phe into MS2

Radioactive F-Phe was added to an E. coli culture 60 minutes prior and at the time of infection with MS2 virus and the virus was purified by differential centrifugation. Table IV shows presumptive evidence that the radioactivity from F-Phe is incorporated into MS2 virus. Uninfected cultures of E. coli were also incubated with ^{14}C-F-Phe and treated by the same procedures. More radioactivity sediments at 12,000 rpm with the uninfected cultures whereas when the preparations were centrifuged at 40,000 rpm more radioactivity was found in the precipitate of the infected cultures. This is what would be expected if virus infection inhibited host protein synthesis with the virus sedimenting at a higher speed of centrifugation. The following experiment better

TABLE II

SPECIFIC INFECTIVITY

Virus type	A_{257}/ml	Titer, PFU/ml	PFU/A_{257}
Normal	15.7	5.1×10^{10}	3.2×10^{9}
F-Phe Grown	0.231	2.2×10^{8}	9.5×10^{8}

Samples of MS2 virus grown in TPG medium and TPG medium + 10 μg/ml F-Phe were diluted and the absorbance was determined at 257 mμ. The titer of virus in the stock solutions was determined as described in the text.

TABLE III

EFFECT OF INCUBATION OF MS2 WITH RIBONUCLEASE

Time of Incubation with Ribonuclease	PFU/ml	
	Normal Phage	F-Phe Grown Phage
0	3.5×10^5	3.5×10^6
20	5×10^5	1×10^6
40	1.4×10^6	1×10^6
90	6×10^5	2.5×10^6
120	6×10^5	1.0×10^6

Normal MS2 virus and phage grown in the presence of 10 μg/ml F-Phe were incubated in TPG with 2.5 μg/ml RNase at 37°. No decrease in titer of control incubation mixtures without RNase occurred under these conditions.

TABLE IV

INCORPORATION OF RADIOACTIVITY FROM ^{14}C F-PHE INTO MS2 PHAGE

Type of Cells	Time of F-Phe Addition, Minutes	12,000 RPM Precipitate	40,000 RPM Precipitate
Infected	-60	1787	329
Uninfected	-60	2214	98
Infected	0	1954	461
Uninfected	0	3002	64

At the time of infection and at 60 minutes prior to infection, 105.6 mμg of ^{14}C F-Phe (0.5 μc) was added to replicate cultures of E. coli. MS2 6 x 10^3 PFU/ml was added at T=0. The culture was quick chilled after 120 minutes, centrifuged at 12,000 RPM for 15 minutes and the precipitate was taken up in one ml of broth and counted as described in the Methods. The supernatant solution was centrifuged at 40,000 rpm for 4 hours and treated as before.

characterizes the radioactivity being incorporated into coat protein.

Sucrose Density Gradient Centrifugation of Radioactive Phage

In Figure 12 MS2 virus grown in the presence of ^{14}C-Phe and ^{3}H-F-Phe in replicate cultures were layered on 5 ml of a linear 5-20% w/v buffered sucrose gradient and were centrifuged. The radioactivity in both types of phage preparation sediment to a similar density position in the gradient which is also the position occupied by MS2 as detected by absorbance at 260 mμ and infectivity. The later data is not shown in Figure 12 but was calculated from Figure 6. The radioactivity is thus probably incorporated into the virus coat.

RNA from MS2

Characterization

Ultraviolet Spectrum of MS2 RNA

The ultraviolet adsorption spectrum of a sample of MS2 RNA prepared by the sodium perchlorate procedure is shown in Figure 13. There is a maximum at 257 mμ and a minimum at 232 mμ. This spectrum is similar to that published by Strauss and Sinsheimer (45) for RNA prepared by the phenol procedure.

Melting Curve of MS2 RNA

MS2 RNA has an ordered structure which is lost on heating (45). The effect of increasing temperature on the relative absorbance of a sample of MS2 RNA is shown in Figure 14. A lower ionic strength accounts for the lower Tm of 62° observed in these experiments than that measured

Figure 12

Sucrose Density Gradient with Radioactive Phage

On a 5 ml linear density gradients of 5-20% w/v sucrose buffered with 0.1 M NaCl, 0.05 M Tris, and 0.01 M EDTA, pH 7.6, was layered 0.3 ml MS2 virus grown in the presence of ^{14}C-Phe, O and ^{3}H-F-Phe, ●. The gradient was centrifuged 3 hours at 35,000 rpm and 4°. Three drop fractions were collected in counting vials and the radioactivity was determined as described in the text.

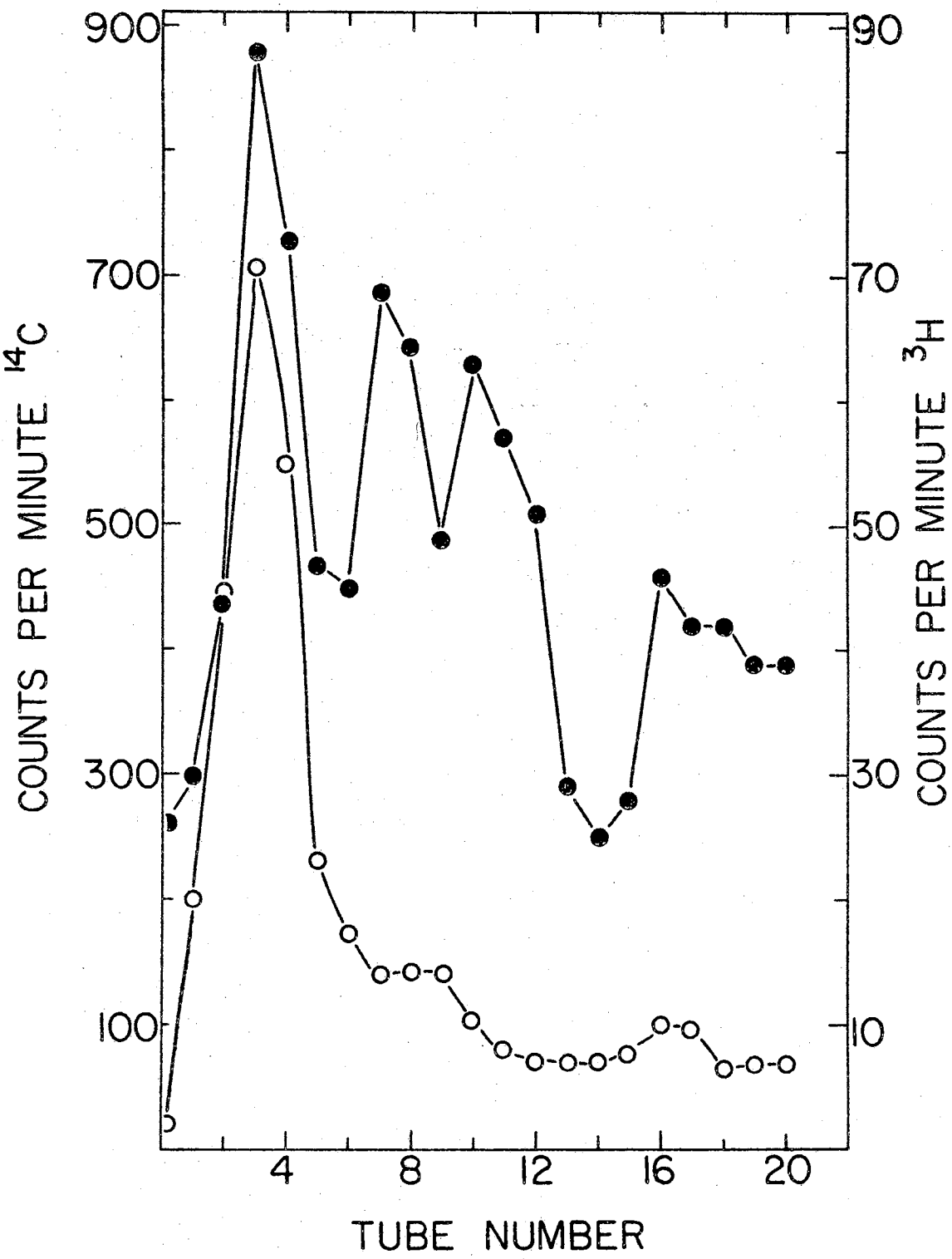

900
700
500
300
100
90
70
50
30
10
COUNTS PER MINUTE ^{14}C
COUNTS PER MINUTE ^{3}H
4
8
12
16
20
TUBE NUMBER

Figure 13

Ultraviolet Absorption Spectrum of MS2 RNA in 0.05 M Tris, pH 7.0 at 25°

Concentration of the RNA was 20 μg/ml.

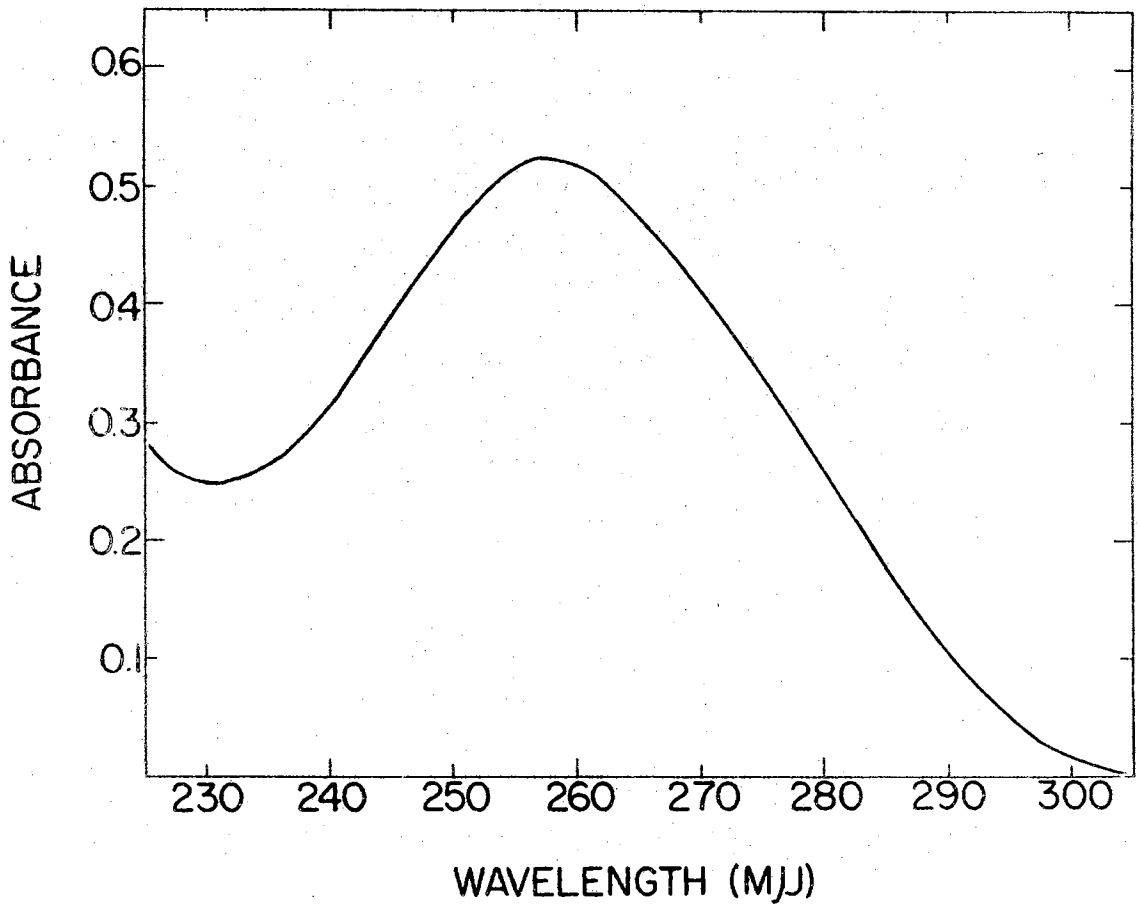
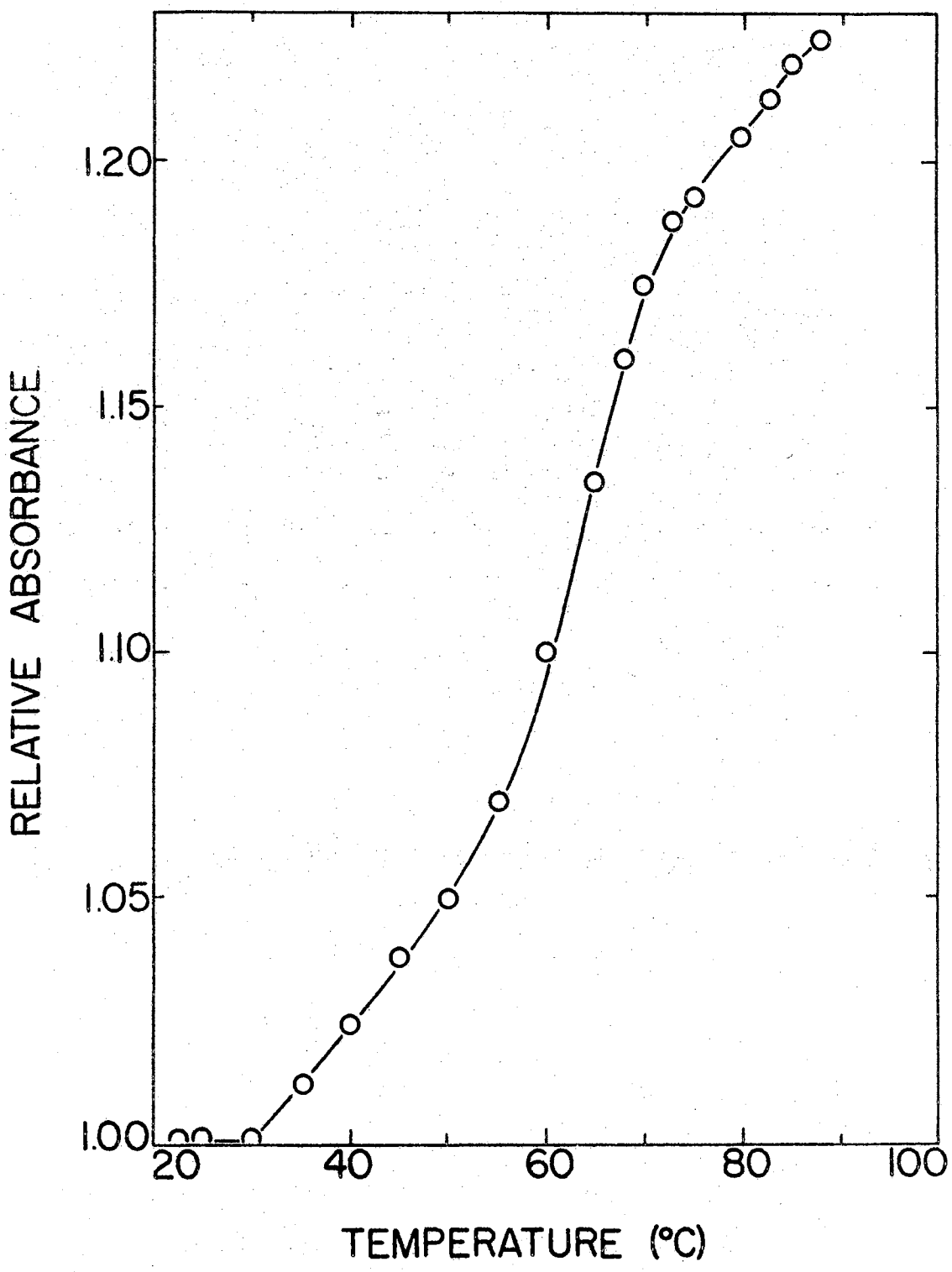

ABSORBANCE
0.6
0.5
0.4
0.3
0.2
0.1
230
240
250
260
270
280
290
300
WAVELENGTH (MJJ)

Figure 14

Melting Curve of MS2 RNA

A sample of MS2 RNA prepared by the sodium perchlorate procedure was diluted in 0.132 M NaCl and 0.01 M tris, pH 7.0 and the variation of absorbance at 257 mμ was determined using a thermostated Gilford-Beckman spectrophotometer. The relative absorbance in the absorbance at the indicated temperature, relative to the absorbance at 23°.


RELATIVE ABSORBANCE
1.20
1.15
1.10
1.05
1.00
20
40
60
80
100
TEMPERATURE (°C)

by Strauss and Sinsheimer (45). The total hyperchromic effect suggests that both samples of RNA have the same degree of ordered structure.

Base Composition

Table V shows the base composition of the MS2 RNA prepared in this study along with that determined by Strauss and Sinsheimer for comparison. Although different analytical procedures were used, the results appear to be comparable.

Sedimentation Pattern

The sedimentation pattern of a sample of MS2 RNA prepared by the sodium perchlorate procedure was obtained using ultraviolet optics and the analytical ultracentrifuge. The densitimeteric trace of a photograph made after 27 minutes sedimentation is shown in Figure 15. Significant heterogeneity is observed from the three distinct sedimenting boundaries. It is possible that the higher temperature of this ultracentrifuge experiment is responsible for the breakdown of the RNA. Strauss and Sinsheimer obtained samples in which 70% of the material sedimented in a fairly sharp boundary while the rest of the material trailed (45).

Function of MS2 RNA as Messenger RNA

When tested in a cell-free protein synthesizing (radioactive amino acid incorporating) system from *E. coli*, MS2 RNA stimulated the incorporation of Phe, F-Phe and radioactivity from a mixture of amino acid as is seen in Table VI. Synthetic poly uridylate is included as a control to establish the capabilities of this system. There is more than a 70-

TABLE V

BASE COMPOSITION OF MS2 RNA

Base	Mole Fraction	
	Ref. (45)	This Study
Guanine	0.275	0.263
Uracil	0.256	0.260
Cytosine	0.245	0.251
Adenine	0.224	0.226

A 400 μg sample of MS2 RNA prepared by the sodium perchlorate procedure described in the text was hydrolyzed by 24 hour incubation in 3 N KOH. The base ratios were determined by the procedure described in the text.

Figure 15

Sedimentation Pattern of MS2 RNA

A sample of MS2 RNA (36 μg/ml) in 0.1 M tris buffer, pH 7.0 was centrifuged in a 12 mm Kel F cell at 56,100 rpm in the AN D rotor of the Spinco Model E Ultracentrifuge at 20°. Photographs were made at 4 or 8 minute intervals using ultraviolet absorption optics and examined with the Beckman Analatrol. The trace of the 27 minute photograph is shown. Sedimentation is from left to right.

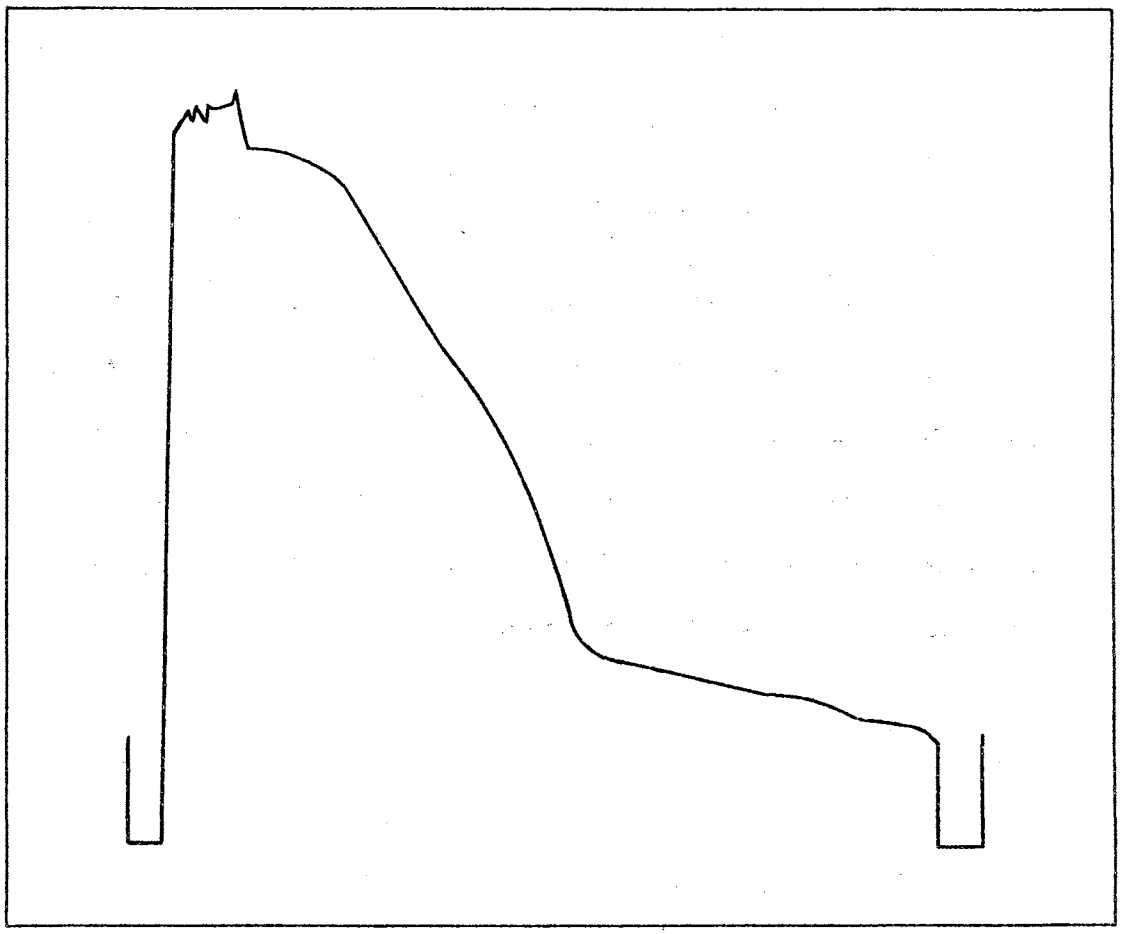


TABLE VI

FUNCTION OF MS2 RNA AS MESSENGER IN E. coli CELL-FREE SYSTEM

Messenger		Additions	Counts per minute incorporated		
			Amino Acid		
Type	μg		Phe	F-Phe	Mix.
	0	complete	789	301	409
Poly U	10	complete	35,936	6,086	1,051
Poly U	50	complete	63,209	11,447	961
Poly U	50	- energy	216	119	320
Poly U	50	+ puromycin	680	373	330
MS-2	10	complete	1,499	426	443
MS-2	50	complete	2,190	508	643
MS-2	50	- energy	199	125	352
MS-2	50	+ puromycin	321	166	318

The complete reaction system contained in 0.25 ml the following in μmoles: Tris pH 7.8, 25; NH_4Cl, 12.5; magnesium acetate, 2.5; β-mercaptoethanol, 1.0; ATP, 0.5; GTP, 0.1; phosphoenolpyruvate, 1.0; 20 amino acid mix without Phe, 0.0025 each; 10 μg of pyruvate kinase; and 2.94 mg of E. coli 30,000 x g supernatant solution. Where indicated 0.02 μ moles of puromycin was added and GTP, ATP, phosphoenolpyruvate and pyruvate kinase were omitted for the-energy samples. Radioactive substrates used were: 0.2 μ C of L-Phe-UL-^{14}C, 1.55 μμ moles; 1.0 μ C of ^{3}H F-Phe, 11 μμ mole; and 0.08 μ C of ^{14}C-amino acid mix from hydrolyzed chorella protein. Messenger RNA was present as indicated. After 30 minutes incubation at 37°, 0.1 ml samples were placed on 2.3 cm Whatman 3MM and dried

TABLE VI (CONTINUED)

in a stream of warm air. The discs were immersed in cold 5% trichloroacetic acid-0.25% sodium tungstate solution and allowed to stand for 10 minutes. Then the discs were treated for 7 minutes with 90° 5% trichloroacetic acid - 0.25% sodium tungstate solution, washed twice with 95% ethyl alcohol, and finally dried with diethyl ether. Radioactivity was determined in Bray's scintillating fluid.

fold stimulation of Phe incorporation by 50 μg poly uridylate and almost a 40-fold stimulation of the incorporation of F-Phe. As expected the system is energy dependent and inhibited by puromycin. MS2 RNA stimulation of incorporation has the same characteristics. The extent of stimulation of incorporation is much less since many amino acids must be incorporated with MS2 RNA as a messenger. However, significant incorporation is observed suggesting that the MS2 RNA is biologically active. The small F-Phe incorporation with the MS2 messenger precludes the use of the cell-free system for the synthesis of F-Phe - containing phage proteins which would be characterized by standard biochemical techniques.

CHAPTER IV

DISCUSSION

The availability of RNA containing bacteriophages makes possible the utilization of natural polycistronic mRNA for protein synthesis in a microbial system.

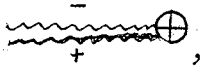
Figure 16 shows a diagramatic scheme patterned after one by Ochoa, Weissman, Borst, Burdon, and Billeter (71) to account for the mechanism of the replication of MS2 replication.

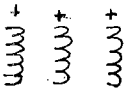
1) Viral RNA is injected into the cell, leaving coat proteins behind.
2) This parental RNA acts as a messenger and combines with ribosomes to form polysomes.
3) The first protein synthesized is RNA replicase (Spiegelman's enzyme) which makes the minus strand and then converts its product with the parental RNA into the double stranded replicative form (72).
4) Then an RNA polymerase is formed. Studies by Ochoa's group (73) indicated the use of RF as a template for synthesis of more viral RNA by a semiconservative mechanism in which the progeny strands displacing parental strands from the duplex is formed.
5) Coat protein subunits are also synthesized on the ribosomes using a portion of the viral RNA as messenger.

Figure 16

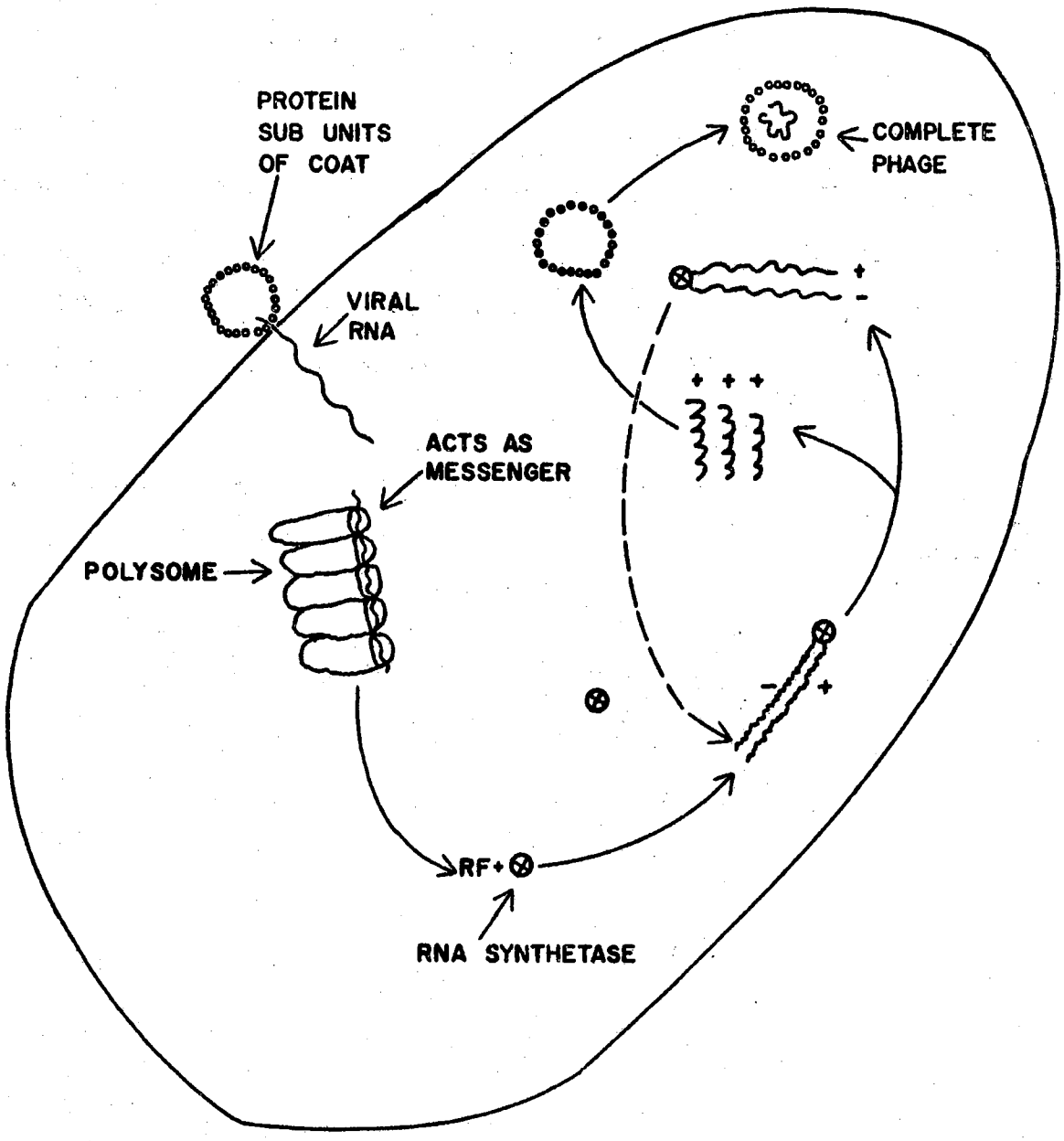
Replication of Bacteriophage MS2

A schematic drawing to account for the mechanism of the replication of an RNA phage which accounts for the four proteins involved in MS2 replication.

RF, replicative form of viral RNA; 

, the RF-RNA synthetase complex; 

, the plus strands of RNA which are then placed inside of the coat proteins; 
, polysome containing anticodons.


PROTEIN
SUB UNITS
OF COAT
COMPLETE
PHAGE
VIRAL
RNA
ACTS AS
MESSENGER
POLYSOME
RF+
RNA SYNTHETASE

6) Finally there is a finishing enzyme required for the conversion of viral RNA into an infective form as evidenced by the effect of chloramphenicol addition after most of the viral RNA has been made, but it is not infective.

In a study of the incorporation of Phe and F-Phe by an E. coli cell-free preparation. Dunn and Leach (75) found that a given amount of sRNA had a greater capacity for Phe than for F-Phe. Using various poly UC polymers they obtained results consistent with the supposition that Phe responds to both UUU and UUC triplets and that F-Phe can be incorporated only by the UUU codewords. Hoskinson and Khorana (76) have separated two Phe sRNA's from yeast and Doctor (77) has obtained two Phe sRNA's from E. coli by counter current distribution.

The selectivity with F-Phe for UUU codewords gives a probe for examining degeneracy. If F-Phe can be incorporated into a protein, it is probable that that phenylalanyl residue was coded for by UUU.

There are three phenylalanyl residues per capsomer of MS2 coat protein (78) and it might be possible to examine these separately by finger printing techniques.

In order to examine the specific early protein whose synthesis of functioning is being inhibited by F-Phe, assays for each of the enzymes and all of the viral precursors and products should be made. At the present state of understanding of the mechanism of MS2 replication this examination is not possible. Even the relationship between Spiegleman's and Ochoa's enzymes is unclear (79) and the process of viral RNA synthesis is more complicated as evidenced by the demonstration of other types of RNA during replication (80).

CHAPTER V

SUMMARY

The presence of F-Phe in the medium during growth of the RNA bacteriophage results in an increased latent period and a decreased burst size. A study of the effect of time of addition revealed that F-Phe is most effective in inhibiting the synthesis or function of one of the early proteins; however, there is also inhibition of one or more of the late proteins. With chloramphenicol there are at least three different inhibitions observed and these do not correspond to those of F-Phe. While the specific infectivity of F-Phe grown virus is slightly decreased, there is no measurable sensitization to RNase treatment. Radioactivity from F-Phe is apparently incorporated into the coat protein of MS2 virus and RNA isolated from MS2 functions as messenger RNA in an E. coli cell-free system. The approaches described are preparatory to examining Phe and F-Phe degeneracy in the MS2 coat protein.

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