Subjugation of Host Macromolecular Synthesis by R17 Bacteriophage During Infection of *Escherichia coli*

by

John Walter Scott, Jr.

A THESIS
Presented to the Department of Microbiology
and the Graduate Division of the University of Oregon Medical School
in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

June 1975

APPROVED:

[redacted]

Professor In Charge of Thesis

[redacted]

Chairman, Graduate Council

TABLE OF CONTENTS

	Page
Title page	i
Approval page	ii
Table of contents	iii
List of figures	v
List of tables	vii
INTRODUCTION	
Statement of the problem	1
Literature review	2
1. Infectious particle	2
2. Attachment and entry of the viral genome	4
3. Viral RNA replication	5
4. Phage specific protein synthesis	7
5. Inhibition of host macromolecular synthesis	9
A. Inhibition of host nucleic acid synthesis	10
B. Inhibition of host protein synthesis	11
MATERIALS AND METHODS	
Materials	14
Media	14
Bacteria	15
Standard agar overlay technique	16
Bacteriophages	17
Pulse-labeling procedure	20
Polyribosomes	20
Beta-galactosidase	22
Hybridization	23
RESULTS	
Lytic infection of *Escherichia coli* with R17 bacteriophage	26
Continuous labeling experiments	26

Page

Pulse labeling experiments 26
Verification of early inhibition 33
1. Dilution of labeled amino acids 33
2. Inhibition of amino acid transport 34
3. Inhibition of polypeptide release 37
Induction and recovery from early inhibition 39
1. Early inhibition is temperature independent 39
2. Massive addition of phage 41
3. Repeated infection with R17 phage suspension 41
4. Inoculation with phage particles which can not adsorb to the F pili 43
Identification of the agent responsible for early inhibition 46
1. R17 bacteriophage 47
2. Host contaminant 51
3. Tryptone broth and TCG1-t synthetic medium 53
Late inhibition 56
Messenger RNA specific for β-galactosidase 57
Synthesis of β-galactosidase enzyme 59
Functional half-life of β-galactosidase m-RNA 59
Inhibition of Qβ phage production 62

DISCUSSION

Early inhibition 68
1. Confirmation of early inhibition 68
2. Early inhibition is not a function of viral infection 70
3. Possible mechanism for early inhibition 73
Late inhibition 74
1. Inhibition of host macromolecular synthesis 74
2. Inhibition of Qβ phage replication 76

SUMMARY AND CONCLUSION 79

REFERENCES 81

LIST OF FIGURES

Figure		Page
1	Lysis of infected cells	27
2	Early inhibition of protein synthesis upon infection with R17 bacteriophage, continuous labeling	28
3	Early inhibition of protein synthesis upon infection with R17 bacteriophage, pulse labeling	30
4	Inhibition of uptake of ^{14}C amino acids during early inhibition	31
5	Examination of polyribosome profiles during early inhibition	35
6	Alteration of polyribosome profiles during amino starvation	36
7	Release of polypeptides from polysomes during early inhibition	38
8	The influence of temperature on early inhibition and recovery	40
9	Infection of *E. coli* with R17 bacteriophage (300 PFU/cell)	42
10	Repeated infection of *E. coli* with R17 bacteriophage	44
11	Infection of *E. coli* with R17 phage purified on sucrose gradients	48
12	Effect of early inhibition on the eclipse of R17 bacteriophage	52
13	Infection of *E. coli* with a crude lysate of R17 bacteriophage	54
14	Early inhibition of protein synthesis upon inoculation with tryptone broth and TCG1-t medium	55

Figure		Page
15	Detection of messenger RNA specific for β-galactosidase in R17 phage infected cells	58
16	Beta-galactosidase synthesis in *E. coli* during infection with various amber mutants of R17 bacteriophage	60
17	Half-life of functional m-RNA in *E. coli* 3000 infected with R17 bacteriophage	63
18	Inhibition of final Qβ phage titer by superinfection with am B22	64
19	Protection of *E. coli* S26 cells from Qβ phage infection by simultaneous infection with am C16	67

LIST OF TABLES

Table		Page
1	Properties of RNA bacteriophages	3
2	Early inhibition in F- culture or incomplete particles	45
3	Factors causing early inhibition of host protein synthesis	49
4	Virus production of *E. coli* 3000 infected with R17 phage	50
5	Beta-galactosidase synthesis in *E. coli* during infection with various amber mutants of R17 bacteriophage	61
6	The Qβ phage-burst size of Qβ infected cells superinfected with amber mutants of R17 phage	66

INTRODUCTION

Statement of the Problem

The objective of this research has been to investigate R17 phage control of host macromolecular synthesis. Studies with both animal viruses and bacteriophages have shown that various viral infections may inhibit host DNA, RNA and/or protein synthesis. For example, infection with Poliovirus inhibits host protein synthesis by the production of double-stranded viral RNA (23,52). In a bacterial system, infection of *Escherichia coli* with T4 bacteriophage inhibits host DNA, RNA and protein synthesis (72), the host DNA is degraded (10,106) and RNA synthesis is terminated within 2 min post infection (2,60).

In 1968 Watanabe (108,109) demonstrated that infection of *E. coli* with an RNA bacteriophage resulted in "early and late" stages of inhibition of host protein synthesis. The primary questions asked in this thesis were:

1. What event of phage infection was responsible for early inhibition?
2. What influence does early inhibition exert on the production of virus in infected cells?
3. During infection of *E. coli* with R17 bacteriophage does any host transcription and translation occur during late inhibition?

4. Was there some property unique to R17 bacteriophage which allowed replication of the R17 phage genome during late inhibition, or could a similar phage be replicated at this time?

Literature Review

1. Infectious Particle

Although the RNA bacteriophages of *E. coli* have been separated into three serological groups (111,112), their physical and chemical properties are very similar (92). The infectious particle consists of a single-stranded ribonucleic acid genome which codes for three phage-specific proteins (4.,42,45,46). The RNA genome is encased in an icosahedran composed of coat protein (25). Each infectious particle contains one molecule of maturation protein (M.W. 35-40,000) (47,85, 93).

The temperature sensitive and amber mutants of RNA phages belong to one of three complementation groups (46,101). The amber mutants can be replicated in an amber suppressor strain such as *E. coli* S26RIE which inserts serine with 65% efficiency (94). Group A (am A31) has been shown to code for the maturation protein; Group C (am C16) codes for the RNA-dependent RNA polymerase (synthetase) which is synthesized after entry of the viral genome into the host cell and is not found in association with the infectious particle. Group B, am B22, is a non-polar coat protein mutant which allows large amounts of synthetase to

Table 1

Properties of RNA Bacteriophages

Serological groups (112,92)	Molecular Weight 10^6		Density (g/ml)
	RNA	Protein	
Group I			
R17	1.3 (9)		1.33
MS2	1.0	3.6	1.46 (111)
R23			
M12		3.6 (33)	
f2			
Group II			
Qβ	1.5 (9)	4.2	1.46 (111)
GA			1.44 (111)
MC			
SB			
Group III			
fr		4.1	
f4			
β			

be translated. The mutant am B24 is a polar mutant and restricts the translation of viral synthetase.

2. Attachment and Entry of the Viral Genome

Although it was known from the original description that RNA bacteriophages were male specific (68), the actual association of these phages with the F pili was not demonstrated until 1964 (18). Valentine and Strand (103) then showed that phage adsorbed to purified F pili.

Paranchych has divided the infectious process into the following events:

1. Association of the phage particle with the F pili
2. Alteration of the maturation protein
3. Entry of the viral genome into the host cell
4. Replication

At sufficient ionic strength (19) the association of the phage particle is a temperature independent process (80). The phage particles possessing a maturation protein can attach to the F pili with an association constant of 1.37 (80). At 4 C, attachment is reversible and does not alter the infective particle. However, at 37 C the host provides energy (10 kcal/mole) and the maturation protein is irreversibly altered (20). It has been shown that the alteration of the maturation protein obeys first order kinetics (78). After the maturation protein of the attached virus has been altered, the viral genome and at

least some of the maturation protein enter the host cell where replication begins (61,63). Then particles, deficient in maturation protein (78), elute from the F pili (80).

3. Viral RNA Replication

The replication of viral RNA is independent of DNA synthesis (16, 21,88) and the host DNA-dependent-RNA polymerase (44). Thus, the viral RNA must direct the synthesis of its own progeny RNA molecule. A probable mechanism of RNA replication was suggested by the discovery that ϕ174 single-stranded phage DNA entered a double-stranded "replicative form" (89). Subsequent studies of *E. coli* infected with RNA bacteriophage yielded a linear double-stranded "replicative form" of viral RNA sedimenting at 16S. Although this replicative form was not infectious by itself, infectious viral RNA was obtained upon heat denaturation (57,113).

Further study revealed the presence of another viral specific RNA, the "replicative intermediate" (3,29). Although this form of the viral RNA was also a linear double-stranded molecule, sedimentation through a sucrose gradient revealed a heterogenous profile of viral RNA sedimenting between 16-23S (26,29,57). Upon treatment of the replicative intermediate with ribonuclease only a homogenous 14S peak was observed (27). Thus it was proposed that the replicative intermediate consisted of a double-stranded core with one or more nascent single-

stranded phage RNA attached. Kelley and Sinsheimer (58) have shown that conservative and semi-conservative replication occurs, and both the positive and minus strands can serve as templates (59).

Synthesis of the complementary minus strand (56) yields the replicative form (30,69,75) which is the precursor of the replicative intermediate. Pulse-labeling experiments show label entering the heterogenous profile of the replicative intermediate which can be chased into the 27S phage RNA (26). The completed RNA strands are incorporated into virus particles 10-15 min after leaving the replicative intermediate (17,24).

Infection of *E. coli* with a Qβ phage produces a viral specific RNA-synthesizing enzyme, which has been isolated and purified (5,115). The Qβ synthetase has a molecular weight of 150,000 and is composed of four subunits only one of which is coded for by the phage genome (65,000) (64). Two of these host factors have been identified as the elongation factors EF-tu and EF-ts (8); the third component is the translation control factor i (40).

Viral synthetase is very specific and only accepts its own RNA (43). Evidence that this enzyme was replicating infectious RNA molecules was provided by Spiegelman et al. (91) and Pace and Spiegelman (76). A small amount of Qβ RNA was added to the first of a series of tubes containing the Qβ synthetase reaction mixture. After incubation a small aliquot of the first tube was transferred to the second tube, etc. After 15 successive transfers the amount of parental RNA was less

than one molecule per tube and considerably less than the 1.4×10^{12} new strands which had been produced by the enzyme.

One of the difficulties in accepting the replicative intermediate as the precursor of viral RNA synthesis has been the fact that the purified replicative form and intermediate are inactive as templates *in vitro* (70). Weissmann has suggested that the replicative form and replicative intermediates do not exist in the infected cell. Ribonuclease resistant structures are not detectable during the synthesis of Qβ RNA *in vitro* (114). Also, phenol extraction of the *in vitro* product generates polynucleotides with the properties of the replicative form and intermediate. Thus it is suggested that the template and products are single-stranded, except for a brief region of hydrogen bonding near the growing point. Recently, this point has been supported by the studies of Thach and Thach (100), who examined the replication of R17 bacteriophage with an electron microscope. The double-stranded viral replicating complexes described by Granboulan and Franklin (39) were only observed after treatment with phenol. If the viral nucleic acid was extracted with lysozyme-EDTA, only polysomes and polysomal aggregates were observed.

4. Phage Specific Protein Synthesis

After entry of the viral genome into the host cell, the plus strand viral RNA is translated and found associated with the polysomes

(28,36); the minus strand does not code for viral proteins (54). At 25 min post infection the replicative intermediate was found in the heavy polysomes, hexamers or larger (35,48,82,102). The single-stranded viral RNA predominated in the smaller polyribosomes (35).

Transcription of the R17 RNA-dependent-RNA polymerase (synthetase) begins 5-15 min after the viral genome enters the host cell (32). The rate of synthesis of the viral synthetase increases until 25 min post infection, when further synthesis is either discontinued or drastically reduced (32,95). If the cell is infected with a temperature-sensitive synthetase mutant at the non-permissive temperature, the viral synthetase will be transcribed from the parental genome. Since coat and maturation proteins are not detected, it appears that they are transcribed from progeny viral RNA (62).

Coat protein is an important factor in controlling phage replication. During infection of *E. coli* by wild-type R17 phage, coat protein synthesis begins after the appearance of synthetase and continues to be translated at a maximum rate throughout phage replication (71,105). If a non-permissive strain of *E. coli* is infected with a non-polar coat protein mutant, excessive amounts of synthetase will be produced (41, 46,66,67). Conversely, infection with a polar mutant results in little or no synthetase production. From the position of the nonsense codon, it appears that part of the coat protein must be translated before synthetase production begins. The presence of coat protein inhibits

synthetase production; thus, in the absence of functional coat protein, synthetase production was not inhibited during the late part of infection. This was verified in an *in vitro* protein synthesizing system in which it was shown that the addition of coat protein inhibited the translation of synthetase (22,84,95,96,97,98).

The synthesis of maturation protein begins later than synthetase and continues later into the infectious cycle. Since maturation protein is transcribed from progeny viral RNA, the presence of viable functional synthetase is essential for the production of maturation protein (71). However, there is no alteration in maturation protein synthesis when large amounts of synthetase are produced by infection of a non-permissive strain of *E. coli* with a non-polar coat protein mutant. Thus, translation of maturation protein appears to be independent of the other two genes. Also, maturation protein mutants do not alter the synthesis of synthetase or coat proteins.

5. Inhibition of Host Macromolecular Synthesis

The subjugation of *E. coli* host macromolecular synthesis by infection with R17 phage was demonstrated by using high resolution autoradiographic techniques (39). At 45 min post infection with R17 bacteriophage, DNA synthesis was strongly inhibited. At 20 min post infection, RNA synthesis occurred predominately in the cytoplasm instead of the nucleoid, and by 45 min post infection, labeled RNA was localized

in the polar regions. At 25 min post infection, protein synthesis was depressed and shifted from a generalized cytoplasmic incorporation of labeled amino acids to a polar focus.

A. Inhibition of Host Nucleic Acid Synthesis

The inhibition of DNA synthesis was first demonstrated by Paranchych and Graham (79) who showed that at 60 min post infection with R17 phage, DNA synthesis was inhibited while both RNA and protein synthesis continued. This inhibition of DNA synthesis was confirmed by Bishop (6) and Granboulan and Franklin (39). Watanabe (110) has shown that infection with the RNA phages Qβ and f2 did not inhibit DNA synthesis to the extent observed with R23 phage.

In addition to the inhibition of DNA synthesis, Paranchych's laboratory observed that r-RNA synthesis was inhibited during the first 15 min of infection with R17 bacteriophage (24,50). This finding was verified by Bishop (6,7) who studied the infection of *E. coli* by ZIK/1 bacteriophage and by the work of Watanabe (110) with R23 bacteriophage. In further studies with amber mutants of R17 phage, Spangler and Iglewski (90) have shown that production of viral synthetase is essential for the inhibition of r-RNA synthesis.

Work with ^{14}C guanine (50) has shown a 30% inhibition of t-RNA synthesis at 30 min post infection with R17 phage. This inhibition has been verified by Bishop (7), Igarashi (53) and Watanabe (110).

Hung and Overby (51) have demonstrated that t-RNA extracted from Qβ phage infected cells showed a decreased ability to support translation of polycytidylic acid. This alteration of prolyl-t RNA was specific for Qβ phage and was not observed with cells infected with MS2 phage.

B. Inhibition of Host Protein Synthesis

Sugiyama and Stone (99) have shown that during infection with MS2 phage there is a shift from host protein synthesis to viral specific proteins. The decrease in host protein synthesis was followed by observing the accumulation of β-galactosidase enzyme or by examining the profiles of labeled proteins on polyacrylamide gels. By following the synthesis of the host enzyme, β-galactosidase, the Watanabe's (108, 109) demonstrated two stages of inhibition of host protein synthesis. The first stage of inhibition, "early inhibition," was encountered immediately after inoculating the culture with the phage preparation. Studies with UV irradiated phage showed that this inhibition was independent of the expression of the viral genome. At 10 min post infection early inhibition had been relieved and the infected culture accumulated β-galactosidase enzyme at the same rate observed in the control culture. By observing early inhibition with ^{14}C amino acid pulse labeling studies, Yamazaki (116) demonstrated that early inhibition of host protein synthesis was the result of an inhibition of amino acid transport into the host cell, and independent of the entry of the

viral genome into the host cell. Thus it was assumed that early inhibition was the result of an early event in viral infection. Therefore Paranchych (20) suggested that amino acid transport and the alteration of the maturation protein competed for a common energy intermediate. The alteration of the maturation protein in the phage would deplete the host of energy required for amino acid transport into the host cell.

The second phase of inhibition of host protein synthesis, "late inhibition," occurred at 25-30 min post infection when the synthesis of β-galactosidase was either discontinued or drastically reduced. Late inhibition was also observed upon infection of constitutive mutants of *E. coli*. Further, at 25 min post infection a decrease in the heavy polyribosome region can be observed; as infection proceeds the decrease in the heavy polyribosomes becomes more pronounced and there is an increase in the monomer, dimer and trimer region (48). This alteration in the polyribosome profile was also dependent on the production of viral synthetase (82).

By using *E. coli* cells grown in a minimal medium, it was possible to inhibit host protein synthesis immediately after the addition of R17 phage (53). Since r-RNA synthesis was not inhibited until 30 min post infection, it appears that host protein synthesis is the primary site of inhibition by R17 phage infection. Also, with the use of a temperature sensitive mutant of the viral synthetase Igarashi (53) has demonstrated the inhibition of host protein synthesis without the production

of viral RNA. Thus, translation of the R17 synthetase polypeptide rather than viral RNA was involved in late inhibition.

MATERIALS AND METHODS

Materials

Omnifluor scintillation fluid and ^{14}C amino acid mixture were purchased from New England Nuclear, ^{3}H uridine and sucrose (enzymatic grade) from Schwarz/Mann, Orangeburg, New York. With the exception of the hybridization on nitrocellulose filters (type B6, Schleicher and Schnell; Keene, New Hampshire), all filtrations were done on Millipore filters type HA purchased from the Millipore Corporation, Bedford, Mass. ONGP (o-nitro-phenyl-β-D-galactoside), IPTG (isopropyl-β-D-thiogalactopyranoside) and calf thymus DNA were purchased from Sigma Chemical Company, St. Louis, Mo. Petri dishes (100 x 15 mm) were obtained from Falcon Plastics, Oxnard, Calif.

Media

TCG1-t medium was a modification of the TCG1 medium described by Franklin (36) which was supplemented with thiamine: NaCl 0.6 g, KCl 8 g, NH_4Cl 1.1 g, $MgCl_2 \cdot 6H_2O$ 0.2 g, KH_2PO_4 0.023 g, tris (hydroxymethyl)-aminomethane 1.66 g, tris (hydroxymethyl) aminomethane hydrochloride 5.72 g, and casamino acids 1.5 g per liter. After autoclaving $CaCl_2$ .55 g, $FeCl_3 \cdot 6H_2O$ 1 µg, sodium pyruvate 0.8 g, thiamine hydrochloride 0.01 g, and glycerol 0.2% were added.

Mineral salts-glucose medium was described by Lichstein and Oginsky (65): KH_2PO_4 1 g, K_2HPO_4 1 g, NaCl 1 g, $MgSO_4 \cdot 7H_2O$ 0.7 g, $(NH_4)_2SO_4$ 4 g, and sodium citrate$\cdot 2H_2O$ 0.5 g; after autoclaving 5 g glucose was added.

Tryptone broth was used as described by Loeb and Zinder (68): 10 g Bacto-tryptone, 1 g Bacto-yeast extract, 8 g NaCl; after autoclaving 1 g glucose and 0.005 M $CaCl_2$ were added.

Agar was prepared by adding Bacto-agar, 8 g per liter for top agar or 10 g per liter for bottom agar, to a liter of tryptone broth before autoclaving. Bottom agar was allowed to cool to 47 C before $CaCl_2$ and glucose were added. Petri plates were made by dispensing 15 ml of bottom agar into a 100 x 15 mm petri dish. Top agar was stored without $CaCl_2$ or glucose at room temperature.

Bacteria

Escherichia coli 3000 is an Hfr strain which was obtained from R. Sinsheimer of the California Institute of Technology. *E. coli* S26RIE and S26 which are permissive and non-permissive for amber mutants of R17 bacteriophage were generously provided by M. Capecchi of Harvard University. *E. coli* CA5004 which is lysogenic for $\lambda CI_{857} h^{80}$ plus $\lambda CI_{857} lac^+$ a defective $\phi 80$ phage was a generous gift of D. Kennell of Washington University School of Medicine, St. Louis, Mo. *E. coli* AB531 (F-) was originally obtained from V. Bryson of Rutgers

University, New Brunswick, New Jersey. *Salmonella typhimurium* LT2, M4, *fla* 52, was graciously provided by T. M. Joys of the University of Oregon Medical School, Portland, Oregon.

Standard Agar Overlay Technique

1. Phage Titer

   A. Preparation of top agar: Top agar was melted and cooled to 45 C before adding $CaCl_2$ and glucose.

   B. *E. coli* cells: An *E. coli* culture was grown to 10^8 cells/ml in tryptone broth in a shaking water bath at 37 C.

   C. Plaquing: The number of infective particles was determined by making a series of 1/10 dilutions in tryptone broth. Aliquots of phage (100 µl) were taken from each dilution, mixed with 0.2 ml of an *E. coli* culture and 2 ml of the prepared top agar, then spread on top of an agar plate. After the agar solidified the petri dishes were incubated at 37 C.

2. Assay for Infected Cells

The number of infected cells was determined by diluting 10 µl of an infected culture in 10 ml of tryptone broth at 4 C. The cells were collected by centrifugation (10,000 g for 10 min at 5 C) and resuspended in 10 ml of cold tryptone broth. The washed cells were then serially diluted and plaqued as described in the previous section (1).

Bacteriophages

R17 bacteriophage was originally provided by M. Capecchi of Harvard University. RNA bacteriophages were purified according to the following procedure adapted from Vasquez, Granboulan and Franklin (104). Crude lysates were obtained by infecting *E. coli* 3000 (5 PFU/cell) grown in tryptone broth in a shaking water bath at 37 C. After a 3 hr incubation, ammonium sulfate (350 g per liter) was added to the crude lysate and stirred overnight at 4 C. The precipitate was collected by centrifugation at 10,000 g for 20 min. The pellet was resuspended in 50 ml of ST buffer (0.1 M NaCl, 0.05 M Tris, pH 7.6 at 25 C) and cell debris was removed by centrifugation at 10,000 g for 30 min. The phage was pelleted by centrifugation in a Beckman type 30 rotor at 29,000 rpm for 3 hr. The pellet was resuspended in 5 ml of ST buffer containing 1 mM MgCl and treated with ribonuclease (5 µg/ml) and deoxyribonuclease (5 µg/ml) for 30 min at 37 C. Following a final 30 min treatment with pronase (25 µg/ml), the phage was again pelleted by centrifugation and resuspended in ST buffer. The virus was banded in a CsCl gradient (0.625 g/ml) by centrifugation to equilibrium for 48 hr at 12 C. The virus was twice dialyzed against 100 vol of ST buffer at 4 C and suspended in either tryptone broth (Yamazaki, personal communication) or phosphate buffer (0.1 M sodium phosphate, pH 7.2 at 25 C). The final titer of virus suspended in tryptone broth was

adjusted to 1.5 x 10^{12} PFU/ml for storage at 4 C. Before using the virus in an experiment, the phage was diluted to 3 x 10^{11} PFU/ml with fresh TCG1-t medium (R17 phage suspension).

The amber mutants of R17 bacteriophage (am A31, am B22, am B24 and am C16) were obtained from R. Kamen of Harvard University. Mutants were replicated by using the techniques of Tooze and Weber (101). The mutant stock was plated on *E. coli* S26RIE by using the agar overlay technique (1). One to ten small plaques, which suggested the presence of mutant phage, were isolated and used to inoculate 20 ml of *E. coli* S26RIE culture (grown to 2 x 10^8 cells/ml in tryptone broth) which was incubated in a shaking water bath for 2.5 hr. Reversion to wild-type phage was checked by plaque assay on the non-permissive strain of *E. coli* S26.

Viral particles deficient in maturation protein were obtained by replicating am A31 in *E. coli* 3000 (2 x 10^8 cells/ml in tryptone broth). Cell debris was removed by centrifugation at 10,000 g for 10 min, then the phage particles were pelleted by centrifugation in a Beckman type 30 rotor at 29,000 rpm at 12 C for 190 min. Finally, the incomplete particles were banded in a CsCl gradient (0.625 g/ml) by centrifugation in a Beckman SW 39 rotor at 35,000 rpm at 12 C for 48 hr. The incomplete particles were twice dialyzed against 100 vol of ST buffer and suspended in tryptone broth.

The defective lysogen $\phi 80$ *plac* phage was prepared from a culture of *E. coli* CA5004 grown to 2 x 10^8 cells/ml in tryptone broth at 30 C in a shaking water bath. The lytic cycle of the lysogenic phage was induced by heating to 40 C for 10 min, then incubated at 37 C for 4 hr. The phage titer was determined with the standard agar overlay technique using *E. coli* 3000 as an indicator. Phage was purified (13) by adding Carbowax 6000 (0.0709 g/ml), dextran sulfate (0.002 g/ml) and NaCl (0.0199 g/ml) to the lysate and allowed to stand overnight at 4 C. After decanting the supernatant, the viscuous sediment was centrifuged at 1,000 g for 15 min. The interphase was collected and resuspended in 1% dextran sulfate. The dextran sulfate was precipitated by adding 0.15 ml of 3 M KCl/ml. After 2 hr at 40 C the precipitate was removed by centrifugation at 1,000 g for 10 min. The Mg^{++} concentration was adjusted to 1 mM with Mg acetate and treated with DNase (25 µg/ml) and RNase (25 µg/ml) for 30 min at 37 C or until viscosity decreased. Cell debris was removed by centrifugation at 14,000 g for 10 min then the phage was pelleted by centrifugation at 21,000 g for 3 hr at 12 C. The phage was then centrifuged to equilibrium in CsCl (0.825 g/ml, 48 hr at 35,000 rpm at 12 C) in a Beckman SW 39 rotor. The two phage bands were collected by slowly removing the CsCl from the top of the gradient with a Pasteur pipet (60). The phage was dialyzed for 2 days against four changes of 200 vol of 0.01X SSC (Standard Sodium Citrate buffer: NaCl 8.766 g, Na Citrate 4.411 g per liter).

Pulse-Labeling Procedure

Control and test cultures of *E. coli* 3000 were grown to 2 x 10^8 cells/ml in TCG1-t medium. Before and after infection, multiple samples were taken from each culture and pulse-labeled with a ^{14}C amino acid mixture at 1 μCi/ml for 1.5 min. Incorporation was terminated with trichloroacetic acid (TCA) 10% final concentration, and the samples were heated at 90 C for 15 min; precipitates were collected on Millipore filters and washed with 5% TCA and 95% ethanol. The filters were dried at room temperature and suspended in Omnifluor-toluene scintillation fluid (4 g Omnifluor per liter of toluene). Radioactivity was counted in a Beckman LS-200 B liquid scintillation counter.

Polyribosomes

The method used to obtain polyribosomes was a modification of the procedure originally described by Hotham-Iglewski and Franklin (48). Polyribosomes were extracted from *E. coli* 3000 grown to 2 x 10^8 cells/ml in TCG1-t medium in a shaking water bath at 37 C. Before collecting the cells by centrifugation at 10,000 g for 5 min, they were treated with chloramphenicol (200 μg/ml), Na Azide (5 mM) and iced TCG1-t medium. All further procedures were done at 4 C. The cells were washed once with 20% tris-sucrose (20% sucrose - ribonuclease free, 0.1 M tris pH 7.7 at 25 C) then resuspended in 1.8 ml of 20% tris-sucrose. Spheroplasts were made by treating the cells with 0.5 mg

lysozyme (20 mg lysozyme dissolved in 5 ml of 20% tris-sucrose) and 0.5 mM ethylenedinitrilo-tetraacetic acid (EDTA) for 4 min. The lysozyme reaction was stopped by adding 0.01 M $MgSO_4$ and spheroplasts were collected by centrifugation at 10,000 g for 5 min. Spheroplasts were lysed when suspended in TMS buffer (0.005 M tris, pH 7.4 at 5 C, 0.06 M NaCl and 0.01 M $MgSO_4$). The lysates were treated with deoxyribonuclease (1 µg/ml) for 1 min and the brij 58 for an additional minute. Cell debris was removed by centrifugation at 12,000 g for 5 min and polyribosomes were sedimented through 10-40% sucrose gradients at 40,000 rpm in an SW 41 rotor for 70 min at 5 C (no brake). Optical density patterns were obtained by continuous monitoring of the gradient at 260 nm with a Beckman spectrophotometer containing a flow-through cell.

Labeled polyribosomes were obtained from an *E. coli* 3000 culture grown in TCG1-t medium. The cells were harvested by centrifugation at 10,000 g for 5 min then resuspended in the original volume of fresh TCG1-t medium containing 1/100 the normal casamino acid concentration. After incubating at 37 C for 10 min in a shaking water bath, the cultures were pulse-labeled with ^{14}C amino acid mixture (0.1 µCi/ml) for 1 min and then chased for 2 min with a hundredfold excess of casamino acids. At the end of the pulse or pulse-chase procedures, incorporation of labeled amino acids was terminated by adding iced TCG1-t medium, chloramphenicol (200 µg/ml) and 0.005 M Na Azide.

Polyribosomes were extracted and sedimented through 10-40% sucrose gradients as described previously. After fractionating the gradients, the incorporation of label into hot TCA precipitates was determined.

Beta-galactosidase

An *E. coli* 3000 culture grown in TCG1-t medium was induced to produce β-galactosidase by adding IPTG (5×10^{-4} M). At various times after induction samples were removed and suspended in cold tryptone broth (4 C) with chloramphenicol (100 µg/ml). Cells were collected by centrifugation at 10,000 g for 5 min and resuspended in 1 ml of STE buffer. The cells were then lysed upon treatment with lysozyme (0.5 mg/ml for 30 minutes at 28 C) (36). The substrate, ONPG (1 mM final concentration), was added and incubated at 28 C until am amber color appeared. The reaction was terminated with Na_2CO_3 (0.1 M final concentration) and cell debris was removed by centrifugation. The optical density was determined at 420 nm. One enzymatic unit produced 1 mµ-mole per ml o-nitrophenol per minute at 28 C, pH 7.0; 1 mµ-mole/ml o-nitrophenol has the optical density of 0.0075 at 420 nm in a 10 mm light path (81).

If the functional half-life was to be observed, the synthesis of the specific messenger was terminated by washing the cells on Millipore filters and resuspending them in an inducer-free medium.

Hybridization

The DNA-RNA hybridization procedure was a modification of the method developed by Gillespie and Spiegelman (34) which has been described by Kennell (60, and personal communications).

1. Preparation of Labeled RNA

E. coli 3000 cells (2×10^8 cells/ml in TCG1-t medium) were induced for β-galactosidase enzyme production with IPTG for 5 min before labeling with ^3H uridine (10 μCi/ml). After 30 sec, the incorporation of label was terminated with iced TCG1-t medium and chloramphenicol (100 μg/ml). The cells were collected by centrifugation (10,000 g for 5 min) and resuspended in 10 ml of ST buffer. The nucleic acid was extracted with 2% SDS and an equal volume of freshly distilled phenol saturated with ST buffer, pH adjusted to 7.2. After shaking for 10 min, samples were centrifuged at 10,000 g for 5 min, nucleic acid was in the aqueous phase. Extractions with buffer-saturated phenol were repeated until the protein interphase was eliminated (87). The nucleic acid was precipitated from the aqueous phase by adjusting the NaCl concentration to 0.25 M and adding 2 vol of absolute ethanol. After storage at -20 C for 2 hr, the precipitate was collected by centrifugation at 12,000 g for 15 min at 5 C and resuspended in ST buffer supplemented with Mg^{++} acetate (10 mM). DNA was removed by treatment with DNase (5 μg/ml) for 30 min at 37 C. DNase was removed

by another phenol extraction and ethanol precipitation. Finally the labeled precipitated RNA was resuspended in 2X SSC (250 μg/ml) and stored at -20 C until hybridization.

2. Preparation of DNA Filters

Purified ϕ80 *plac* phage was suspended in ST buffer and phenol extracted with freshly distilled phenol saturated with ST buffer and the pH was adjusted to 8.2 with sodium hydroxide. The aqueous phase was dialyzed against 0.01 SSC for 48 hr (5 changes of 200 vol). The DNA (8 μg/ml) was then alkali denatured with 0.5 N NaOH for 1 hr at 5 C, neutralized with HCl, and the salt concentration was adjusted to 2X SSC with 20X SSC.

Nitrocellulose filters were soaked in 2X SSC before placing the filters in a Millipore filtering apparatus. Filters were washed with 5 ml of 2X SSC before adding the DNA (50 μg/ml). The filters were again washed with 5 ml of buffer and allowed to dry for 4 hr at room temperature. Finally, the filters were baked at 80 C in an evacuated desiccator for 2 hr and stored at -20 C.

3. Hybridization

The DNA filters were suspended in 2X SSC buffer (2 ml) to which various concentrations of labeled RNA had been added and hybridized at 66 C for 12-15 hr. Filters were then washed with 20 ml of 2X SSC and

treated with pancreatic RNase (20 µg/ml) for 1 hr at room temperature. After five washings with 2X SSC (20 ml) the filters were dried at room temperature and suspended in Omnifluor-toluene scintillation fluid for counting in a Beckman liquid scintillation counter (73,74).

RESULTS

Lytic Infection of *Escherichia coli* with R17 Bacteriophage

Infection of *Escherichia coli* with R17 bacteriophage has been shown to inhibit host protein synthesis and to result ultimately in the lysis of the infected host (120). In Fig. 1 the growth and lysis of *E. coli* 3000 was observed by monitoring the turbidity at 610 nm in a spectrophotometer. A parent culture of *E. coli* (2 x 10^8 cells/ml grown in TCG1-t medium) was divided into infected and control cultures. One of these subcultures was infected with R17 bacteriophage (10 PFU/cell) while the other remained uninfected as a control. Samples were taken as the cultures were incubated at 37 C in a shaking water bath. When the turbidity of the infected culture was compared with the control, no alteration could be observed until thirty to forty min post infection. The increasing turbidity of the infected culture continued to lag behind the control until sixty min post infection when no further growth (increase in turbidity) was observed. Finally lysis began and the turbidity decreased as the cells ruptured.

Continuous Labeling Experiments

In order to detect alterations in host protein synthesis during infection, the incorporation of labeled amino acids into hot TCA precipitates was followed. In Fig. 2, a ^{14}C amino acid mixture

Figure 1

Lysis of infected cells. One of two identical *E. coli* 3000 cultures was infected with R17 bacteriophage (10 PFU/cell). Samples were taken as the cultures were incubated in a shaking water bath (37 C) and the optical density was determined at 610 nm; control (○), infected with R17 bacteriophage (■).

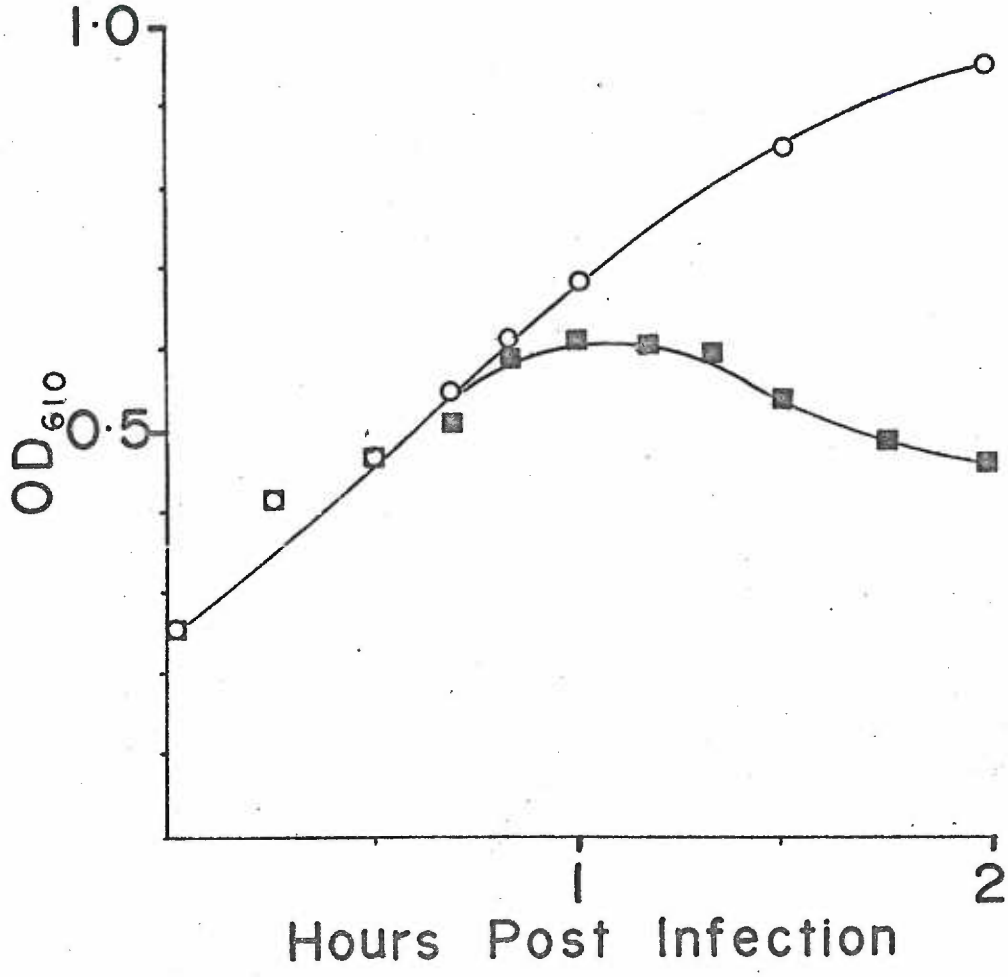
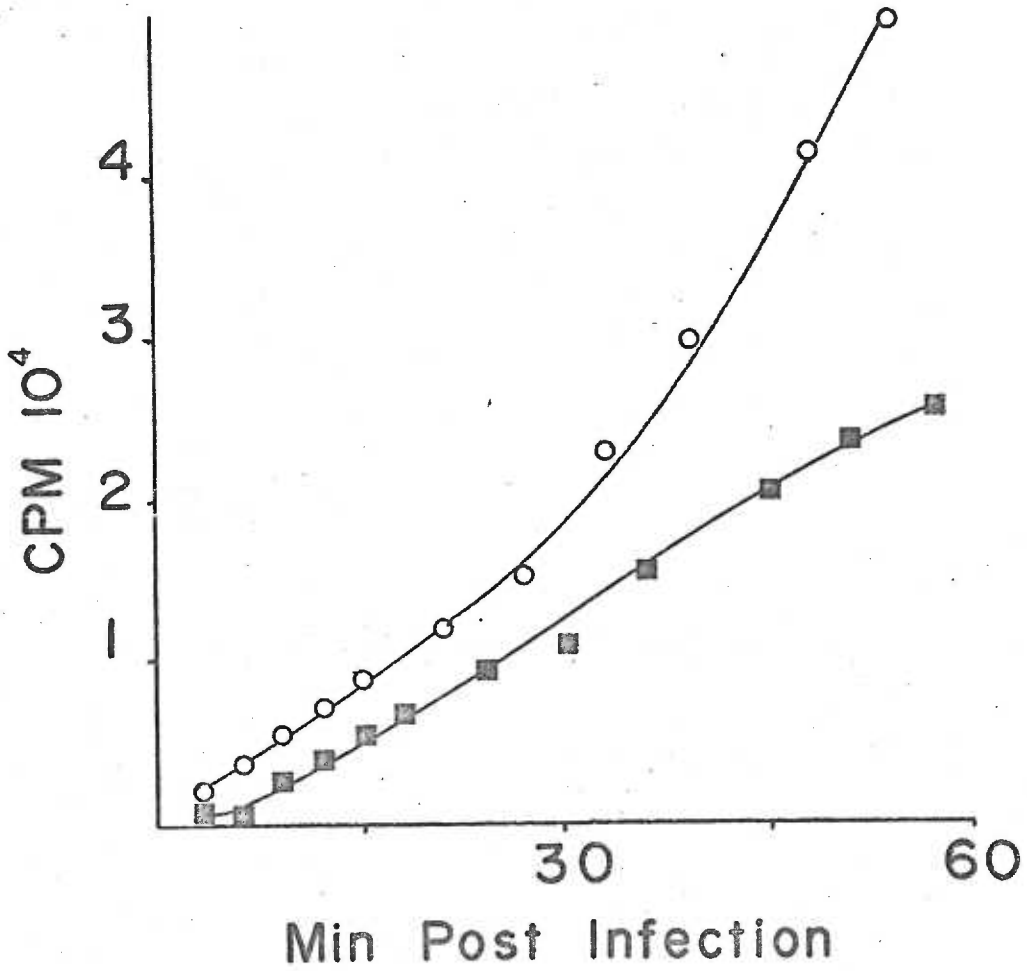

1·0
0·5
OD610
1
2
Hours Post Infection

Figure 2

Early inhibition of protein synthesis upon infection with R17 bacteriophage, continuous labeling. An *E. coli* 3000 culture was divided into control and test cultures. The control culture remained untreated (control ○) while the test culture (■) was inoculated with R17 phage (30 PFU/cell) suspended in tryptone broth (1.5×10^{12} PFU/ml) and diluted with TCG1-t medium (3×10^{11} PFU/ml). At the time of infection both cultures were labeled with ^{14}C amino acid mixture (0.01 μCi/ml). At various times post infection samples were withdrawn and incorporation was terminated with 5% TCA (final concentration). The incorporation of labeled amino acids was determined by heating samples at 90 C for 15 min then collecting the precipitates on membrane filters and washing with 5% TCA and 95% ethanol. After drying the filters were suspended in Omnifluor-toluene scintillation fluid. Radioactivity was counted in a Beckman liquid scintillation counter.


CPM 10^4
4
3
2
1
30
60
Min Post Infection

(0.1 μCi/ml) was added to control and infected cultures of *E. coli* 3000 grown in TCG1-t medium. At various times post infection, samples were removed and incorporation terminated with 5% TCA (final concentration). The samples were heated to 90 C for 15 min, precipitates were collected on Millipore filters and washed with 5% TCA and 95% ethanol. The dried filters were suspended in Omnifluor-toluene scintillation fluid. Radioactivity was counted in a Beckman LS-200 B liquid scintillation counter. Immediately after infection there was a decrease in the incorporation of labeled amino acids into hot TCA precipitates; this decrease has been termed "early inhibition" (108,109). Inhibition was maximal, since increasing the phage inoculation did not increase or prolong the inhibition (116). Inhibition was reversed during the subsequent 10 min of infection, and the incorporation of label paralleled that of the control culture, until late inhibition was encountered at 25-35 min post infection. These results agreed with those of Watanabe (108,109) in which he studied the synthesis of β-galactosidase during infection of *E. coli* with R23 bacteriophage.

Pulse-Labeling Experiments

Using a modification of the pulse-labeling procedures described by Yamazaki (116), early inhibition and recovery were examined (Fig. 3). Control and test cultures of *E. coli* were grown to 2 x 10^8 cells/ml in TCG1-t medium. Before and after infection, samples were taken from

Figure 3

Early inhibition of protein synthesis upon infection with R17 phage, pulse-labeling. An *E. coli* 3000 culture was divided into control and test cultures. The control culture remained untreated (control-baseline ■) while the test culture (O) was inoculated with R17 phage suspended in tryptone broth and TCG1-t medium. Samples were withdrawn at various times and pulse-labeled with ^{14}C amino acid mixture (1 μCi/ml) for 1.5 min. Pulse-labeling was terminated with 10% TCA and incorporation of label into hot TCA precipitates was determined as described in Fig. 2.

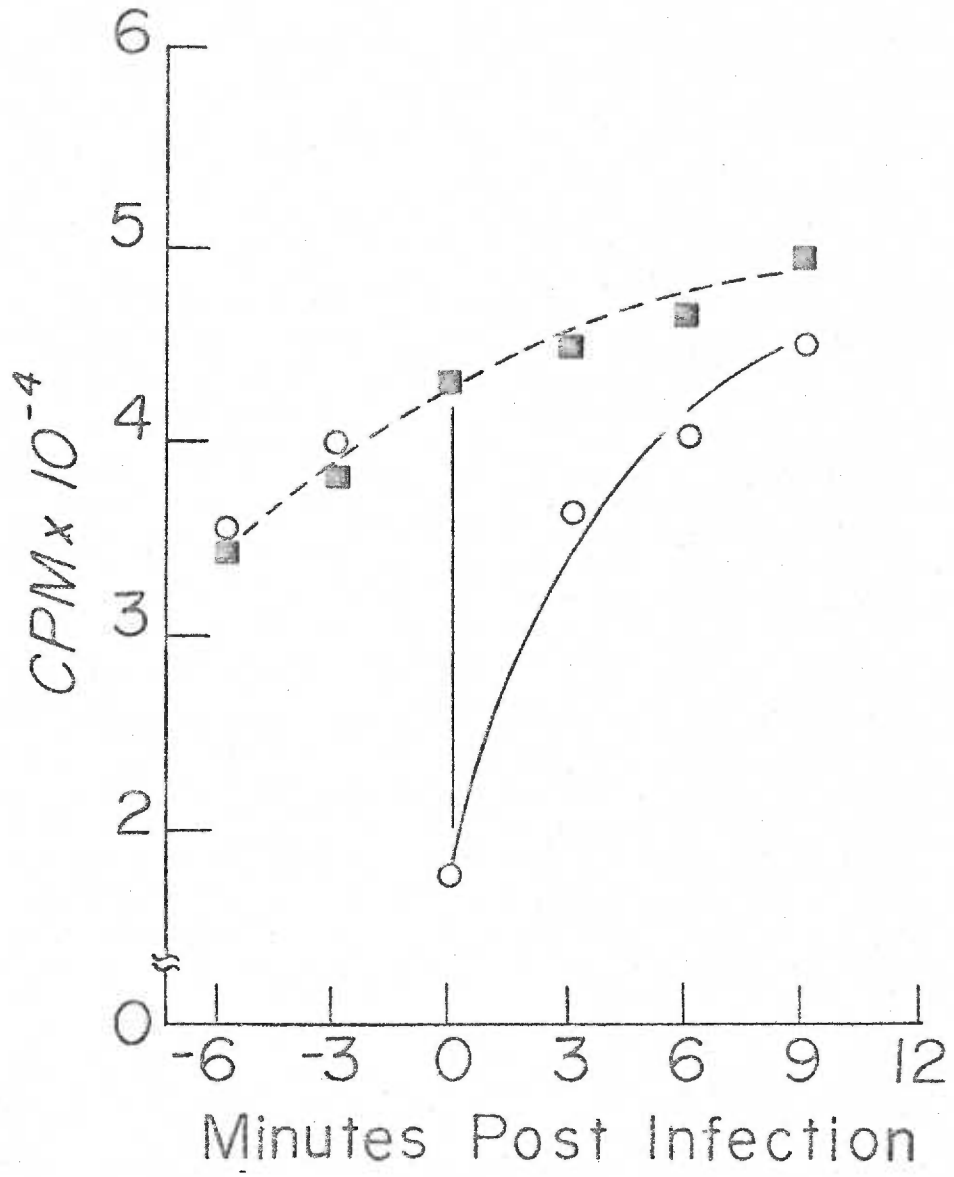
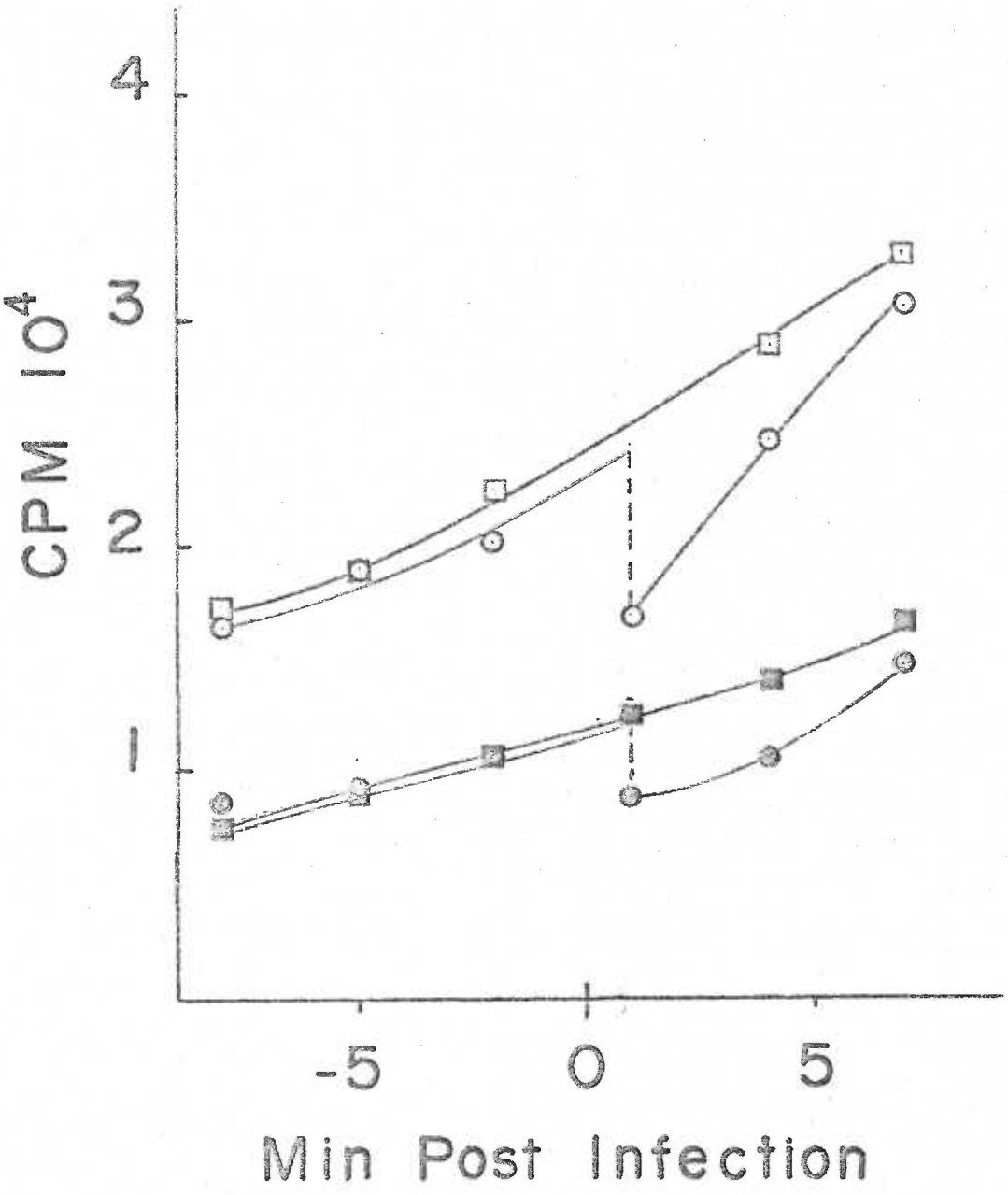

6
5
4
3
2
0
CPM x 10⁻⁴
-6
-3
0
3
6
9
12
Minutes Post Infection

Figure 4

Inhibition of uptake of ^{14}C amino acids during early inhibition. Before and after inoculation with R17 phage suspension, control (□ ■) and test (○ ●) cultures of *E. coli* 3000 were pulse-labeled with ^{14}C amino acid mixture (1 μCi/ml) for 1.5 min. Incorporation was terminated with either TCG1-wash (○ □ Fig. 4) or 10% TCA (● ■ Fig. 3).


CPM 10^4
4
3
2
1
-5
0
5
Min Post Infection

each culture and pulse-labeled with a ^{14}C amino acid mixture (1 μCi/ml) for 1.5 min; incorporation was terminated with 10% TCA (final concentration). The incorporation of label was determined according to the procedures described for the continuous labeling experiment (see Materials and Methods). Immediately after the addition of the phage preparation, there was a 55% decrease in the incorporation of labeled amino acids into hot TCA precipitates (Fig. 3). Recovery occurred as infection proceeded; the rate of incorporation returned to the level observed in the control by 10 min post infection.

It was further demonstrated that inoculation of the *E. coli* culture with the R17 phage suspension inhibited the transport of amino acids into the host cell. Amino acid uptake was estimated by labeling an *E. coli* culture and washing the cells on Millipore filters with TCG1-t medium from which the energy compounds, sodium pyruvate and glycerol, had been omitted (TCG1-wash; 83,107). The pulse-labeling experiment of Fig. 3 was modified; in addition to the hot TCA precipitation the uptake of label was terminated by washing duplicate samples on Millipore membrane filters with the TCG1-wash. The results showed that the amount of label taken up by the cells exceeded that which was incorporated into the hot TCA precipitates (Fig. 4). Immediately after infection both the amino acid uptake and incorporation of label into hot TCA precipitates decreased; as recovery occurred the rates of uptake and incorporation returned to control levels.

Verification of Early Inhibition

There are three possible interpretations of the experiments in Fig. 2, 3 and 4 in which early inhibition was observed.

1. Neither host protein synthesis or amino acid transport had been inhibited and the decreased amount of label entering the hot TCA precipitates was the result of diluting the specific activity of the labeled amino acids with the amino acids in the R17 phage suspension.
2. There was no inhibition of host protein synthesis and the decreased amount of labeled amino acids entering the hot TCA precipitates was the result of an inhibition of amino acid transport into the host cell.
3. Host protein synthesis had been temporarily interrupted.

1. Dilution of Labeled Amino Acids

The viral inoculum consisted of R17 bacteriophage suspended in tryptone broth and TCG1-t medium. Calculations of total nitrogen of the ^{14}C amino acid mixture by the addition of amino acids in the R17 phage suspension show that the dilution should be less than 5%.

$$\text{Dilution} = \frac{\text{Nitrogen in inoculum}}{\text{Nitrogen in medium}} \times 100\%$$

$$= \frac{\text{\% Nitrogen x Tryptone + \% Nitrogen x Casamino Acids}}{\text{\% Nitrogen Casamino Acids}}$$

$$= \frac{1.0 \text{ mg} \times 0.13 + 0.6 \text{ mg} \times 0.1}{37.5 \text{ mg} \times 0.1} \times 100\% = 5\%$$

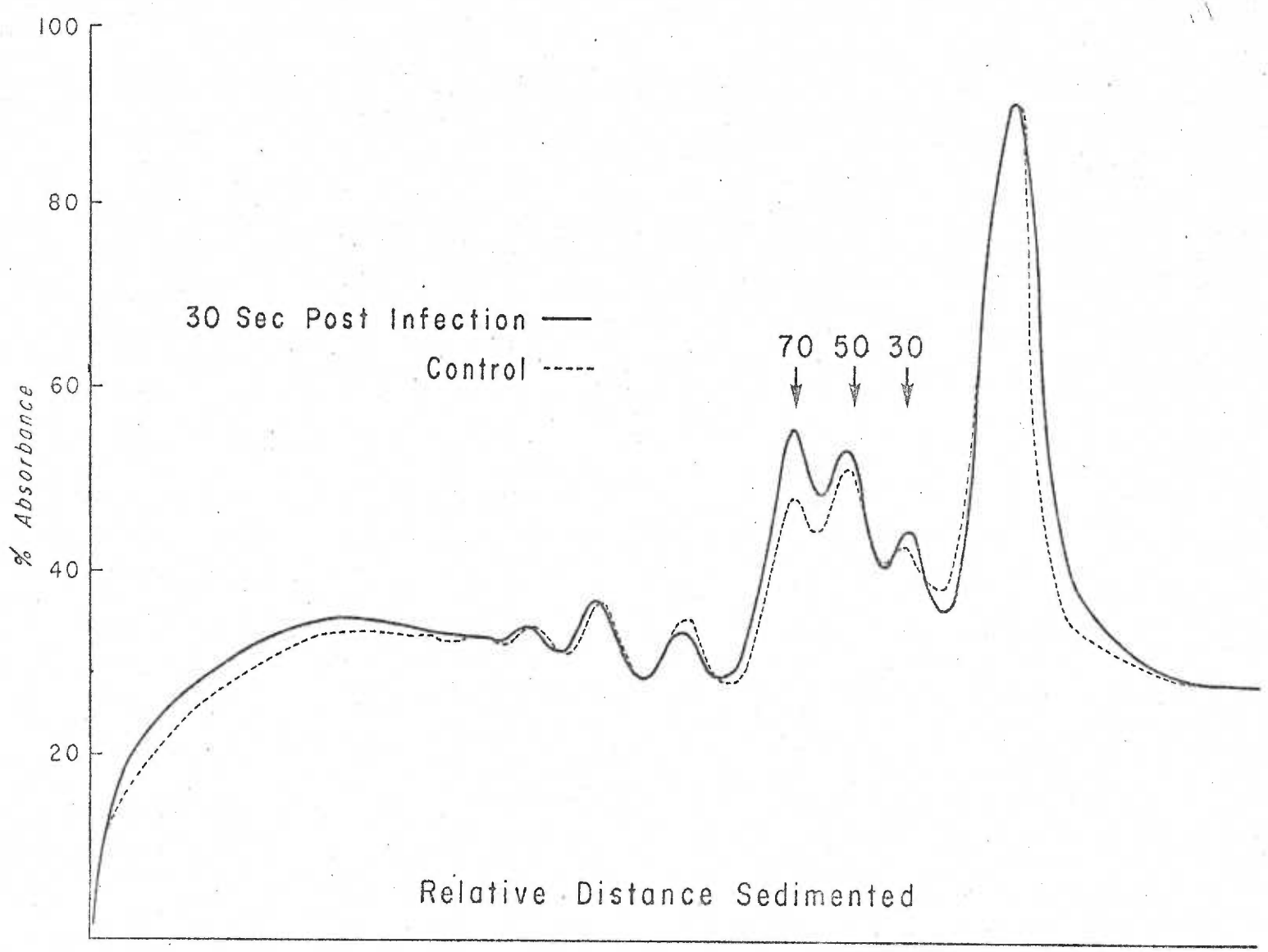
The inhibition observed in Fig. 2, 3 and 4 cannot be explained as a dilution of the specific activity of the labeled amino acids when the phage suspension containing amino acids was used to inoculate the host culture.

2. Inhibition of Amino Acid Transport

A study of the polyribosome profiles immediately after infection with the R17 phage suspension confirmed the inhibition of amino acid transport during early inhibition of protein synthesis. Polyribosomes were extracted from infected and control cultures of *E. coli* 3000 at 0.5 and 13 min post infection according to the procedures described in Materials and Methods. The results shown in Fig. 5 revealed that immediately after the infection of the culture with the phage suspension there was a small, consistent rise in the monomer peak. As infection proceeded, the monomer peak returned to the level observed in the control. There was, however, no reproducible alteration in the heavy polyribosome profile.

Figure 5

Examination of polyribosome profiles during early inhibition. *E. coli* 3000 cells were grown in TCG1-t medium (2×10^8 cells/ml). At either 30 sec (b) or 13 min (c) post infection polyribosomes were extracted from control (a) and infected cultures and sedimented in 10-40% sucrose gradients.


100
80
60
40
20
% Absorbance
30 Sec Post Infection
Control
70 50 30
Relative Distance Sedimented

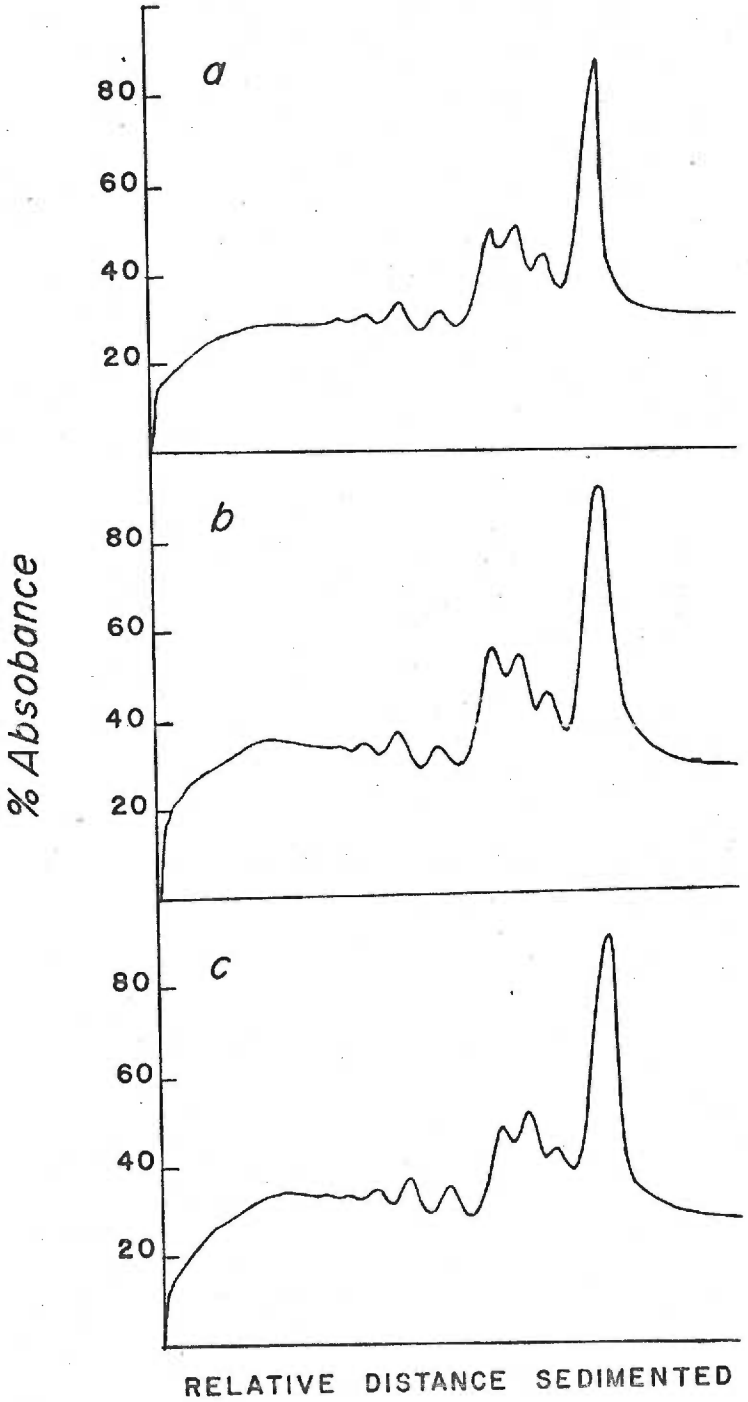
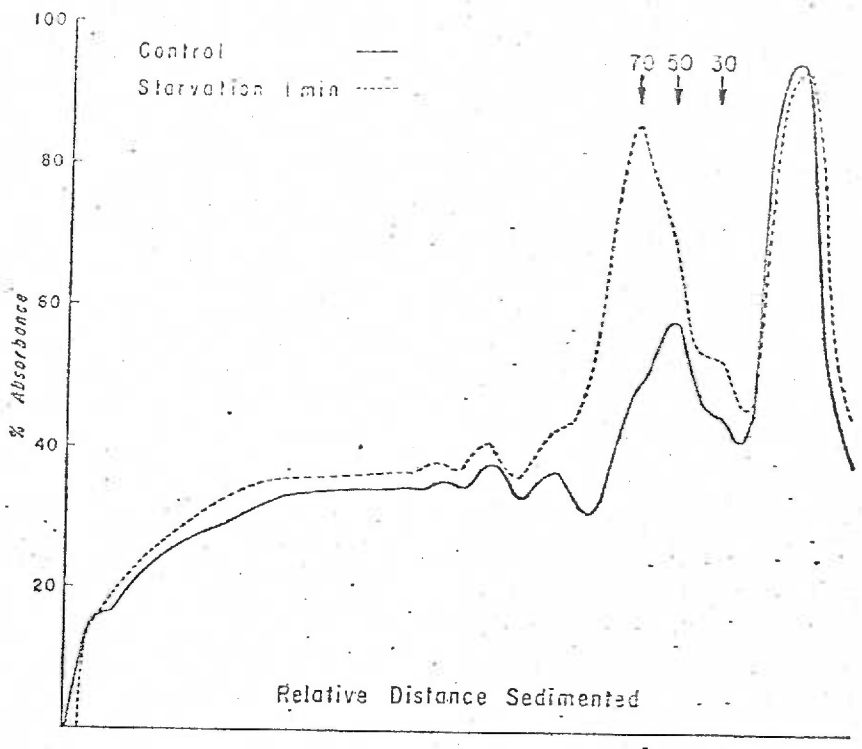
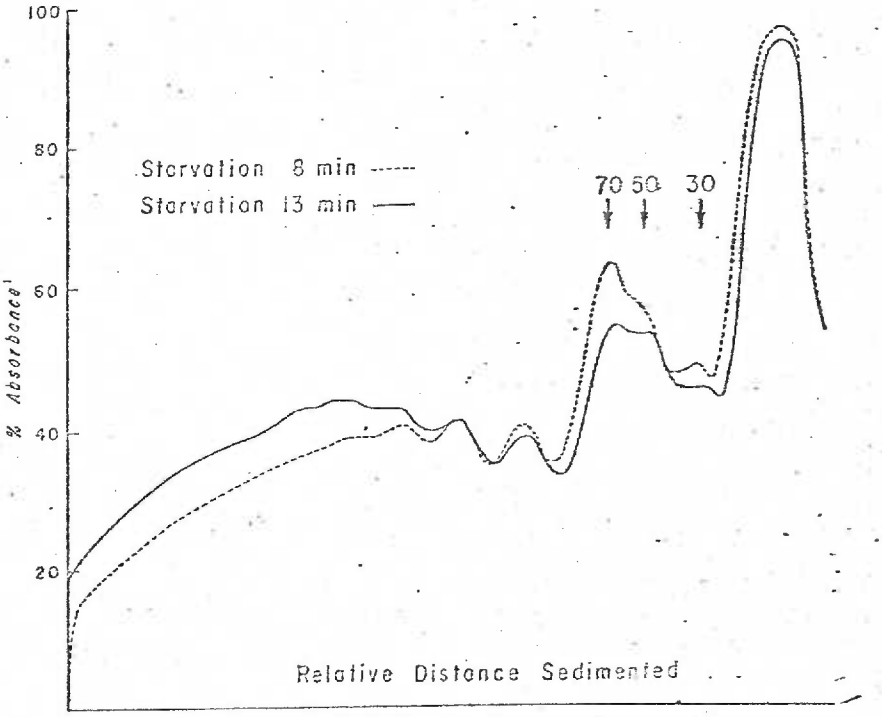

a
b
c
80
60
40
20
% Absobance
RELATIVE DISTANCE SEDIMENTED

Figure 6

Alteration of polyribosome profiles during amino acid starvation. *E. coli* 3000 cells were grown in TCG1-t medium and resuspended in either TCG1-t medium from which the casamino acids had been omitted or TCG1-t medium (control). Polyribosomes were extracted at the indicated times.


100
80
60
40
20
% Absorbance
Control
Starvation 1 min
70 50 30
Relative Distance Sedimented


100
80
60
40
20
% Absorbance
Starvation 8 min
Starvation 13 min
70 50 30
Relative Distance Sedimented

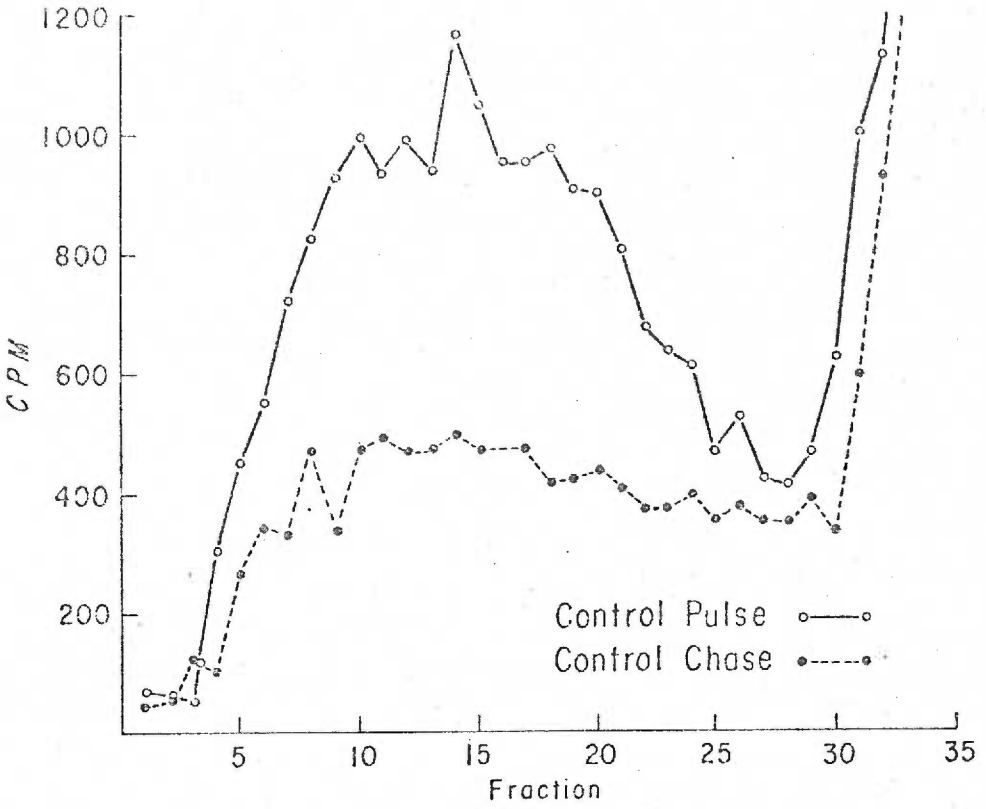
The next experiment demonstrated that the rise in the monomer peak accompanied amino acid starvation. *E. coli* 3000 cells were grown to 2 x 10^8 cells/ml in TCG1-t medium. The cells were pelleted by centrifugation and resuspended in TCG1-t medium from which the casamino acids had been omitted. At various times after suspension in an amino acid-free medium, the polyribosomes were extracted as before. The results (Fig. 6) showed that 1 min after total amino acid starvation there was an increase in the monomer peak and no alteration in the heavy polyribosome profile. This finding was later published by Ron (86) who showed that amino acid starvation increased the monomer peak without altering the heavy polyribosome profile.

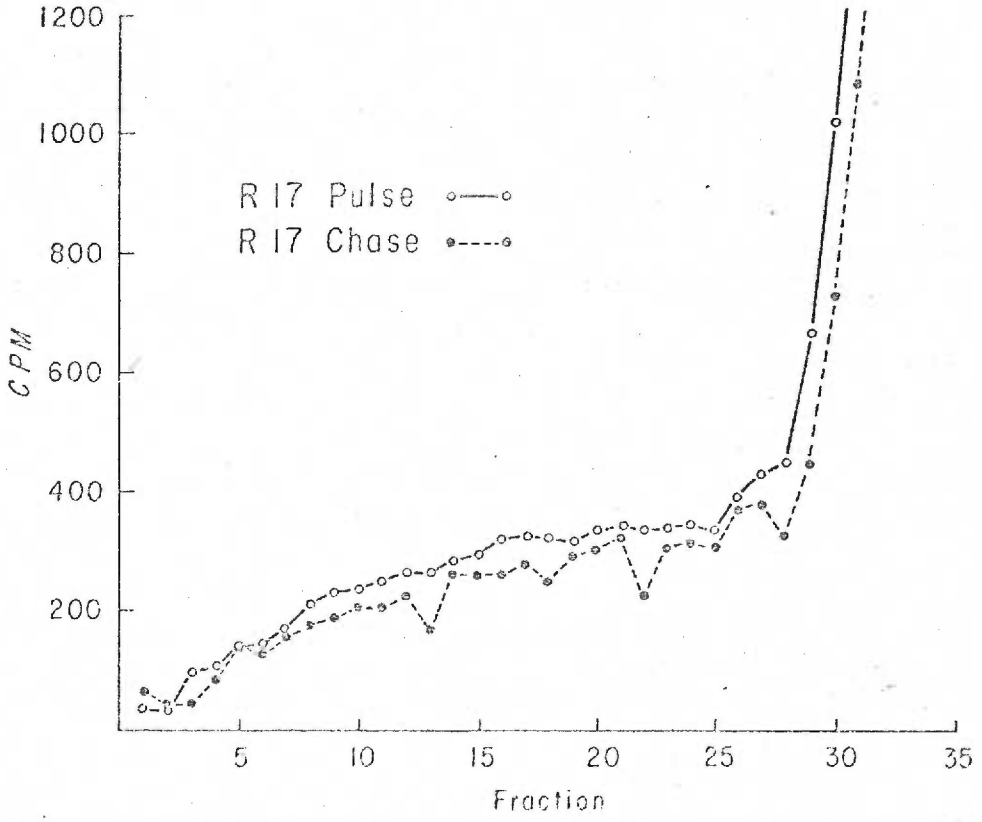
3. Inhibition of Polypeptide Release

Inhibition of polypeptide formation was observed by pulse-chase experiments (Fig. 7) in which label was removed from the polyribosome region during early inhibition. An *E. coli* 3000 culture was harvested by centrifugation and resuspended in TCG1-t medium (1/100 the normal casamino acid concentration). Two sets of control and infected cultures were pulse-labeled with ^{14}C amino acid mixture for 1 min beginning at 0.5 min post infection. At this time, polyribosomes were extracted from the first set of control and infected cultures. The second set was chased with a hundredfold excess of cold amino acids for 2 min and polyribosomes were extracted. The gradients were

Figure 7 (A,B)

Release of polypeptides from polysomes during early inhibition. An *E. coli* 3000 culture was grown to 2 x 10^8 cells/ml in TCG1-t medium and harvested by centrifugation. The cells were resuspended in TCG1-t medium with one hundredth the normal casamino acid concentration. Control and test cultures were incubated in a shaking water bath for 5-10 min before infection with the R17 bacteriophage suspension. Thirty sec after inoculation with R17 phage suspension, control and test cultures of *E. coli* 3000 were labeled with ^{14}C amino acid mixture (0.1 μCi/ml) for 1 min then chased with a hundredfold excess of cold amino acids for 2 min. At the end of the pulse and pulse-chase periods polysomes were extracted and sedimented on sucrose gradients. The gradients were fractionated and the incorporation of label into hot TCA precipitates was determined as described in Fig. 2.


1200
1000
800
600
400
200
CPM
Control Pulse
Control Chase
5
10
15
20
25
30
35
Fraction


1200
1000
800
600
400
200
CPM
R 17 Pulse
R 17 Chase
5
10
15
20
25
30
35
Fraction

fractionated and the amount of label incorporated into hot TCA precipitates was determined. As expected in the control, more labeled amino acids were incorporated into the polyribosome region than were observed in the infected culture. While chasing the control, 50% of the labeled amino acids were removed. In the infected culture only 15% of the label was removed. Thus, it appears that amino acid starvation inhibited the release of polypeptides from polyribosomes. This system is independent of the dilution of the specific activity of the labeled amino acids by the phage inoculum and supports the conclusion that amino acid starvation inhibited protein synthesis (116).

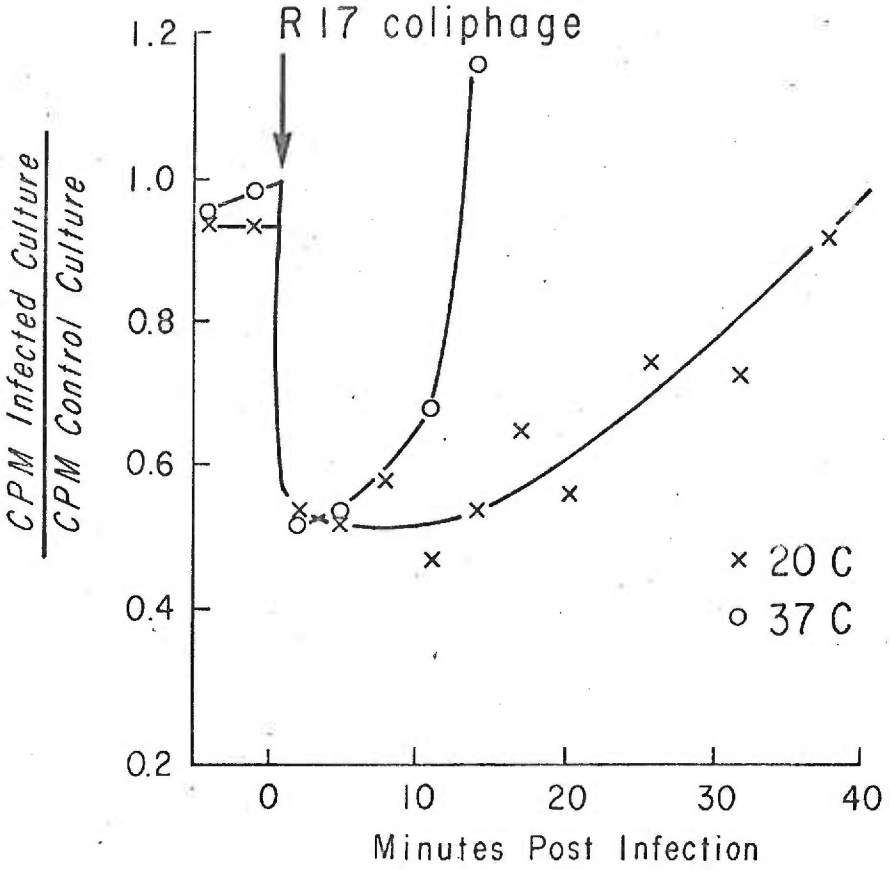
Induction and Recovery from Early Inhibition

1. Early Inhibition is Temperature Independent

Preliminary experiments were designed to determine those factors which alter inhibition and recovery during early inhibition of protein synthesis. First, it was shown that early inhibition is temperature independent. Viral attachment is a unique event of infection being temperature independent (10). The basic pulse-labeling experiment demonstrating early inhibition was repeated at 37 and 20 C; the data is presented in Fig. 8. At both temperatures, inhibition occurred immediately after infection, and the same degree of inhibition (CPM infected culture/CPM control culture) was observed. This result demonstrated that early inhibition was a temperature independent event.

Figure 8

The influence of temperature on early inhibition and recovery. *E. coli* 3000 cultures were grown to 2 x 10^8 cells/ml in TCG1-t medium at 20 C and 37 C. The test cultures at 20 and 37 C were inoculated with the R17 phage suspension and the ^{14}C amino acid pulse-labeling procedure of Fig. 3 was used to observe early inhibition.


R17 coliphage
1.2
1.0
0.8
0.6
0.4
0.2
0
10
20
30
40
Minutes Post Infection
CPM Infected Culture / CPM Control Culture
× 20 C
o 37 C

At the lower temperature, recovery was significantly prolonged, which was consistent with the reduced metabolic rate of the host cells at 20 C.

2. Massive Addition of Phage

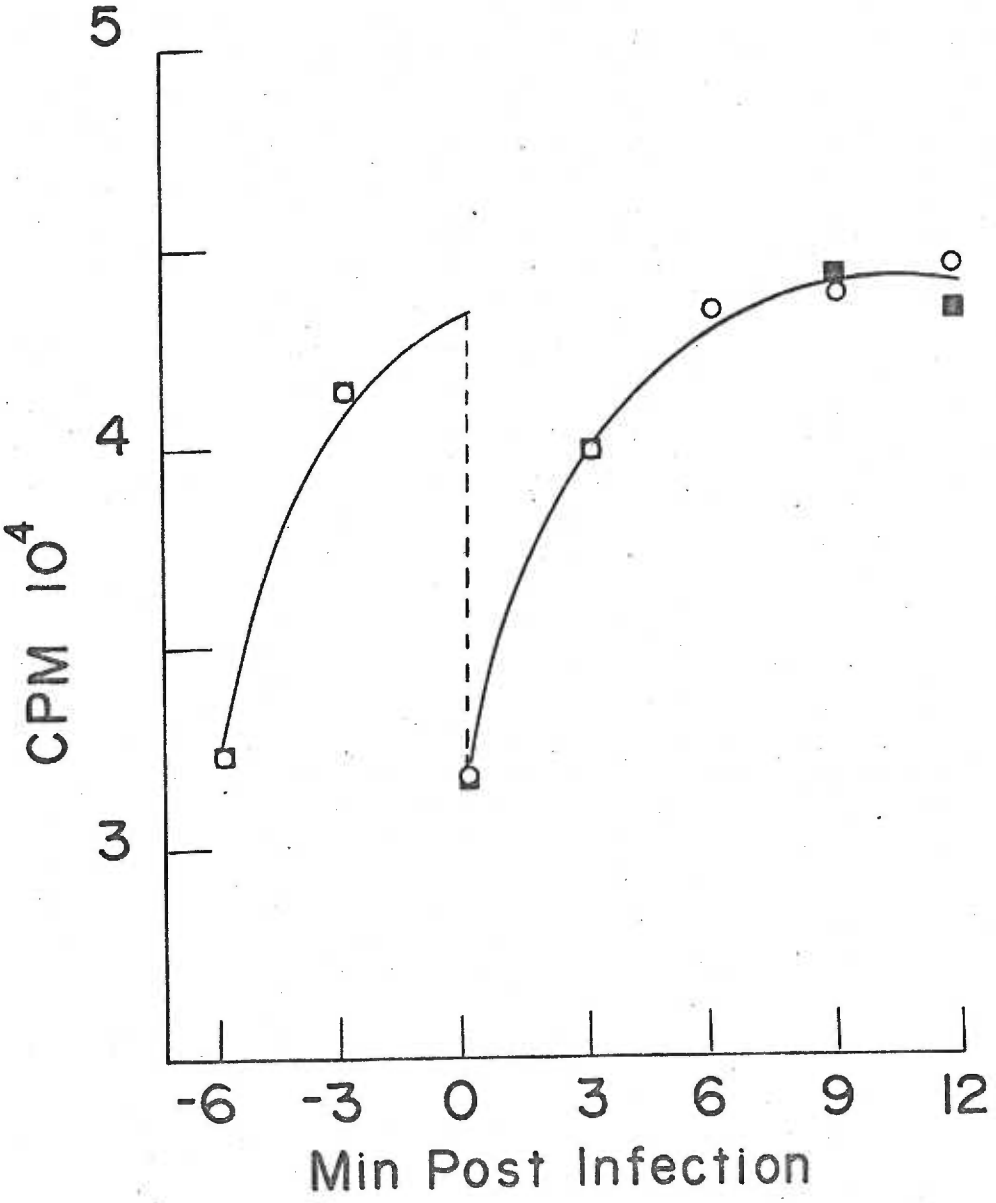
Although it was known that inhibition is maximum at 30 PFU/cell (116), it had not been determined if additions of more phage would extend the recovery time. Therefore, the basic pulse-labeling experiment was modified, the control was infected with R17 phage (30 PFU/cell) and the test culture was inoculated with 300 PFU/cell. This experiment (Fig. 9) verified that the inhibition was maximal at 30 PFU/cell, and that recovery was not prolonged by larger additions of phage.

3. Repeated Infection with the R17 Phage Suspension

The basic pulse-labeling experiment shown in Fig. 3 demonstrated that after infection of an *E. coli* culture, the cells regained their ability to incorporate labeled amino acids into hot TCA precipitates. Recovery was complete when the rate of amino acid incorporation of the test culture was equivalent to the uninfected control culture. However, it was not known if the host was again susceptible to early inhibition. In examining this possibility the basic pulse-labeling experiment was again modified. Both the control and test cultures were infected at time 0. After recovery occurred (10 min), the test

Figure 9

Infection of *E. coli* with R17 bacteriophage (300 PFU/cell). Identical cultures of *E. coli* 3000 were infected with R17 bacteriophage either 30 PFU/cell (■) or 300 PFU/cell (○). Before and after infection cultures were pulse-labeled with a ^{14}C amino acid mixture. The pulse was terminated with 10% TCA and the incorporation of label into hot TCA precipitates was determined as described in Fig. 3.


5
4
3
CPM 10^4
-6
-3
0
3
6
9
12
Min Post Infection

culture was again inoculated with the R17 phage suspension (30 PFU/cell). A second inhibition was observed when the test culture was again infected with the R17 phage suspension (Fig. 10). It was surprising that a second inhibition occurred at all. According to the kinetic studies of phage attachment (78), at the time of the second inoculation viable phage should still have been present in the culture. Recovery had occurred in the presence of infectious phage particles, and after recovery was complete the culture was again susceptible to early inhibition even though infectious phage particles from the first infection were still present. Thus the property which was responsible for early inhibition was not always associated with viable phage.

4. Inoculation with Phage Particles which can not Adsorb to the F Pili

It was confirmed that early inhibition was not associated with phage adsorption. Non-attaching particles were obtained by infecting *E. coli* 3000 (a non-permissive strain) with amA_{31}, a mutant that does not synthesize the complete maturation protein which is responsible for attachment. The purified defective particles were suspended in tryptone broth and TCG1-t medium, then inoculated into a test culture of the pulse-labeling experiment described in Fig. 3. The results in Table 2 show that the defective particles were capable of inducing early inhibition.

Figure 10

Repeated infection of *E. coli* with R17 bacteriophage. Identical cultures of *E. coli* 3000 were infected with R17 bacteriophage (30 PFU/cell ○). After 9 min the test culture was re-inoculated with R17 phage (30 PFU/cell ■). Before and after infection cultures were pulse-labeled with a ^{14}C amino acid mixture. The pulse was terminated with 10% TCA and the incorporation of label into hot TCA precipitates was determined as described in Fig. 3).

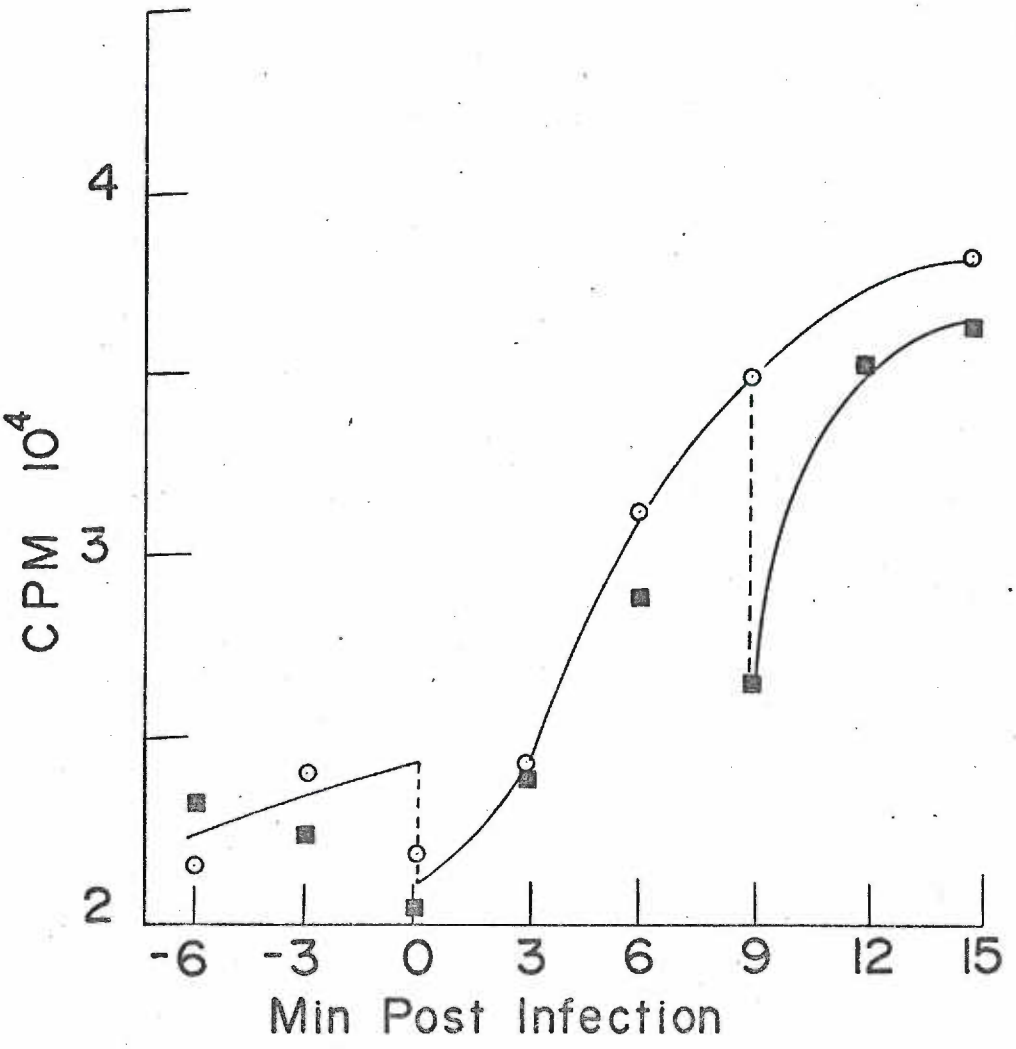

CPM 10^4
4
3
2
-6
-3
0
3
6
9
12
15
Min Post Infection

Table 2

The degree of inhibition was determined according to the procedures described in Fig. 3. Incomplete particles were purified from a crude lysate obtained by infecting *E. coli* 3000 (non-permissive) with the amber mutant am A31.

Table 2

Early Inhibition in F- Culture or Incomplete Particles

Phage Suspension		Culture	Degree Inhibition
Incomplete particles	→	*E. coli* 3000	57 %
R17 phage suspension	→	*E. coli* F-	46 %
R17 phage suspension	→	*S. typhimurium*	54 %

The possibility that early inhibition was mediated by virus attachment to the F pili was eliminated by infecting F- cultures of *E. coli* or *Salmonella typhimurium*. Upon inoculation with the phage suspension early inhibition was observed in both cultures (Table 2). Thus early inhibition was independent of the maturation protein and F pili.

From the repeated infection experiments described in Fig. 11, it appeared possible for infective particles to be present without inducing early inhibition. Further investigation was undertaken to determine if a non-viral agent(s) was responsible for early inhibition.

Identification of the Agent Responsible for Early Inhibition

Since the previous work has shown that recovery occurs in the presence of viable phage particles, it was decided to examine each of the components of the virus inoculum in order to identify the agent responsible for early inhibition. The R17 phage suspension consisted of:

1. R17 bacteriophage
2. Possible unknown host contaminants
3. Tryptone broth and TCG1-t medium

1. R17 Bacteriophages

Studies with purified R17 phage showed that early inhibition was not a function associated with R17 phage infection. The phage was further purified by sedimenting the R17 phage suspension through a 5-20% sucrose gradient; the sucrose was removed by dialysis against 0.1 M ST buffer. The results in Fig. 11 showed that no early inhibition was observed when this purified virus was used to inoculate the test culture of the pulse-labeling experiment described in Fig. 3. Identical results were obtained with virus which had been purified on a CsCl gradient and suspended in phosphate buffer (Fig. 14 and Table 3).

Although the agent responsible for early inhibition could be separated from the phage particle, it was still possible that this agent was required for phage infectivity. The infectivity of purified virus suspended in phosphate buffer (0.1 M, pH 7.2) was examined. Plaque assays revealed no appreciable loss of infectivity during these experiments. In Table 4, *E. coli* cultures were infected with either R17 phage suspended in phosphate buffer or the R17 phage suspension. Virus production was examined 2 hr after infection. Plaque assays revealed that the presence or absence of early inhibition had no influence on the amount of virus produced by infected cells. It was also shown that the presence of early inhibition did not modify the early stages of infection. After the virus attaches to the F pili,

Figure 11

Infection of *E. coli* with R17 phage purified on sucrose gradients. The R17 phage suspension was sedimented on a 5-20% sucrose gradient. Fractions containing virus were dialyzed against 0.1 M ST buffer (■). This virus preparation was then used to infect *E. coli* in the pulse-labeling experiment (30 PFU/cell) described in Fig. 3. Control (○).

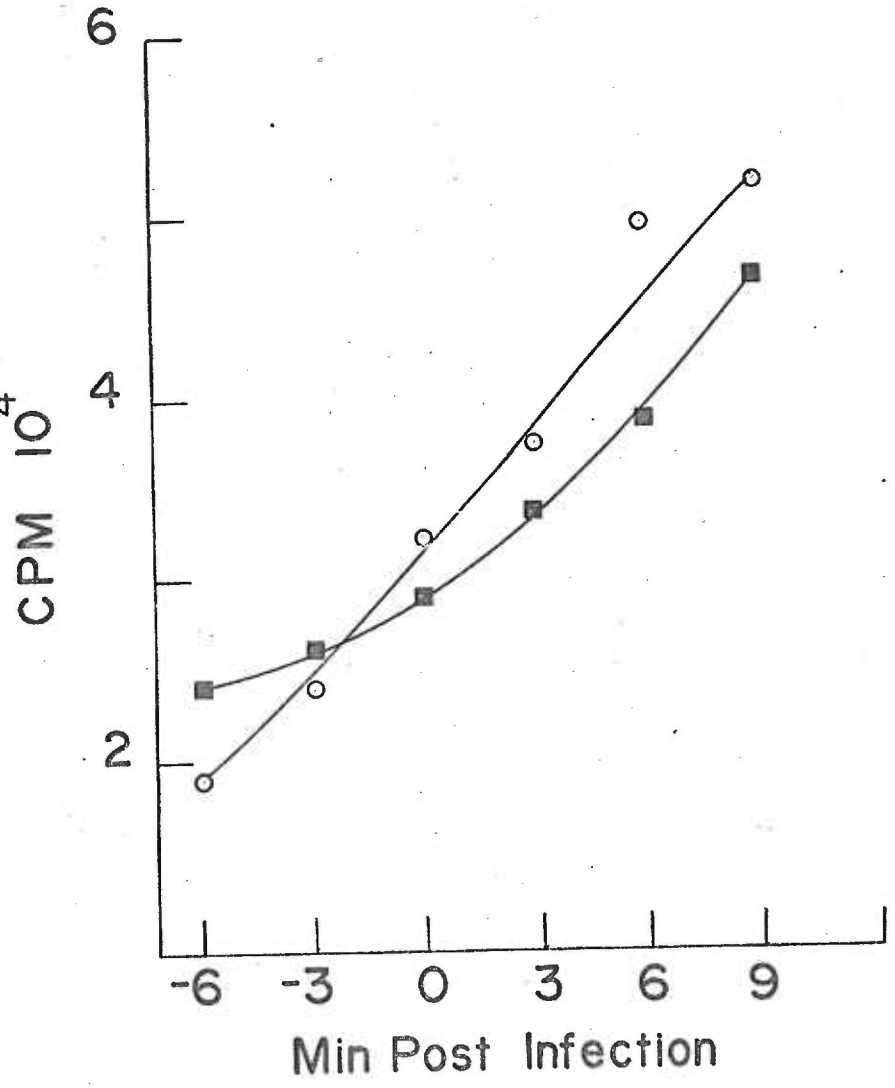

6
4
2
CPM 10^4
-6
-3
0
3
6
9
Min Post Infection

Table 3

The degree of inhibition was determined according to the procedures in Fig. 1.

$$\text{Maximum degree of inhibition} = 1 - \frac{\text{CPM test culture}}{\text{CPM control-baseline}} \times 100\%$$

[1]R17 bacteriophage total lysate was prepared by infecting a culture of *E. coli* 3000 grown to 2 x 10^8 cells/ml in TCG1-t medium.

[2]TCG1-t medium was prepared immediately prior to use.

[3]Depleted TCG1-t medium was obtained from *E. coli* 3000 culture grown to 2 x 10^8 cells/ml in TCG1-t medium, cells were removed by centrifugation. Protein determinations (7) revealed that the amino acid concentration was depleted by less than 1% by the growing *E. coli* 3000 culture.

[4]Nineteen of the essential amino acids (0.1 mM - cysteine omitted) were suspended in distilled water, pH adjusted to 7.2.

Table 3

Factors Causing Early Inhibition Of Protein Synthesis

Samples added to *E. coli* 3000 culture (25 ml)	Degree of inhibition
1. R17 bacteriophage (30 PFU/cell) in TCG1-t medium containing tryptone broth	30 - 55%
2. R17 bacteriophage (30 PFU/cell) in phosphate buffer	0
3. R17 bacteriophage total lysate (30 PFU/cell)[1]	0
4. TCG1-t medium containing tryptone broth	30 - 55%
5. Tryptone broth	30 - 55%
6. TCG1-t medium[2]	30 - 55%
7. TCG1-t medium (depleted)[3]	0
8. 19 amino acids[4]	70%
9. TCG1-t medium with casamino acids omitted	0

Table 4

Virus production of *E. coli* 3000 infected with R17 phage. Two cultures of *E. coli* 3000 were inoculated with R17 phage (10 PFU/cell) suspended in either phosphate buffer (10^{11} PFU/ml) or a mixture of tryptone broth (5 x 10^{11} PFU/ml) diluted with TCG1-t medium to a titer of 10^{11} PFU/ml. Two hr post infection samples were withdrawn, treated with chloroform and phage titer was determined by the standard agar overlay technique (1).

Table 4

R17 Bacteriophage suspended in	Early Inhibition	Titer (PFU/ml)
Phosphate buffer	-	1.2×10^{12}
TCG1-t medium containing tryptone broth	+	1.2×10^{12}

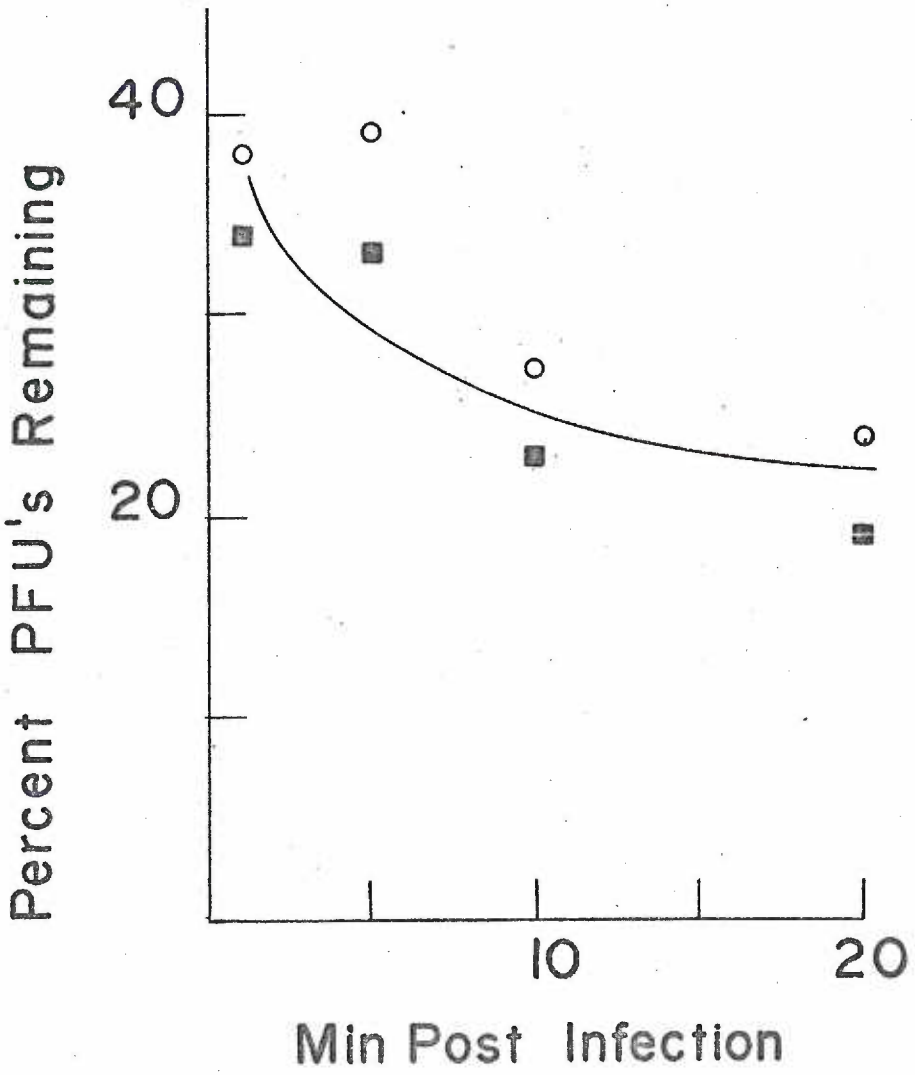
the maturation protein is altered and the viral RNA is then sensitive to RNase until the nucleic acid enters the host cell (77,118). Thus it is possible to examine the kinetics of eclipsing of phage infectivity by inoculating virus in the presence of RNase. At various times post infection samples were withdrawn and assayed for infectious virus. Using this experimental procedure, virus suspended in ST buffer was compared with the R17 phage suspension which produced early inhibition. It was determined that early inhibition did not alter the eclipse of R17 phage (Fig. 12). Therefore purified virus does not induce early inhibition, and the agent responsible for early inhibition does not influence viral infectivity.

2. Host Contaminant

The possibility of some unknown host contaminant being responsible for early inhibition was eliminated when it was observed that inoculating an *E. coli* culture with a crude lysate containing R17 phage did not induce early inhibition. Watanabe (108,109) observed early inhibition when an *E. coli* culture was inoculated with a crude lysate of R23 phage obtained by infection of *E. coli* grown in tryptone broth. Since tryptone broth was one of the components of the R17 phage suspension which was being studied, crude lysates were obtained by replicating R17 phage in either *E. coli* 3000 grown in TCG1-t medium or *E. coli* 209 grown in a Minimal Salts Medium. When test cultures from

Figure 12

Effect of early inhibition on the eclipse of R17 bacteriophage. R17 phage was suspended in either 0.1 M ST buffer (■) or tryptone broth and TCG1-t medium (○) and used to infect *E. coli* 3000 in the presence of RNase (50 µg/ml). At various times post infection samples were withdrawn and assayed for infective units by plaquing on *E. coli* 3000.


40
20
Percent PFU's Remaining
10
20
Min Post Infection

the pulse-labeling experiment described in Fig. 3 were inoculated with these lysates no early inhibition was observed (Fig. 13 and Table 3).

3. Tryptone Broth and TCG1-t Synthetic Medium

In the work of Yamazaki (116), Watanabe (108,109), and in the previous experiments in this paper, early inhibition was always observed when an *E. coli* culture was inoculated with tryptone broth. In order to determine if tryptone broth was the agent responsible for early inhibition, the test culture of the pulse-labeling experiment described in Fig. 3 was inoculated with tryptone broth and TCG1-t medium at a concentration equivalent to that of the virus suspension. The results (Fig. 14) showed that in the absence of phage, the media induced early inhibition. When tryptone broth and TCG1-t medium were examined separately, both induced early inhibition in the pulse-labeling experiment (Table 3).

The coincidence that both media induced early inhibition was fortunate since tryptone broth does not lend itself to fractionation procedures. Drop-out experiments with TCG1-t medium revealed that inhibition was only encountered when casamino acids were present. When the test cultures were inoculated with an amino acid mixture (19 amino acids 0.1 mM), a 70% inhibition of host protein synthesis was observed (Table 3). The data presented in Table 3 shows that early inhibition was observed upon the addition of TCG1-t medium to

Figure 13

Infection of *E. coli* with a crude lysate of R17 bacteriophage. A crude lysate of R17 phage was prepared from an *E. coli* 3000 culture grown in TCG1-t medium. The culture was inoculated with the R17 phage suspension (10 PFU/cell) and incubated at 37 C in a shaking water bath for 2 hr. At time 0, the test culture (■) was infected with the crude lysate (30 PFU/cell). The rate of incorporation of ^{14}C amino acids into hot TCA precipitates was observed with the pulse-labeling experiment described in Fig. 3 and compared to the control culture (○).

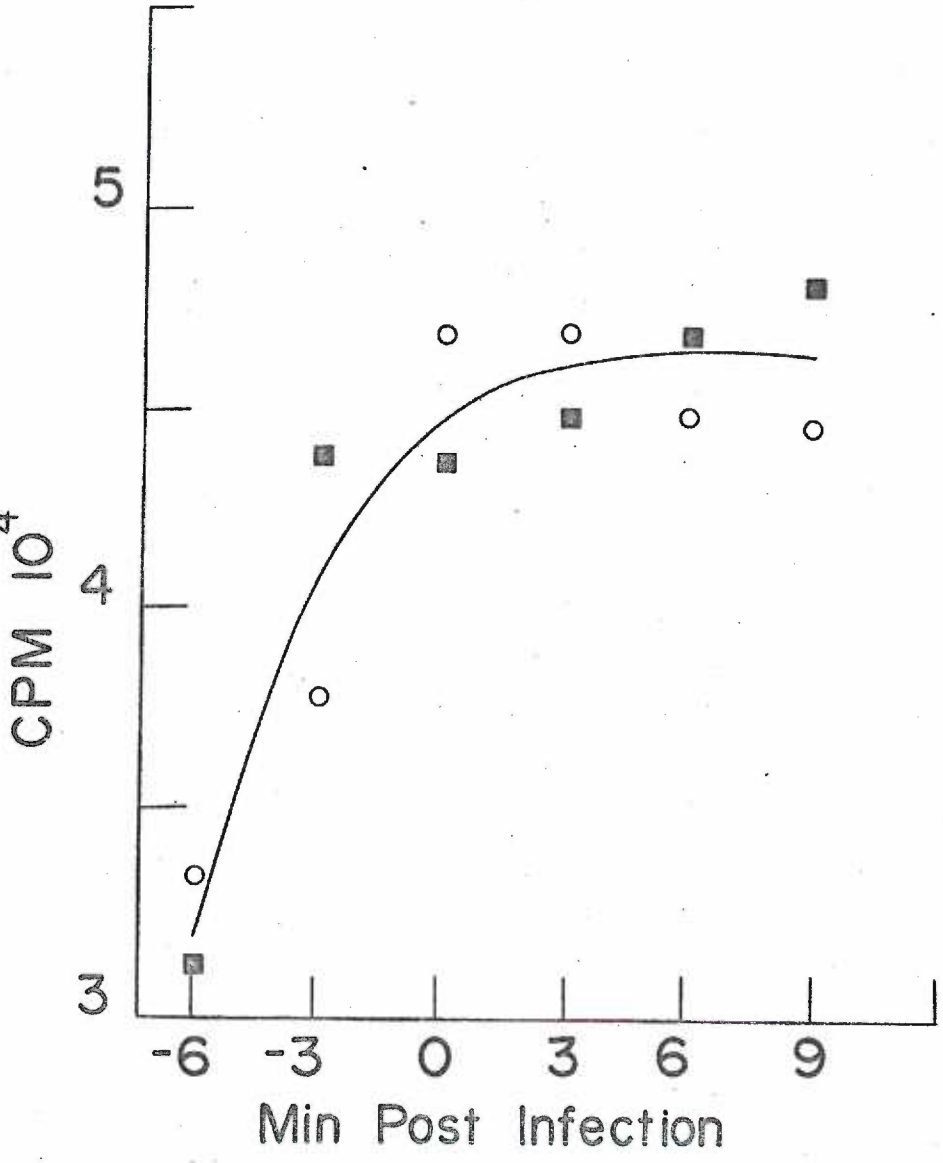
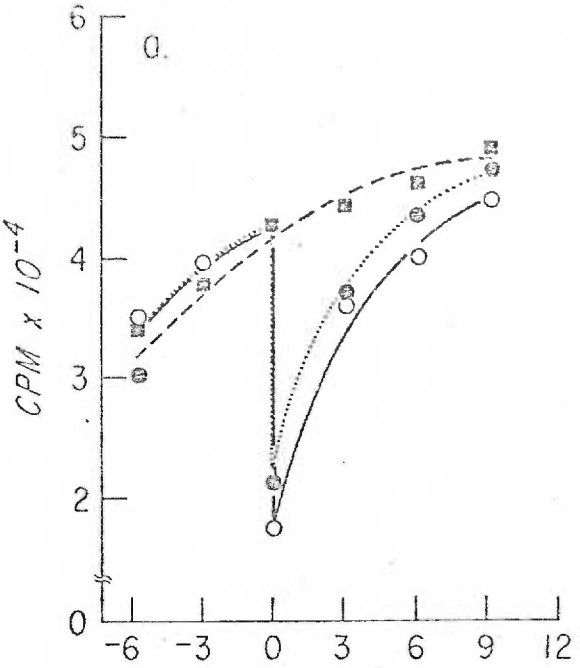
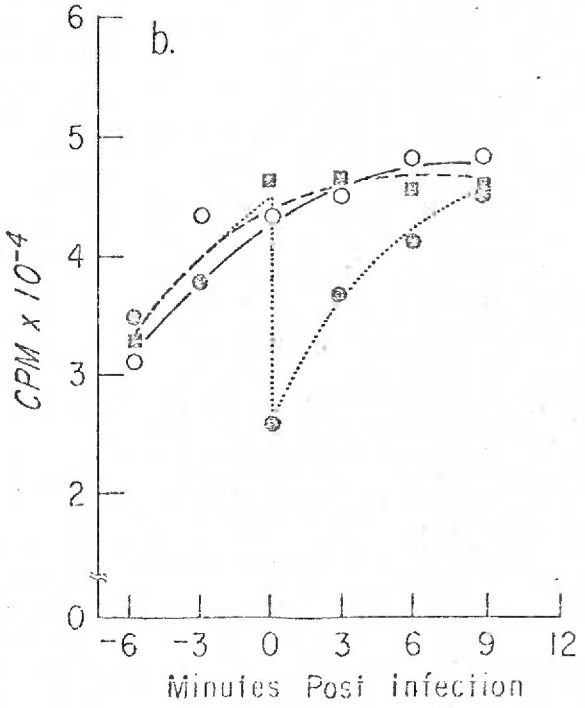

5
4
3
CPM 10^4
-6
-3
0
3
6
9
Min Post Infection

Figure 14

Early inhibition of protein synthesis upon inoculation with tryptone broth and TCG1-t medium. An *E. coli* 3000 culture was divided into control and test cultures. The control culture remained untreated (control ■). The trp-TCG1 suspension test mixture received a volume of tryptone broth and TCG1 synthetic medium equivalent to that used to suspend the phage (tryptone-TCG1 ●). The test culture (○) was inoculated with R17 phage suspended in either (a) tryptone broth and TCG1-t medium, or (b) sodium phosphate buffer. Early inhibition was observed according to the procedures described in Fig. 3.


a.
CPM x 10⁻⁴
0
2
3
4
5
6
-6
-3
0
3
6
9
12


b.
CPM x 10⁻⁴
0
2
3
4
5
6
-6
-3
0
3
6
9
12
Minutes Post infection

E. coli cells growing in TCG1-t medium. The process of growing the *E. coli* culture to the concentration of 2×10^8 cells/ml made a subtle alteration in the medium. The amino acid concentration had been decreased by less than 1% but the addition of fresh TCG1-t medium immediately inhibited protein synthesis in the *E. coli* culture by 55%.

The preceding experiments have demonstrated that infection of *E. coli* with R17 phage does not induce early inhibition of protein synthesis (Fig. 14). The agent responsible for early inhibition has been identified as tryptone broth (Table 3). Apparently the mechanism of inhibition involves a subtle alteration in the concentration of an amino acid(s).

Late Inhibition

Previous work has indicated that phage infection interrupts host protein synthesis (38,99), the normal polyribosome distribution (48) and the synthesis of ribosomal RNA (50). Watanabe (108) studied the synthesis of β-galactosidase after infection with R23 bacteriophage, a close relative of R17 phage. His results showed that synthesis of the viral synthetase and possibly other viral proteins was required to inhibit the synthesis of β-galactosidase. Previous work by Iglewski (55) identified heterogenous pulse-labeled RNA entering the heavy polyribosomes during late inhibition. This strongly suggested that host-specific messenger RNA was transcribed and translated during late

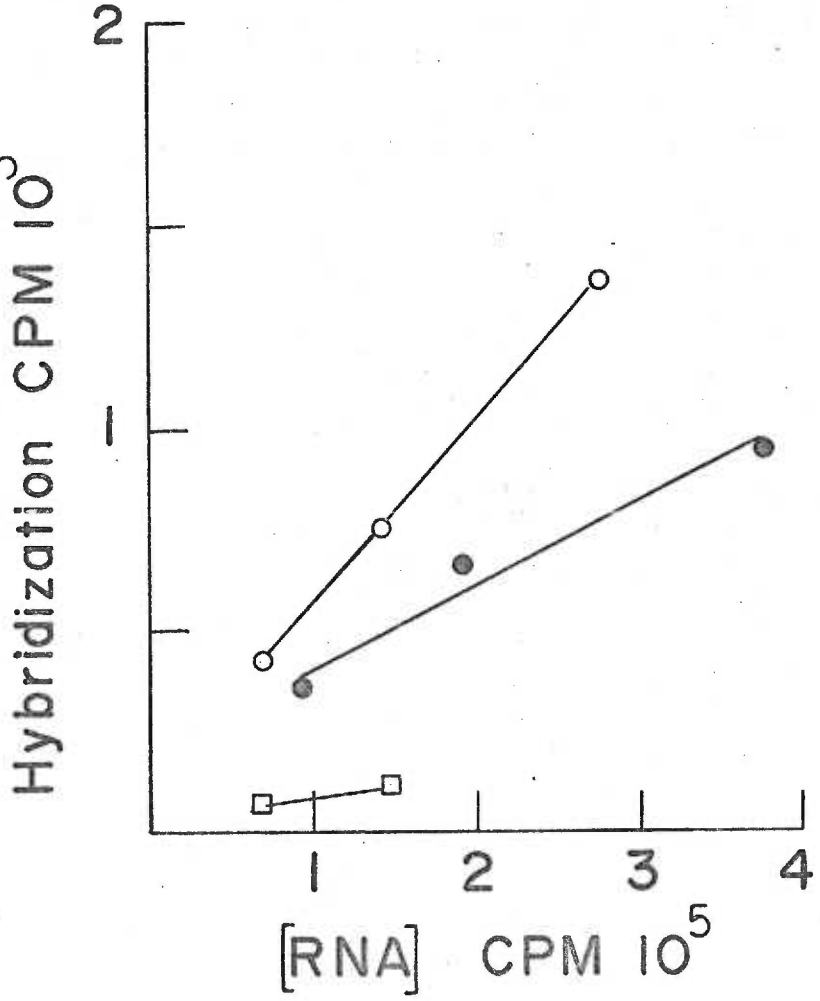
inhibition. The following experiments demonstrated that the host messenger RNA specific for β-galactosidase was transcribed and translated during late inhibition. Preliminary work in the characterization of late inhibition verified the inhibition of β-galactosidase synthesis and showed that the functional half-life of the β-galactosidase messenger RNA had not been significantly altered.

Messenger RNA Specific for β-galactosidase

The results in Fig. 15 showed the presence of messenger RNA specific for β-galactosidase in infected cells during late inhibition. Beta-galactosidase messenger RNA was induced in infected and control cells by adding IPTG (isopropyl-thiogalactoside) at 30 min post infection. After 5 min the cells were labeled with ^{3}H uridine for 0.5 min. Further incorporation of label was terminated with iced TCG1-t medium. RNA for hybridization was obtained by phenol extraction of the control and infected cultures (see Materials and Methods). Beta-galactosidase m-RNA was specifically identified by hybridization to ϕ80 *plac* DNA. The defective lysogen ϕ80 *plac* contains virtually the entire *lac* operon (60), since *E. coli* 3000 is not lysogenic for ϕ80 bacteriophage. The RNA extracted from *E. coli* 3000 will only hybridize to the *lac* operon and not to ϕ80 DNA. The results described in Fig. 15 showed that labeled RNA specifically hybridized to ϕ80 *plac* DNA. Thus messenger RNA specific for β-galactosidase was present in both infected and control cells during late inhibition.

Figure 15

Detection of messenger RNA specific for β-galactosidase in R17 phage infected cells. At the time of infection the test culture was inoculated with 10 PFU/cell of R17 bacteriophage. Thirty min later the cultures were induced for β-galactosidase with IPTG. After 5 min of incubation, the cells were pulse labeled with ^{3}H uridine (10 μCi/ml) for 0.5 min. Further incorporation was terminated with iced TCG1-t medium and chloroamphenicol (200 μg/ml). RNA was obtained by phenol extraction and hybridized to ϕ80 *plac* DNA (see Materials and Methods): Control culture (○), R17 phage infected culture (●), Calf thymus DNA control (□).


Hybridization CPM 10^3
2
1
1
2
3
4
[RNA] CPM 10^5

Synthesis of β-galactosidase Enzyme

In addition to demonstrating the presence of a specific messenger RNA it is also possible to identify the protein transcribed from this m-RNA (81). Several of the amber mutants do not lyse host cells since coat protein is not produced (am C16, am B22, and am B24) (119,120). Using these amber mutants it was possible to observe the accumulation of β-galactosidase in infected cells without terminating enzymatic synthesis by rupturing the host cell. *E. coli* cultures were infected with these mutants, then thirty min after infection, each culture was induced for β-galactosidase with IPTG. The results in Fig. 16 showed that the synthetase mutant am C16 did not inhibit the synthesis of β-galactosidase. Also, am B24 which produced a reduced amount of synthetase showed only marginal inhibition. The greatest inhibition of β-galactosidase synthesis was associated with infection by the mutant which produced the greatest amount of viral synthetase, am B22. The results in Table 5 suggest that the degree of inhibition is related to the translation of the viral synthetase.

Functional Half-Life of β-galactosidase m-RNA

If infection with R17 phage inhibited host protein synthesis during translation, it was possible that the functional half-life of this messenger RNA would have been altered. Since R17 phage infection did not completely inhibit host protein synthesis, it was possible to

Figure 16

Beta-galactosidase synthesis in *E. coli* during infection with various amber mutants of R17 bacteriophage. *E. coli* 3000 was infected with amber mutants of R17: am B22 and am B24 (coat protein ● and ▲); and am C16 (polymerase ■) at 10 PFU/cell; control (○). Thirty min post infection the infected and control cultures were induced for β-galactosidase production with IPTG. Samples were collected at various times post infection and assayed for enzyme production (see Materials and Methods).

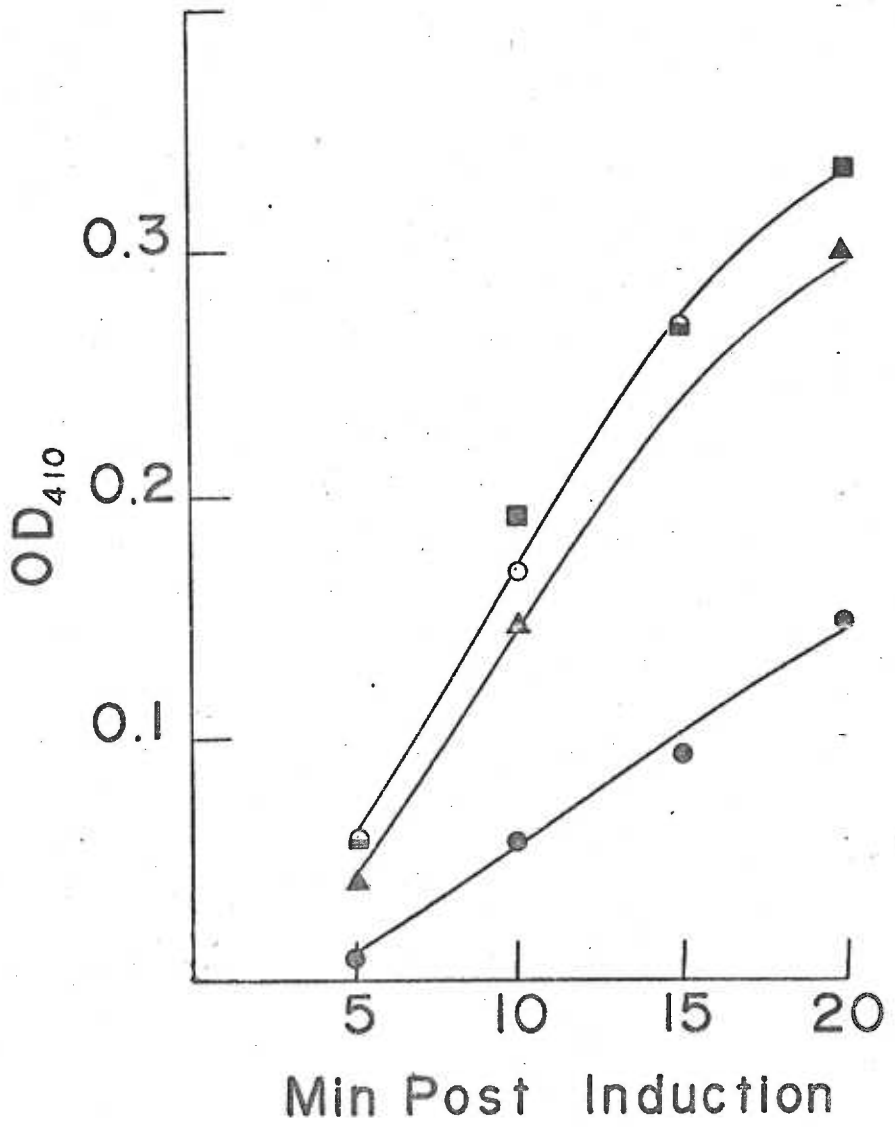

0.3
0.2
0.1
OD410
5
10
15
20
Min Post Induction

Table 5

Beta-galactosidase synthesis in *E. coli* during infection with various amber mutants of R17 bacteriophage. Data from Fig. 18 and Philips et al. (82).

Table 5

	Defective viral protein	Synthetase	Inhibition of β-galactosidase
R17 phage	none	normal	not done
am A31	maturation protein	normal	not done
am B22	coat protein	>normal	60%
am B24	coat protein	<normal	10%
am C16	synthetase	none	0%

examine the functional half-life of the m-RNA specific for β-galactosidase. This was done by inducing cells for 1 min with IPTG then removing the inducer by filtration. The cells were resuspended in medium devoid of inducer, and samples removed periodically to assay for enzyme production. By plotting the production of new enzyme against time, it was possible to determine the rate of degradation of the m-RNA translating β-galactosidase (functional half-life). When comparing the functional half-lives of β-galactosidase m-RNA in infected and control cells only a marginal inhibition was observed (Fig. 17). This suggested that inhibition of host protein synthesis did not involve the degradation of messenger RNA actually involved in the translation of protein synthesis.

Inhibition of Qβ Phage Production

During infection, R17 phage specific protein synthesis occurs within a host cell in which the synthesis of host proteins has been impaired (99). It was not known if the R17 phage was only capable of insuring its own replication in the infected cell or if a similar phage such as Qβ phage would have also been replicated. To answer this question, an *E. coli* S26 culture was superinfected with am B22 at various times after infection with Qβ phage. After two hr the titer of Qβ phage was determined. The results in Fig. 18 showed that during the first 30 min of Qβ infection, superinfection by am B22 inhibited Qβ phage production.

Figure 17

Half-life of functional m-RNA in *E. coli* 3000 infected with R17 bacteriophage. Thirty-five min post infection with R17 phage, an *E. coli* culture was induced for β-galactosidase with IPTG. The cells were rapidly filtered on Millipore filters and resuspended in TCG1-t medium. At various times post infection samples were withdrawn and assayed for enzyme production (control ○ ; R17 phage ●).

Emax = Maximum production of β-galactosidase.

Et = β-galactosidase at time of assay.

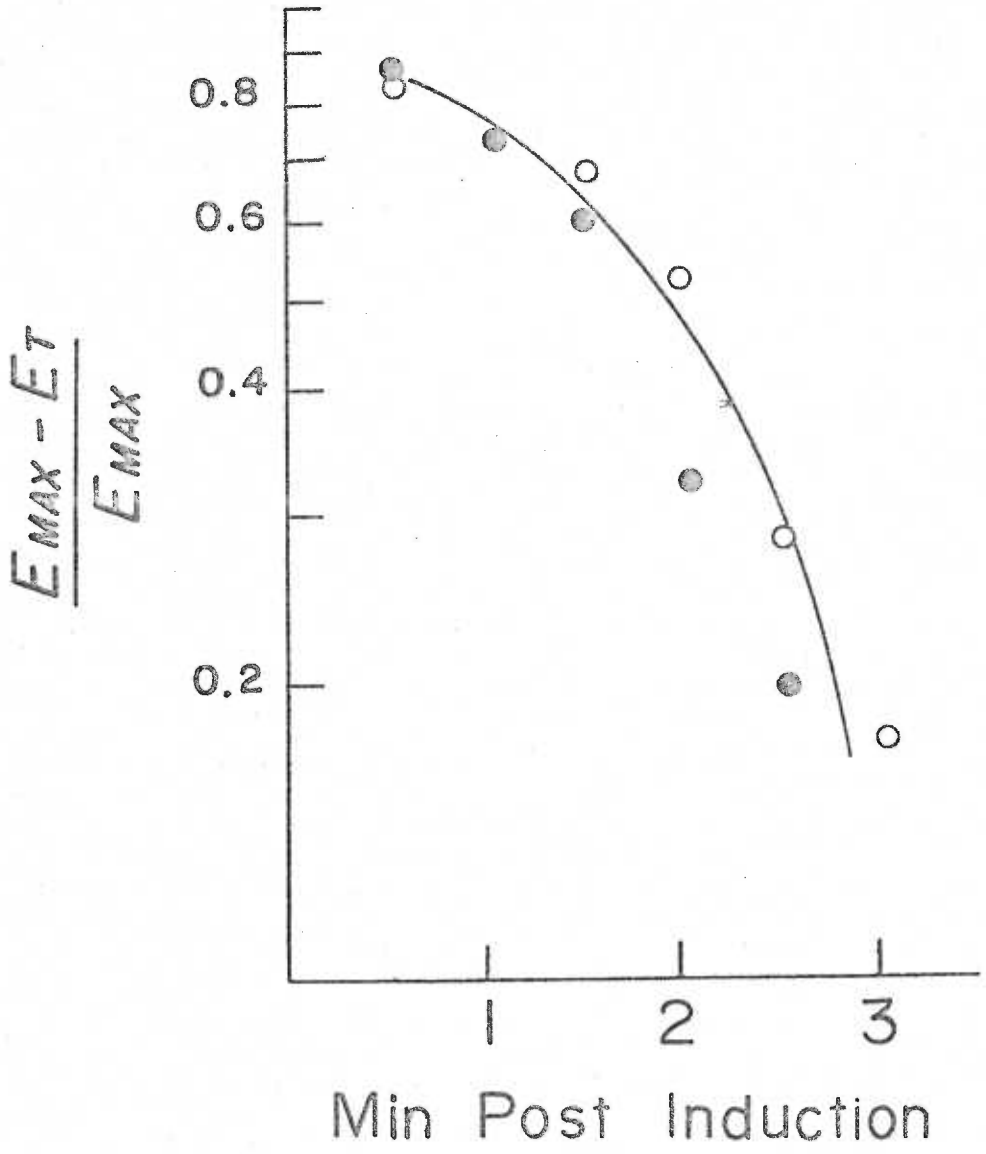
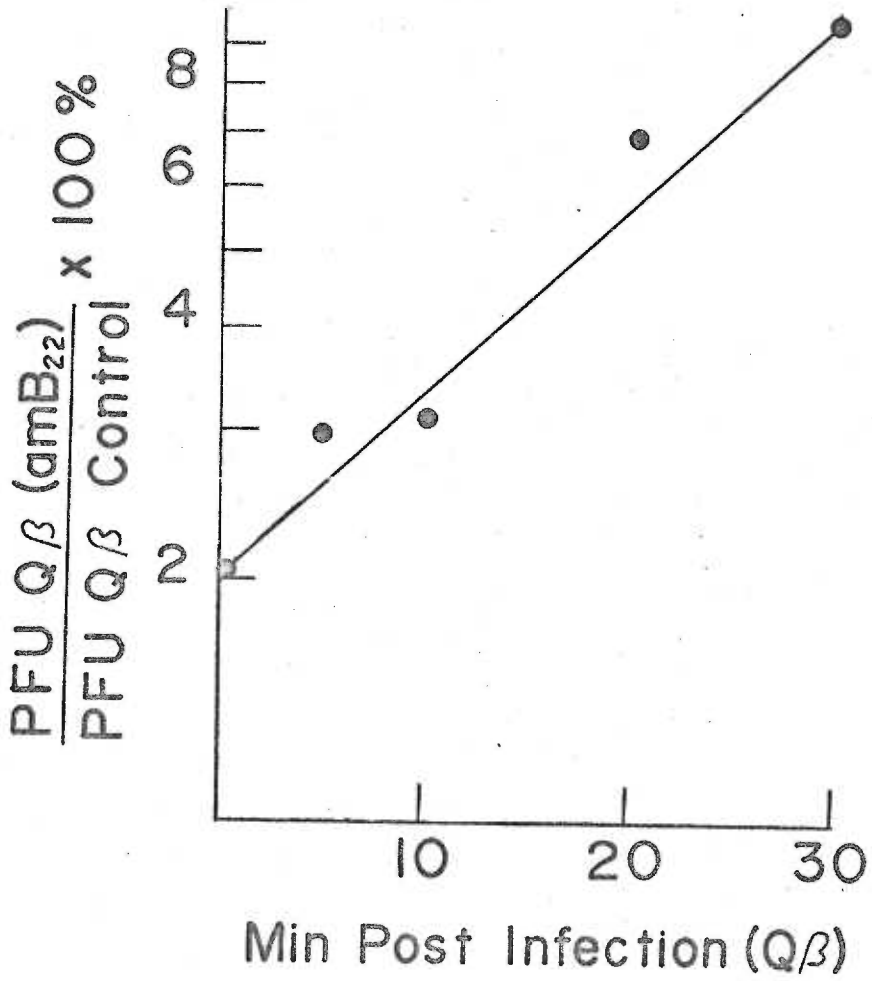

$\frac{E_{MAX} - E_T}{E_{MAX}}$
0.8
0.6
0.4
0.2
1
2
3
Min Post Induction

Figure 18

Inhibition of final Qβ phage titer by superinfection with am B22. Non-permissive (for am B22) *E. coli* 3000 cells were infected with Qβ phage (10 PFU/cell) at time 0. At various times post infection the Qβ phage infected cultures were superinfected with am B22 (10 PFU/cell). After a 2 hr incubation, all cultures were lysed with chloroform and the final Qβ phage titer determined by plaque assay with *E. coli* 3000. The results are compared to a control culture which was only infected with Qβ phage.


PFU Qβ (amB22) / PFU Qβ Control x 100 %
8
6
4
2
10
20
30
Min Post Infection (Qβ)

In order to determine which viral protein was responsible for inhibition of Qβ phage replication, host cells were infected with Qβ phage for 5 min then superinfected with mutants of R17 phage. After two hr of incubation at 37 C, the final Qβ phage titer was determined on *E. coli* S26. In Table 6, an examination of the burst size showed that Qβ phage production was inhibited by superinfection with mutants of the coat and maturation proteins. Inhibition was always observed if the viral synthetase genome was translated.

In addition to inhibiting the replication of Qβ phage, the R17 phage particle also competed with the Qβ phage particle for viral attachment sites on the host cell. This phenomenon was observed by inoculating *E. coli* S26 cultures with either Qβ phage or a mixture of Qβ phage and am C16, a mutant of R17 phage. As infection proceeded, samples were withdrawn and the number of Qβ infected cells was determined with the standard agar overlay technique described by Adams (1). The results in Fig. 19 showed that fewer host cells were infected with Qβ phage when the host culture was inoculated with both Qβ phage and am C16, the polymerase mutant of R17 phage. The ability of the mutant of R17 phage to protect host cells from Qβ phage infection is similar to the report by Zavada and Koutecha (117) in which UV irradiated R17 phage protected cells from infection by viable R17 phage.

Table 6

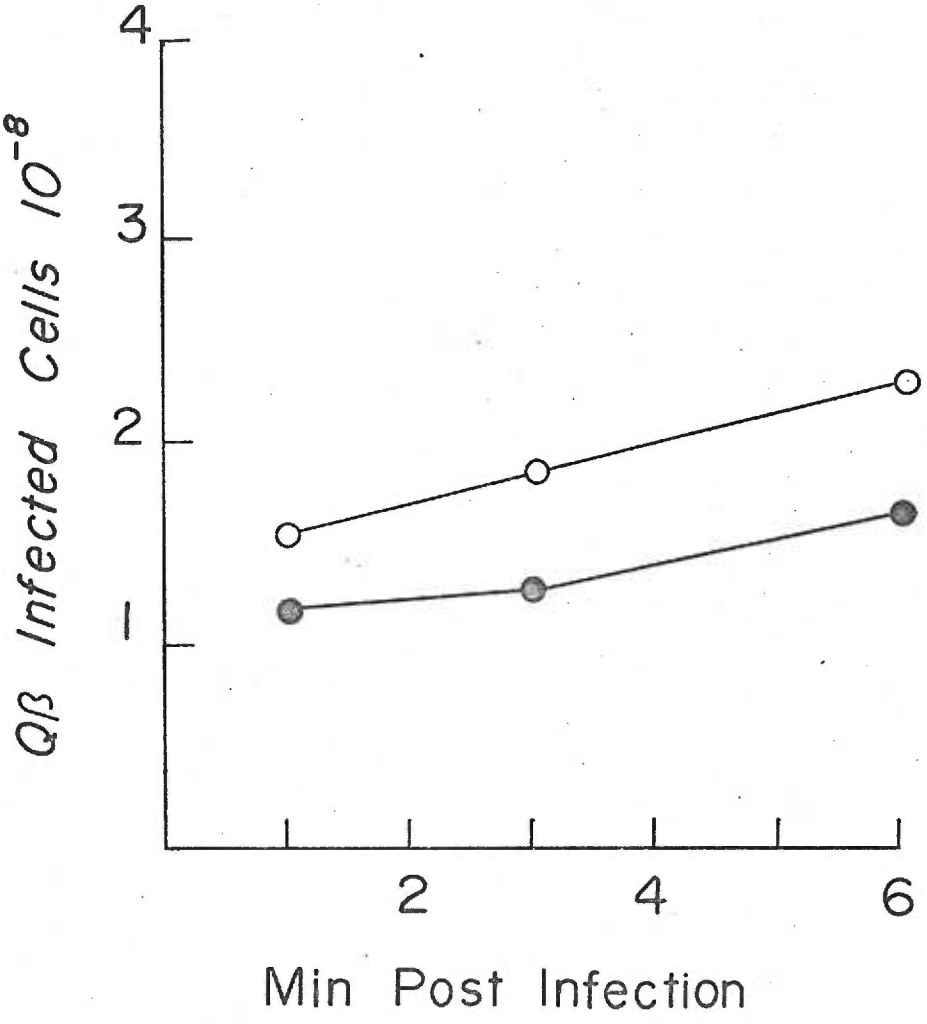
The Qβ phage-burst size of Qβ infected cells superinfected with amber mutants of R17 phage. *E. coli* S26 cultures were grown to 2 x 10^8 cells/ml in tryptone broth and infected with Qβ phage (10 PFU/cell), then 5 min post infection these cultures were superinfected with amber mutants of R17 phage. Samples were removed at 15 min post infection and the number of infected cells was determined by plaquing on *E. coli* S26. Total Qβ phage production was determined at 120 min post infection by titering the Qβ phage with *E. coli* S26 in the standard agar overlay technique (1).

Table 6

Superinfecting Phage	Qβ Bacteriophage			
	Infectious Centers 10^8	Titer 10^{11}	Burst Size	Inhibition of Phage Production
Qβ	2.6	2.4	935	-
am A31	1.0	2.0	500	46%
am B22	2.8	0.16	58	94%
am C16	1.5	1.5	962	0%

Figure 19

Protection of *E. coli* S26 cells from Qβ phage infection by simultaneous infection with am C16. *E. coli* S26 cultures (2×10^8 cells/ml in tryptone broth) were infected with either Qβ phage (10 PFU/cell ○) or a mixture of Qβ phage and am C16 (10 PFU/cell each ◉). At various times post infection samples were diluted a thousandfold in cold tryptone broth and collected by centrifugation. The number of infected cells was determined with the standard agar overlay technique described by Adams (1).


Qβ Infected Cells 10^{-8}
4
3
2
1
2
4
6
Min Post Infection

DISCUSSION

Early Inhibition

Infection of *E. coli* with R23 bacteriophage results in "early and late" stages of inhibition of host protein synthesis (108,109). In the original description Watanabe demonstrated that early inhibition was:

1. Encountered immediately after infection
2. Reversed after 10-15 min with no apparent damage to the host cell
3. Independent of the expression of the viral genome

Using amino acid pulse-labeling experiments, Yamazaki (116) demonstrated that the inhibition of host protein synthesis was the result of an inhibition of amino acid transport into the host cell. Since inhibition was independent of the expression of the viral genome, it was assumed that early inhibition was involved in some early stage of phage infection.

1. Confirmation of Early Inhibition

Yamazaki's (116) basic pulse-labeling experiment was repeated except a control was added which served as a baseline. In the experiment described in Fig. 3, it was established that during early inhibition there was a 50% decrease in the rate of incorporation of labeled amino acids into hot TCA precipitates, and recovery required approximately 10 min at 37 C.

Both the inhibition of amino acid transport and protein synthesis were verified in polyribosome studies. Amino acid starvation of an *E. coli* culture increased the monomer region without altering the heavy polyribosomes (Fig. 6); this observation was later confirmed by Ron (86). An examination of the polyribosome profile immediately after infection with the R17 phage suspension (R17 phage, tryptone broth and TCG1-t medium) revealed an increased monomer peak which subsided as recovery occurred (Fig. 5). This is consistent with Yamazaki's conclusion that inoculation of an *E. coli* culture with the R17 phage suspension decreased the rate of amino acid transport into the host cell.

Direct confirmation of the inhibition of protein synthesis was accomplished by observing the release of ^{14}C labeled polypeptides from heavy polyribosomes. Polyribosomes were extracted from infected and control cultures of *E. coli* which had been pulse-labeled with ^{14}C amino acids, then chased with a hundredfold excess of casamino acids. Chasing the control culture resulted in a 50% loss of labeled polypeptides associated with polyribosomes (Fig. 7a), while in the infected culture (Fig. 7b) chasing removed 15% of the label from the polyribosome region. Therefore, during early inhibition there was a 65% decrease in the release of nascent polypeptides from polyribosomes which is in agreement with the pulse-labeling experiment in Fig. 3 showing 50% inhibition of incorporation of labeled amino acids into hot TCA precipitates.

Because of the presence of tryptone broth in the viral suspension, it could have been argued that the observed inhibition was the result of a dilution of the specific activity of a labeled amino acid(s). This argument is unlikely, since inoculation of host cells with the R17 phage suspension inhibited the release of ^{14}C labeled polypeptides from the polyribosome region and altered the monomer peak. Both of these observations are independent of the specific activity of the amino acids present and are in total agreement with the work of Yamazaki (110) who demonstrated that a decreased rate of amino acid transport resulted in an inhibition of protein synthesis.

2. Early Inhibition is not a Function of Viral Infection

It is difficult to imagine that attachment of the virus particle to the F pili, a thin tubular structure protruding from the host cell (11), can inhibit the amino acid transport systems located in the cell membrane. Paranchych et al. (20) offered the hypothesis that there was a common energy intermediate required for both the transport of amino acids and for the alteration of the maturation protein during infection. Thus the inhibition of amino acid transport was explained in terms of an "energy crisis" in which altering the maturation protein depleted the host of the energy intermediate required to transport amino acids into the cell. Although this idea was in agreement with the published data, it did not explain the observation in Fig. 8 in

which early inhibition was shown to be temperature independent and thus can be distinguished from the temperature dependent eclipsing of phage activity (20).

Repeated infection with R17 phage (Fig. 10) showed that after recovery occurred, early inhibition could be induced a second time by another addition of the R17 phage suspension. In a study of the alteration of maturation protein Paranchych (20) showed that at 20 min post infection only 50% of the phage was inactivated when an *E. coli* culture was inoculated with R17 phage at 50 PFU/cell. Thus both the first and second recoveries from early inhibition occurred in the presence of viable phage. Therefore, it was conceivable that some component other than R17 phage was the agent responsible for early inhibition. Support for this hypothesis was obtained when early inhibition was observed upon the inoculation of *E. coli* F- and *S. typhimurium* with the R17 phage suspension (Table 2). Finally it was observed that purification of the virus on sucrose gradients removed the agent responsible for early inhibition (Fig. 11).

The importance of tryptone broth and TCG1-t medium in early inhibition was demonstrated in the experiment described in Fig. 14. Early inhibition was observed when *E. coli* cultures were inoculated with only tryptone broth and TCG1-t medium (suspending medium). The addition of R17 phage to the suspending medium had no influence on the degree of inhibition or the time required for recovery, and early inhibition was not observed when cultures were infected with

purified virus suspended in phosphate buffer.

Although early inhibition was not a phage function, it was not known if suspending R17 phage in tryptone broth would affect phage replication. In order to protect phage infectivity, Yamazaki (personal communication) suspended purified phage in tryptone broth. However, R17 phage was suspended in phosphate buffer without inactivating infectious particles (Table 4). Also, an examination of the eclipsing of infectious phage (Fig. 12) failed to reveal any significant difference between virus suspended in either phosphate buffer or tryptone broth. Finally, infection of *E. coli* cultures with phage suspended in either phosphate buffer or tryptone broth resulted in identical yields of progeny phage (Table 5).

Early inhibition was not observed when the phage was suspended in either phosphate or ST buffers (Fig. 11 and 14). Since phosphate buffer did not alter the infectivity of the parental virus or the yield of progeny phage (Table 4), early inhibition was not an intrinsic event associated with phage infection or replication. In the works of Yamazaki (116), Watanabe (108.109) and in all of the previous experiments of this paper early inhibition was always observed when an *E. coli* culture was inoculated with only tryptone broth. The presence of R17 phage had no influence on either the degree of inhibition or the time required for recovery.

3. Possible Mechanism for Early Inhibition

The experiments described in Table 3 demonstrated that both tryptone broth and TCG1-t medium induced the same degree of inhibition and required the same recovery time. It was assumed that tryptone broth and TCG1-t medium induced early inhibition by the same general mechanism. Further examination of TCG1-t medium revealed that its ability to induce early inhibition depended upon the presence of casamino acids (1.5 mg/ml). This suggested that the presence of amino acid in the phage inoculum induced early inhibition. This hypothesis was confirmed when a 70% inhibition was observed upon inoculation of an *E. coli* culture with a mixture of amino acids.

The data presented in Table 3 showed that early inhibition was observed upon the addition of TCG1-t medium to an *E. coli* culture growing in TCG1-t medium. The process of growing the *E. coli* cells to the concentration of 2×10^8 cells/ml made a subtle alteration in the medium. The total amino acid concentration had been decreased by less than 1% but the addition of fresh TCG1-t medium immediately inhibited protein synthesis by 55%. Apparently the difference between fresh and depleted medium was due to the subtle changes in the relative concentrations of the amino acids.

In *E. coli* cells group-specific permeases transport amino acids into the cell (83). For example, the neutral amino acids have been placed into the following groups:

1. alanine, serine, glycine
2. leucine, isoleucine, valine
3. tryptophan, tyrosine, phenylalanine
4. methionine

It has been shown in the case of valine and isoleucine that the alteration of the relative concentrations of these amino acids inhibits protein synthesis (12,14,15). Further, the addition of large amounts of the two amino acids does not alter protein synthesis if their relative concentrations remain the same. It is possible to explain early inhibition of protein synthesis as the result of an alteration in the relative concentration of amino acids in TCG1-t medium when the *E. coli* culture was inoculated with tryptone broth. This mechanism of inhibition of protein synthesis is completely consistent with Yamazaki's report that inoculation of an *E. coli* culture with virus suspended in tryptone broth resulted in an inhibition of amino acid transport (116).

Late Inhibition

1. Inhibition of Host Macromolecular Synthesis

Since early inhibition is a function of tryptone broth in the phage suspension, late inhibition is the only form of inhibition of host protein synthesis associated with phage infection. Sugiyama and Stone (99) have shown that during late inhibition there is a shift

from host to viral protein synthesis. At 25-30 min post infection, the synthesis of β-galactosidase was inhibited 50% (108,109). An examination of polyribosome profiles during late inhibition revealed a decrease in the heavy polyribosomes and an increase in the monomer, dimer and trimer regions (48). Iglewski (55) has shown that during late inhibition pulse-labeled heterogenous RNA enters the heavy polyribosomes. This strongly suggested that host messenger RNA was synthesized and translated into host proteins during late inhibition.

The hybridization experiment (Fig. 15) demonstrated that during late inhibition it was possible to induce the infected cells to synthesize β-galactosidase specific messenger RNA. Also, this messenger RNA could be translated into an enzymatically active protein (Fig. 16). It was possible to demonstrate the inhibition of host protein synthesis by infecting host cells with mutants which did not lyse the cell (99,119). When these infected cells were induced for β-galactosidase, it was observed that the mutants which produced little or no phage synthetase showed only a marginal inhibition of β-galactosidase synthesis. Conversely, the mutant (am B22) which produced the most viral synthetase showed the greatest inhibition of the production of β-galactosidase. Inhibition of host protein synthesis required the translation of R17 phage synthetase genome (53,82,99,108.109). Studies with temperature sensitive and amber mutants have shown that translation of the synthetase polypeptide resulted in an alteration of the polyribosome profile (82) and inhibited:

1. The incorporation of labeled amino acids into hot TCA precipitates (53)
2. Ribosomal RNA synthesis (90)
3. Qβ phage production (Table 5 and Fig. 18)
4. Beta-galactosidase synthesis (Fig. 16)

By using a temperature sensitive polymerase mutant of R17 phage, Igarashi (53) demonstrated that it was possible to inhibit host protein synthesis without producing viral RNA. However, the mechanism by which the phage synthetase polypeptide inhibited host protein synthesis is still unknown.

From the studies on the functional decay of m-RNA in infected cells late inhibition is not simply a degradation of m-RNA which was involved in the translation of host proteins (Fig. 17). Igarashi (53) has also demonstrated that when host cells were grown in minimal media, late inhibition of host protein synthesis occurred shortly after infection and preceded the inhibition of r-RNA synthesis by 30 min. This suggested that phage infection inhibited protein synthesis, then through a feedback control mechanism r-RNA synthesis was inhibited.

2. Inhibition of Qβ Phage Replication

Sugiyama and Stone (99) demonstrated that during infection with MS2 bacteriophage there is a shift from host protein to viral protein synthesis. It was further demonstrated that this was not a simple

shift from host protein synthesis. When the *E. coli* host was infected with a coat protein mutant of MS2 which overproduced viral synthetase, host protein synthesis was still inhibited even though the phage coat protein was not produced (Fig. 16) (99). Thus during phage infection host protein synthesis was inhibited at the same time that the coat protein genome was translated.

The experiments with Qβ phage suggested that the translation of the R17 phage genome was a very specific event which occurred during late inhibition. When Qβ phage infected cells were superinfected with am B22, an amber mutant of R17 phage, Qβ phage replication was inhibited (Fig. 18). This inhibition of Qβ phage replication was associated with the production of R17 viral synthetase. *E. coli* cultures which had been infected with Qβ phage were superinfected with various amber mutants of R17 phage. An examination of the Qβ phage burst size (Table 6) showed that superinfection with am C16, the mutant which does not produce synthetase, did not inhibit Qβ phage replication. Conversely, both am A31 (assembly mutant) and am B22 (coat protein mutant) inhibited Qβ phage replication. The inhibition of Qβ phage production was dependent on the expression of the genome coding for the R17 phage synthetase.

The fact that host protein synthesis and Qβ phage replication both were inhibited when the genome for R17 phage synthetase was translated suggested that the mechanism of inhibition may be the same. If this

assumption is valid, it is possible that information relevant to the inhibition of host protein synthesis may be obtained by studying the fate of Qβ RNA and coat protein in host cells infected with Qβ phage then superinfected with the temperature sensitive polymerase mutant of R17 phage described by Igarashi (53). The possible advantage of this system is that both Qβ RNA and coat protein can be readily identified on polyacrylamide gels. Also, if an *in vitro* system were desired to study the inhibition of host protein synthesis, the Qβ phage offers the advantage of already having a well-studied *in vitro* protein synthesizing system available.

SUMMARY AND CONCLUSION

Only late inhibition of host protein synthesis is associated with R17 bacteriophage infection. Early inhibition was encountered after an *E. coli* culture was inoculated with tryptone broth. From the studies with TCG1-t medium and the mixture of amino acids, it is suggested that early inhibition was the result of a subtle alteration in the relative concentrations of the various amino acids. Previous studies by Cohn (12,14,15) have shown that changing the relative concentrations of isoleucine and valine results in an inhibition of protein synthesis. This mechanism of inhibition is entirely consistent with the data of Yamazaki (116) and Watanabe (108,109).

Late inhibition was quantitatively related to the production of R17 viral synthetase (53,82,90). Igarashi (53) presented evidence that transcription of the R17 synthetase polypeptide directly or indirectly inhibited host protein synthesis. This conclusion was also arrived at by Watanabe (108), who was unable to prevent late inhibition in constituative mutants of *E. coli*.

Late inhibition is not an absolute phenomenon. During late inhibition both β-galactosidase messenger RNA and enzyme were synthesized. Although the total enzyme production was inhibited by 60%, the observed functional half-life of the message had not been significantly altered. Superinfection of Qβ infected *E. coli* cells with amber mutants of R17

phage inhibited the replication of Qβ phage. Although a transcriptional control has not been excluded, it appears that synthetase was capable of making the subtle distinction between R17 and Qβ viral RNA and insured the translation of its own genome.

REFERENCES

1. Adams, M.H. Bacteriophages. New York: Interscience Publishers, Inc. 1959.

2. Adensnik, M. and Levinthal, C. 1970. RNA metabolism in T4 infected *Escherichia coli*. J. Mol. Biol. 48, 187-208.

3. Ammann, J., Delius, H. and Hofschneider, P.H. 1964. Isolation and properties of an intact phage-specific replicative form of RNA phage M12. J. Mol. Biol. 10, 557-561.

4. Artman, M. and Ennis, H.T. 1972. Dissociation of lac messenger RNA transcription from translation during recovery from inhibition of protein synthesis. J. Bact. 110, 652-660.

5. August, J.T., Cooper, S., Shapiro, L. and Zuider, N.D. 1963. RNA phage induced RNA polymerase. Cold Spring Harbor Symp. Quant. Biol. 28, 95-97.

6. Bishop, D.H.L. 1965. RNA synthesis by *E. coli* C 3000/L after infection by the RNA coliphage Z1K/1 and properties of coliphage Z1K/1 RNA. Biochem. J. 97, 17-26.

7. Bishop, D.H.L. 1965. Ribonucleic acid synthesis by *E. coli* C 3000/L after infection by the RNA coliphage Z1K/1 and properties of the coliphage-induced double stranded RNA. Biochem. J. 100, 601-613.

8. Blumenthal, T., Landers, T.A. and Weber, K.A. 1972. Bacteriophage Qβ replicase contains the protein biosynthesis elongation factors EF-tu and EF-ts. Proc. Natl. Acad. Sci. U.S.A. 69, 1313-1317.

9. Boedtker, H. 1971. Conformation independent molecular weight determinations of RNA by gel electrophoresis. Biochim. Biophys. Acta 240, 448-453.

10. Bose, S.K. and Warren, R.J. 1969. Bacteriophage-induced inhibition of host functions. II. Evidence for multiple, sequential bacteriophage-induced deoxyribonucleases responsible for degradation of cellular deoxyribonucleic acid. J. Vir. 3, 549-556.

11. Brinton, C.C. Jr. 1965. The structure, function, synthesis and genetic control of bacterial pili and a molecular model for DNA and RNA transport in gram negative bacteria. Trans. N.Y. Acad. Sci. 27, 1003-1054.

12. Britten, R.J. and McClure, F.T. 1962. The amino acid pool in *Escherichia coli*. Bact. Rev. 26, 292-335.

13. Cantoni, G.L. and Davies, D.R. (Eds.) Procedures in nucleic acid research. New York: Harper and Row, 1966. (page 553)

14. Cohen, G.N. and Monod, J. 1957. Bacterial permeases. Bact. Rev. 21, 169-194.

15. Cohen, G.N. and Rickenberg, H.U. 1956. Concentration specifique reversible des amino acids chez *Escherichia coli*. Ann. de L'Institut Pasteur 91, 693-720.

16. Cooper, S. and Zinder, N. 1962. The growth of an RNA bacteriophage: The role of DNA synthesis. Vir. 18, 405-411.

17. Cooper, S. and Zinder, N. 1963. The growth of an RNA bacteriophage: The role of protein synthesis. Vir. 20, 605-612.

18. Crawford, E.M., Gestland, R.F. 1964. The adsorption of bacteriophage R17. Vir. 22, 165-167.

19. Danziger, R.E. and Paranchych, W. 1970. Stages in phage R17 infection. II. Ionic requirements for phage R17 attachment to F-pili. Vir. 40, 547-553.

20. Danziger, R.E. and Paranchych, W. 1970. Stages in phage R17 infection. III. Energy requirements for the F-pili mediated eclipse of viral infectivity. Vir. 40, 554-564.

21. Doi, R.H. and Spiegelman, S. 1962. Homology test between the nucleic acid of an RNA virus and the DNA in the host cell. Science 138, 1270-1272.

22. Eggens, K. and Nathans, D. 1969. Regulation of protein synthesis directed by coliphage MS2 RNA. II. In vitro repression by phage coat protein. J. Mol. Biol. 39, 293-305.

23. Ehrenfeld, E. and Hunt, R.T. 1971. Double-stranded poliovirus RNA inhibits initiation of protein synthesis by reticulocyte lysates. Proc. Natl. Acad. Sci. U.S.A. 68, 1075-1078.

24. Ellis, D.B. and Paranchych, W. 1963. Synthesis of ribonucleic and protein in bacteria infected with an RNA bacteriophage. J. Cell. Comp. Physiology 62, 207-213.

25. Enger, M.D. and Kaseberg, P. 1965. Comparative studies of the coat proteins of R17 and M12 bacteriophages. J. Mol. Biol. 13, 260-268.

26. Erikson, R.L., Fenwick, M.L. and Franklin, R.M. 1964. Replication of bacteriophage RNA: Studies on the fate of parental RNA. J. Mol. Biol. 10, 519-529.

27. Erikson, R.L., Fenwick, M.L. and Franklin, R.M. 1965. Replication of bacteriophage RNA: Some properties of the parental-labeled replicative intermediate. J. Mol. Biol. 13, 399-406.

28. Erikson, R.L. and Szybalski, W. 1964. The Cs_2SO_4 equilibrium density gradient and its application for the study of seven phage DNA glucosylation and replication. Vir. 22, 111-124.

29. Fenwick, M.L., Erikson, R.L. and Franklin, R.M. 1964. Replication of the RNA of bacteriophage R17. Science 146, 527-530.

30. Francke, B. and Hofschneider, P.H. 1966. Infectious nucleic acids of *Escherichia coli* bacteriophages. IX. Sedimentation constants and strand integrity of infectious M12 phage replicative form RNA. Proc. Natl. Acad. Sci. U.S.A. 56, 1883-1890.

31. Franklin, R.M. 1966. Purification and properties of the replicative intermediate of the RNA bacteriophage R17. Proc. Natl. Acad. Sci. U.S.A. 55, 1504-1511.

32. Fromageot, H.P.M. and Zinder, N.D. 1968. Growth of bacteriophage f2 in *E. coli* treated with rifampicin. Proc. Natl. Acad. Sci. U.S.A. 61, 184-191.

33. Gesteland, R.F. and Boedtker, H. 1964. Some physical properties of bacteriophage R17 and its ribonucleic acid. J. Mol. Biol. 8, 496-507.

34. Gillespie, D. and Spiegelman, S. 1965. A quantitative assay for DNA-RNA hybrids with DNA immobilized on a membrane. J. Mol. Biol. 12, 829-842.

35. Godson, G.N. 1968. Site of synthesis of v-RNA and phage assembly in MS2 infected *Escherichia coli* . J. Mol. Biol. 34, 149-163.

36. Godson, G.N. and Sinsheimer, R.L. 1967. Lysis of *Escherichia coli* with a neutral detergent. Biochim. Biophys. Acta 149, 476-488.

37. Godson, G.N. and Sinsheimer, R.L. 1967. The replication of bacteriophage MS2. VI. Interaction between bacteriophage RNA and cellular components in MS2 infected *Escherichia coli*. J. Mol. Biol. 23, 495-521.

38. Granboulan, N. and Franklin, R.M. 1966. High resolution autoradiography of *E. coli* cells infected with R17. J. Bact. 91, 849-857.

39. Granboulan, N. and Franklin, R.M. 1966. Electromicroscopy of viral RNA, replicative form and replicative intermediate of bacteriophage R17. J. Mol. Biol. 22, 173-177.

40. Groner, Y., Scheps, R., Kamen, R., Kolakofsky, D. and Revel, M. 1972. Host subunit of Qβ replicase translation control factor i. Nature New Biol. 239, 19-20.

41. Gurgo, C., Craig, E., Schlessinger, D. and Afolayan, A. 1971. Polyribosome metabolism in starved for an amino acid. J. Mol. Biol. 62, 525-535.

42. Gussin, G.N. 1966. Three complementation groups in bacteriophage R17. J. Mol. Biol. 21, 435-453.

43. Haraña, I. and Spiegelman, S. 1965. Specific template requirements of RNA replicase. Proc. Natl. Acad. Sci. U.S.A. 54, 579-587.

44. Haywood, A.M. and Sinsheimer, R.L. 1963. Inhibition of protein synthesis in *E. coli* protoplasts by actinomycin D. J. Mol. Biol. 6, 247-249.

45. Horinchi, K., Lodish, H.F. and Zinder, N.D. 1966. Mutants of the bacteriophage f2. VI. Homology of temperature-sensitive and host dependent mutants. Vir. 28, 438-447.

46. Horiuchi, K. and Matsuhashi, S. 1970. Three cistrons in bacteriophage Qβ . Vir. 42, 49-60.

47. Horiuchi, K., Webster, R.E. and Matsuhazhi, S. 1971. Gene products of bacteriophage Qβ. Vir. 45, 429-439.

48. Hotham-Iglewski, B. and Franklin, R.M. 1967. Replication of bacteriophage ribonucleic acid: Alterations of polyribosome patterns and association of double-stranded RNA with polyribosomes in *E. coli* infected with bacteriophage R17. Proc. Natl. Acad. Sci. U.S.A. 58, 743-749.

49. Hotham-Iglewski, B., Phillips, L.A. and Franklin, R.M. 1968. Viral RNA transcription-translation complex in *Escherichia coli* infected with bacteriophage R17. Nat. 219, 700-703.

50. Hudson, B. and Paranchych, W. 1967. Effect of bacteriophage R17 infection on host directed synthesis of ribosomal ribonucleates. J. Virol. 1, 529-537.

51. Hung, P.P. and Overby, L.R. 1968. Alteration of t-RNA (pro) during infection which gave specific advantage to Qβ. J. Biol. Chem. 243, 5525-5531.

52. Hunt, R.T. and Ehrenfeld, E. 1971. Cytoplasm from poliovirus-infected HeLa cells inhibits cell-free haemoglobin synthesis. Nature New Biol. 230, 91-94.

53. Igarashi, S.J., Elliot, J.F. and Bissonnette, R.P. 1970. Temperature sensitive mutation. V. Infection of ts-mutant of RNA phage and host cell metabolism. Can. J. Biochem. 48, 47-54.

54. Iglewski, W.J. 1969. The lack of messenger activity of ribonucleic acid complementary to the viral ribonucleic acid of R17. J. Mol. Biol. 244, 736-743.

55. Iglewski, W.J. 1971. Alterations in the distribution of labeled ribonucleic acid in polyribosomes from *Escherichia coli* infected with bacteriophage R17. J. Virol. 8, 446-454.

56. Iglewski, W.J. and Franklin, R.M. 1967. Denaturation and renaturation of viral RNA. III. Purification of the complementary strand of R17 RNA. Proc. Natl. Acad. Sci. U.S.A. 58, 1019-1024.

57. Kelley, R.B. and Sinsheimer, R.B. 1964. A new RNA component in MS2-infected cells. J. Mol. Biol. 8, 602-605.

58. Kelley, R.B. and Sinsheimer, R.L. 1967. The replication of bacteriophage MS2 structure and replication of the replicate intermediate. J. Mol. Biol. 29, 237-249.

59. Kelley, R.B. and Sinsheimer, R.L. 1967. The replication of bacteriophage MS2. VIII. Fractionation of the "replication intermediate" using a BNC column. J. Mol. Biol. 29, 229-236.

60. Kennell, D. 1970. Inhibition of host protein synthesis during infection of *Escherichia coli* by bacteriophage T4. II. Induction of host messenger ribonucleic acid and its exclusion from polysomes. J. Vir. 6, 208-217.

61. Kozak, M. and Nathans, D. 1971. Fate of maturation protein during infection by coliphage MS2. Nat. N.B. 234, 209-211.

62. Kozak, M. and Nathans, D. 1972. Translation of the genome of a ribonucleic acid bacteriophage. Bact. Rev. 36, 109-134.

63. Krahn, P.M., O'Callaghan, R.J. and Paranchych, W. 1972. Stages in phage R17 infection. VI. Injection of a protein into the host cell. Vir. 47, 628-637.

64. Kondo, M., Gallerani, R. and Weissmann, C. 1970. Subunit structure of Qβ replicase. Nat. 228, 525-527.

65. Lichstein, H.C. and Oginsky, E.L. Experimental microbial physiology. San Francisco: W.H. Freeman and Co., 1965.

66. Lodish, H.F., Cooper, S. and Zinder, N.D. 1964. Host-dependent mutants of the bacteriophage f2. IV. On the biosynthesis of a viral RNA polymerase. Vir. 24, 60-70.

67. Lodish, H.F. and Zinder, N.D. 1966. Mutants of bacteriophage f2. VIII. Control mechanism for phage-specific synthesis. J. Mol. Biol. 19, 333-348.

68. Loeb, T. and Zinder, N.D. 1961. A bacteriophage containing RNA. Proc. Natl. Acad. Sci. U.S.A. 47, 282-289.

69. Mills, D.R., Bishop, D.H.L. and Spiegelman, S. 1968. The mechanism and direction of RNA synthesis templated by free minus strands of a "little" variant of Qβ-RNA. Proc. Natl. Acad. Sci. U.S.A. 60, 713-720.

70. Mills, D.R., Pace, N.R. and Spiegelman, S. 1966. In vitro synthesis of a noninfectious complex containing biologically active viral RNA. Proc. Natl. Acad. Sci. U.S.A. 56, 1778-1785.

71. Nathans, D., Oeschger, M.P., Polmar, S.K. and Eggens, K. 1969. Regulation of protein synthesis directed by coliphage MS2 RNA. I. Phage protein and RNA synthesis in cells infected with suppressible mutants. J. Mol. Biol. 39, 279-292.

72. Nomura, M., Matsubara, K., Okamoto, K. and Fjuimura, R. 1962. Inhibition of host nucleic acid and protein synthesis by bacteriophage T4: Its relation to the physical and functional integrity of host chromosome. J. Mol. Biol. 5, 535-549.

73. Nygaard, A.P. and Hall, B.D. 1963. Formation and properties of RNA-DNA complexes. Biochem. Biophys. Res. Comm. 9, 125-142.

74. Nygaard, A.P. and Hall, B.D. 1963. A method for the detection of RNA-DNA complexes. Biochem. Biophys. Res. Comm. 12, 98-104.

75. Pace, N.R., Bishop, D.H.L. and Spiegelman, S. 1968. The immediate precursor of viral RNA in the Qβ replicase reaction. Proc. Natl. Acad. Sci. U.S.A. 59, 139-144.

76. Pace, N.R. and Spiegelman, S. 1966. In vitro synthesis of an infectious mutant RNA with a normal RNA replicase. Science 153, 64-67.

77. Paranchych, W. 1966. Stages in phage R17 infection: The role of divalent cations. Vir. 28, 90-99.

78. Paranchych, W., Ainsworth, S.K., Dick, A.J. and Krahn, P.M. 1971. Stages in phage R17 infection. V. Phage eclipse and the role of F pili. Vir. 45, 615-628.

79. Paranchych, W. and Graham, A.F. 1962. Isolation and properties of an RNA-containing bacteriophage. J. Cell. Comp. Physiology 60, 199-208.

80. Paranchych, W., Krahn, M. and Bradley, R.D. 1970. Stages in phage R17 infection. Vir. 41, 465-473.

81. Parde, A.B., Jacob, F. and Monod, J. 1959. The genetic control and cytoplasmic expression of "inducibility" in the synthesis of β-galactosidase by *E. coli*. J. Mol. Biol. 1, 165-178.

82. Phillips, L.A., Truden, J.L., Iglewski, W.J., Hotham-Iglewski, B. and Franklin, R.M. 1969. Replication of bacteriophage ribonucleic acid: Alternating in polyribosome patterns in *E. coli* infected with amber mutants of bacteriophage R17. Vir. 39, 781-790.

83. Piperno, J.R. and Oxender, D.L. 1968. Amino acid transport systems in *Escherichia coli* K12. J. Biol. Chem. 243, 5914-5920.

84. Robertson, H.D., Webster, R.D. and Zinder, N.D. 1968. Bacteriophage coat protein as repressor. Nature (London) 218, 533-536.

85. Roberts, J.W. and Steitz, J.A. 1967. The reconstitution of infective bacteriophage R17. Proc. Natl. Acad. Sci. U.S.A. 58, 1416-1421.

86. Ron, E.Z. 1971. Polysome turnover during amino acid starvation in *Escherichia coli*. J. Bacteriol. 108, 263-268.

87. Saito, H. and Miura, K. 1963. Preparation of transforming deoxyribonucleic acid by phenol treatment. Biochim. Biophys. Acta 73, 619-629.

88. Simon, E.H. 1962. Evidence for the nonparticipation of DNA in viral RNA synthesis. Vir. 13, 105-118.

89. Sinsheimer, R.L., Starman, B., Nagler, C. and Guthrie, S. 1962. The process of infection with bacteriophage ØX 174. I. Evidence for a "replicative form." J. Mol. Biol. 4, 142-160.

90. Spangler, V.L. and Iglewski, W.J. 1972. The effect of R17 phage specific proteins on ribosomal RNA synthesis of *Escherichia coli*. J. Vir. 9, 792-799.

91. Spiegelman, S., Haruna, I., Holland, I.B., Beaudreau, G.B. and Mills, D. 1965. The synthesis of a self-propagating and infectious nucleic acid with a purified enzyme. Proc. Natl. Acad. Sci. U.S.A. 54, 919-927.

92. Stavis, R.L. and August, J.T. 1970. The biochemistry of RNA bacteriophage replication. Ann. Rev. Biochem. 527-560.

93. Steitz, J.A. 1968. Identification of the A protein as a structural component of bacteriophage R17. J. Mol. Biol. 33, 923-936.

94. Stretton, A.O.W. and Brenner, S. 1965. Molecular consequences of the amber mutation and its suppression. J. Mol. Biol. 12, 456-465.

95. Sugiyama, T. 1964. Translational control of MS2 RNA cistrons. Cold Spring Harbor Symp. Quant. Biol. 34, 687-694.

96. Sugiyama, T. and Nakada, D. 1967. Control of translation of MS2 RNA cistrons by MS2 coat protein. Proc. Natl. Acad. Sci. U.S.A. 57, 1744-1750.

97. Sugiyama, T. and Nakada, D. 1968. Translational control of bacteriophage MS2 RNA cistrons by MS2 coat protein: Polyacrylamide gel electrophoretic analysis of proteins synthesized in vitro. J. Mol. Biol. 31, 431-440.

98. Sugiyama, T. and Nakada, D. 1970. Translational control of bacteriophage MS2 RNA cistrons by MS2 coat protein: Affinity and specificity of the interaction of MS2 coat protein with MS2 RNA. J. Mol. Biol. 48, 349-355.

99. Sugiyama, T. and Stone, H.O. 1968. Viral and bacterial protein synthesis in MS2 infected *E. coli* cells. J. Mol. Biol. 36, 91-105.

100. Thach, S.S. and Thack, R.E. 1973. Mechanism of viral replication. I. Structure of replication complexes of R17 bacteriophage. J. Mol. Biol. 81, 367-380.

101. Tooze, J. and Weber, K. 1967. Isolation and characterization of amber mutants of bacteriophage R17. J. Mol. Biol. 28, 311-330.

102. Truden, J.L. and Franklin, R.M. 1972. Polysomal location of R17 bacteriophage specific protein synthesis. J. Vir. 9, 75-84.

103. Valentine, R.C. and Strand, M. 1965. Complexes of F- pili and RNA bacteriophage. Science 148, 511-513.

104. Vasquez, C.N., Granboulan, N. and Franklin, R.M. 1966. Structure of the ribonucleic acid bacteriophage R17. J. Bacteriol. 92, 1779-1786.

105. Vinuela, E., Aegranati, I.D., Feix, G., Garves, D., Weissmann, C. and Ochoa, S. 1968. Virus-specific proteins in *Escherichia coli* infected with some amber mutants of phage MS2. Biochim. Biophys. Acta 155, 558-565.

106. Warren, R. and Bose, S.K. 1968. Bacteriophage-induced inhibition of host functions. I. Degradation of deoxyribonucleic acid after T4 infection. J. Vir. 2, 327-334.

107. Wargel, R.J., Chadur, C.A. and Neuhaus, F.C. 1971. Mechanism of D-alanine, D cycloserine and glycine. J. Bacteriol. 105, 1028-1035.

108. Watanabe, H. and Watanabe, M. 1968. Effect of infection with ribonucleic acid bacteriophage R23 on the inducible synthesis of ß-galactosidase in *Escherichia coli*. J. Vir. 2, 1400-1407.

109. Watanabe, H. and Watanabe, M. 1970. Expression of the lac operon in *Escherichia coli* infected with RNA phage R23. Can. J. Microbiol. 16, 713-717.

110. Watanabe, H. and Watanabe, M. 1971. Effect of infection with R23 on DNA and RNA synthesis in *Escherichia coli*. Can. J. Microbiol. 17, 461-466.

111. Watanabe, I., Miyake, T., Saurai, T., Shiba, T. and Ohno, T. 1967. Isolation and grouping of RNa phages. Proc. Japan. Acad. 43, 204-209.

112. Watanabe, I., Nishihara, T., Kaneko, H., Sakurai, T. and Osawa, S. 1967. Group characteristics of RNA phages. Proc. Japan. Acad. 43, 210-213.

113. Weissmann, C. and Borst, P. 1963. Double-stranded ribonucleic acid formation in virto by MS-2 phage-induced RNA synthesis. Science 142, 1188-1191.

114. Weissmann, C., Feix, G. and Slor, H. 1968. In vitro synthesis of phage RNA: The nature of the intermediates. Cold Spring Harbor Symp. Quant. Biol. 33, 83-100.

115. Weissmann, C., Simion, L., Borst, P. and Ochoa, S. 1963. Induction of RNA synthesis in *E. coli* after infection by the RNA phage, MS2. Cold Spring Harbor Symp. Quant. Biol. 28, 99-104.

116. Yamazaki, H. 1969. The mechanism of early inhibition by an RNA phage of protein and RNA synthesis in infected cells. Vir. 37, 429-436.

117. Zavada, V. and Kontecka, E. 1965. Interference effect of non-infectious particles in infection with phage f2. Folia Microbiologica (Praha) 10, 345-351.

118. Zinder, N. 1963. Properties of a bacteriophage containing RNA. Perspectives Virol. 3, 58-67.

119. Zinder, N.D. 1965. RNA phages. Ann. Rev. Micro. 19, 455-472.

120. Zinder, N.D. and Lyons, L.B. 1968. Cell lysis: Another function of coat protein of the bacteriophage f2. Science 159, 84-85.