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PHAGE R17 INFECTION AND THE ROLE OF F-PILI

by

PETER MARTEN KRAHN

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "PHAGE R17 INFECTION AND THE ROLE OF F-PILI", submitted by Peter M. Krahn in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Wm Paranchych
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ABSTRACT

Investigations have been performed to characterize the role of F-pili in the infection of Escherichia coli with an RNA bacteriophage (R17).

An examination of the interaction of highly purified R17 phage preparations with host cells at 4° demonstrated that phage are heterogeneous with respect to attachment, infectious phage being adsorbed preferentially. About 10% of wild-type phage particles were found to be devoid of A protein and exhibited no interaction with host cells.

Studies on the interaction of phage R17 with E. coli at 37° indicated the presence of three phage classes, only one of which contains infectious phage. It was shown that an initially rapid attachment of phage to host cells at this temperature was followed by an elution of empty or partially empty phage capsids into the medium. Further studies revealed that while all phage containing A protein (~90%) interact with cell-associated F-pili, only about 30% of the phage population is capable of injecting its RNA into host bacteria.

Experiments on the eclipse of phage R17 indicate that this reaction is complete by five minutes after phage addition and that the A protein is removed from the phage particle by this process. The total eclipsing capability of a single host cell was found to be about 250 PFU.

Approximately equimolar amounts of phage A protein and RNA were observed to penetrate into host cells during infection suggesting that these two components enter the cell as a complex. Using SDS-polyacrylamide gel electrophoresis, it was demonstrated that the A protein is fragmented into three polypeptides having an estimated molecular weight of 24,000, 15,000 and less than 10,000 daltons. Because these

fragments were found associated with F-pili, it was concluded that F-pili function in the cleavage of the phage A protein.

An analysis of the nucleoside triphosphate levels in cultures before and after infection demonstrated that large amounts of cellular energy are utilized in the phage infective process. The degree of energy consumption was dependent on the multiplicity of infection and appeared to be complete by 5 min after infection.

A direct test of the "F-pili retraction" model for RNA phage infection by a detailed electron-microscope examination of cell-associated F-pili, and phage attachment studies of cultures before and after infection has been conducted. The results of this study showed that while F-pili shorten considerably during infection, this shortening is due to fragmentation of F-pili rather than retraction.

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LIST OF ABBREVIATIONS

DNA	deoxyribonucleic acid
RNA	ribonucleic acid
NMP	nucleoside 5'-monophosphate
AMP	adenosine 5'-monophosphate
UMP	uridine 5'-monophosphate
CMP	cytidine 5'-monophosphate
GMP	guanosine 5'-monophosphate
NTP	nucleoside 5'-triphosphate
ATP	adenosine 5'-triphosphate
UTP	uridine 5'-triphosphate
CTP	cytidine 5'-triphosphate
GTP	guanosine 5'-triphosphate
PFU	plaque forming units
TCA	trichloroacetic acid
Ci	Curie - 2.22×10^{12} disintegrations per minute
g	centrifugal force relative to gravity
SDS	sodium dodecyl sulfate
ME	2-mercaptoethanol
Lys	lysine
Arg	arginine
His	histidine
Ser	serine
Tyr	tyrosine
Asp	aspartic acid
Glu	glutamic acid

EDTA	ethylenediaminetetraacetic acid
rpm	revolutions per minute
cpm	counts per minute
moi	multiplicity of infection
Tris	Tris (hydroxymethyl) aminomethane
NaDS	sodium dextran sulfate
PEG	polyethylene glycol
PEI	polyethyleneimine
RNase	ribonuclease
DNase	deoxyribonuclease
TMM	Tris (hydroxymethyl) aminomethane maleic acid minimal salts medium
TSB	trypticase soy broth
SSC	standard saline citrate 0.15 M NaCl, 0.015 M sodium citrate
TEMED	tetramethylethylenediamine
A_{650} A_{260}	light absorbancy of a solution in a 1 cm light path at 650 mμ (260 mμ)
S (following a number)	sedimentation constant in Svedberg units (10^{-13} sec)

All temperatures are expressed in degrees Centigrade except when specified otherwise.

CHAPTER I

INTRODUCTION

The discovery of the first RNA bacteriophage f_2, a phage specific for male strains of Escherichia coli (Loeb & Zinder, 1961), disproved what had appeared to be a rule that all bacterial viruses must contain DNA. Since that discovery, numerous other closely related members of this family, generally referred to as the f_2 group, have been isolated and characterized throughout the world (Paranchych & Graham, 1962; Davis et al., 1961; Hofschneider, 1963; Hoffmann-Berling et al., 1963; Nonoyama, 1963) and a considerable body of data has now accumulated on their molecular characteristics (see reviews, Zinder, 1965; Hoffmann-Berling et al., 1966; Kaper, 1968; Stavis & August, 1970).

Briefly, RNA phages of this class have a molecular weight of about 3.6×10^6 daltons (Gesteland & Boedtker, 1964) of which approximately 1.1×10^6 daltons are accounted for by the single strand of RNA which is about 3300 nucleotides in length. Native phage particles are comprised of 180 identical coat protein subunits arranged around the compact phage genome in icosahedral symmetry; i.e., 12 pentamers and 20 hexamers (Vasquez et al., 1966). In addition, each phage particle contains a single copy of a minor component (M.W. ~40,000) known as the "A" protein (Steitz, 1968b). The members of the f_2 phage group are probably "mutational variants" of a common ancestor since the only distinguishing feature among them can generally be attributed to minor amino acid replacements in the coat protein (Enger & Kaesberg, 1965; Weber, 1967) or base substitutions in its RNA sequence (Thirion & Kaesberg, 1968, 1970).

The genetic simplicity of the RNA phages, i.e., three cistrons in a single-stranded RNA molecule (Horiuchi et al., 1966; Gussin, 1966) has made them particularly amenable to in vivo and in vitro studies of the molecular aspects of virus replication. This is reflected in the fact that progress in this area has been extremely rapid in the last few years and numerous reviews have now appeared on this subject (Spiegelman et al., 1967; Hofschneider et al., 1967; Weissman, 1967; August et al., 1970; Stavis & August, 1970).

The protein products of the three cistrons (A, B and C) of phage R17 have now been identified and characterized. The proteins, which appear to be encoded by the same cistron in each of the related phages are the following:

(i) The histidine-free coat protein encoded by the B cistron (Notani et al., 1965; Capecchi & Gussin, 1965; Tooze & Weber, 1967);

(ii) The viral RNA synthetase specified by the C cistron (Capecchi, 1966; Lodish & Zinder, 1966; Tooze & Weber, 1967; Gussin, 1966); and

(iii) A minor structural component designated the "A" protein which is synthesized under the direction of the A cistron (Steitz, 1968a, b).

In vitro studies on the translation of phage RNA in extracts of E. coli have shown that all three phage proteins specified by the polycistronic messenger are initiated with N-formylmethionine as their N-terminal amino acid (Lodish, 1968; Lodish & Robertson, 1969). These findings have encouraged numerous studies on the primary structure of phage RNA to elucidate initiation codons (AUG or GUG) and termination

codons (UAA, UAG, UGA) at or near the 5' and 3' ends respectively. It was hoped that in this way the nucleotide sequence of phage RNA might be related by the genetic code to a sequence of amino acids in one of the phage-specified proteins. These studies have been greatly augmented by the elegant RNA sequencing techniques developed by Sanger and his colleagues (Sanger et al., 1965; Brownlee & Sanger, 1967, 1969; Brownlee et al., 1968) which allowed large RNA fragments to be rapidly sequenced. Using these techniques, Steitz (1969) has elucidated the initiation sequences of the three R17 phage proteins from fragments of RNA obtained from R17 RNA-ribosome complexes formed under conditions of polypeptide chain initiation. More recently, Jeppeson et al. (1970) have deduced the gene order of the R17 genome as "5' end - A protein - coat protein - synthetase - 3' end" on the basis of the distribution of phage protein initiation sites between two specific R17 RNA fragments (Spahr & Gesteland, 1968). Similar investigations have revealed the presence of large regions of untranslatable nucleotide sequences at the extremities and somewhat smaller ones in the intracistronic regions (Nichols, 1970). Although little is presently known about the function of such regions it has been suggested that they may facilitate the binding of ribosomes specifically to initiation codons bordering such regions (Steitz, 1969).

Although much information presently exists with respect to physical and chemical properties of RNA phages, as well as the replicative events associated with phage infection, considerably less attention has been directed towards the study of the early extracellular stages of infection of E. coli by RNA phages. The isolation of a number of phages which grew only on male strains (F^+ or Hfr) and did not adsorb

to female (F^-) at all (Loeb, 1960) implied that males contain a receptor substance which allows phage to adsorb and specifically infect only male cells. The Ørskovs (1960) had already established that the male produced a male substance called the F-antigen. Strauss and Sinsheimer (1963) also suggested that male E. coli cells contain some unique feature which confers sensitivity to RNA phages and that this specificity probably lay in the adsorption and penetration steps of infection since spheroplasts of female cells and male cells were equally effective in supporting phage replication when infected with isolated phage RNA. Although Loeb and Zinder (1961) concluded that the general features of RNA phage adsorption and intracellular growth resemble those of DNA phages, investigations by other workers in this area proved to be entirely inconclusive, and the reasons for the widely variable results obtained in different laboratories remained obscure. It was not until 1964, when Crawford and Gesteland examined with the use of the electron microscope the possibility that RNA phages might require a cellular mating site, that systematic studies on the early stages of infection became possible. They observed that when purified phage R17 was added to a culture of E. coli K12 Hfr_1 cells, phage particles adsorbed specifically to long filamentous structures rooted in the cell wall, but showed no special affinity for the cell wall of either male or female cells.

It immediately became obvious that these filaments might be a necessary requirement for phage adsorption and penetration in the normal infective process of male-specific phages. Brinton, who had already devoted several years to the study of various other types of E. coli surface appendages, immediately confirmed the involvement of

these filaments in specific phage attachment, demonstrating that this property was determined genetically by the presence of the fertility (F) factor and named these filaments "F-pili" (Brinton et al., 1964).

Subsequent characterization of F-pili revealed them to be quite fragile and extremely sensitive to shear forces; frequently even a mild centrifugation appeared to break off as much as 50% of the F-pili (Brinton, 1965). Moreover, treatment of cells in a blendor resulted in the total removal of pili from cells as seen by a complete loss of the cells' phage attachment capacity (Brinton, 1966). Physiological studies moreover revealed the degree of piliation to be dependent on the stage of the growth cycle; maximum piliation in all cases occurred during the exponential growth phase. Cells in the stationary phase were only sparsely piliated and supported infection only weakly, if at all (Brinton & Beer, 1967). These observations thus clarified the uncertainties surrounding earlier investigations on the RNA phage infective process. It became clear, for example, that the vigorous washing procedures formerly employed in the removal of unadsorbed phage stripped F-pili from host cells in an unpredictable fashion and hence affected the cells' ability to support the functions associated with phage infection.

F-pili, as characterized by Brinton (1965), are long narrow tubular rods; usually only a single pilus is found associated with each cell. These structures are morphologically defined by their ability to adsorb large numbers of RNA phages along their lateral axis, or a single filamentous DNA phage at their distal end (Caro & Schnös, 1966). They have a diameter of 80-85 Å and appear to have a central axial hole (20-25 Å diameter) running the length of the F-pilus. The length of F-pili

has been observed to vary in electron micrographs, ranging from less than 1μ to as much as 20μ from the cell surface (Valentine & Strand, 1965; Lawn, 1966). Although the function of F-pili in phage attachment may be completely destroyed by treatment of cells with proteolytic enzymes and organic solvents (Wendt et al., 1966; Brinton & Beer, 1967) supporting the idea that they are protein in nature, little is known about their chemistry and this conclusion must await direct chemical analysis. Interestingly, preliminary studies in progress in this laboratory (J. Donelian, personal communication) suggest that they may contain large amounts of carbohydrate material.

Brinton (1965) has proposed a biological function for F-pili suggesting that they serve as hollow "conduction tubes" through which the chromosome of donor (Hfr) cells is transferred to recipient (F^-) cells. It was further postulated that they act as a channel through the cell membrane for the entry of nucleic acids of RNA and DNA male-specific phages into the cell during infection. There has, in fact, been much correlative evidence in support of such a dual involvement of F-pili in bacterial mating and male-specific phage infection:

(i) Appearance of F-pili, fertility and sensitivity to male-specific phages have been demonstrated to be determined by the same genetic element (Brinton et al., 1964).

(ii) Elimination of the F-factor by treatment of cells with acridine dyes totally abolishes their susceptibility to phage and also prevents the formation of mating pairs.

(iii) Mechanical removal of F-pili by blending similarly leads to a temporary loss of these two functions which reappear upon pili regeneration (Novotny et al., 1969a).

(iv) Lastly, this view is supported by the observation that there is a complete inhibition of mating-pair formation when male-specific phages are allowed to adsorb to donor cells prior to mating (Ippen & Valentine, 1967; Knolle, 1967a; Novotny et al., 1968).

Despite the fact that the precise chemical nature of F-pili is largely unknown, and that the presence of nucleic acid in F-pili has not yet been demonstrated, there is presently much reason to believe that F-pili are intimately associated with nucleic acid transport. The F-pilus of E. coli thus lays claim to a unique function unknown to any other cellular organelle.

The early stages of RNA phage infection (operationally defined as those events occurring prior to the onset of intracellular phage replication) are probably a series of dynamic events in which the RNA phage genome is liberated from the enveloping capsid and transported through the cell wall into the host cytoplasm. These early events of the F-pili-mediated RNA phage infective process have been divided into the following stages for experimental purposes in this laboratory:

(i) Adsorption of phage to the F-pilus;

(ii) Phage eclipse. An interaction of phage with cell associated F-pili which sensitizes their RNA to the action of RNase;

(iii) Penetration of phage RNA into host cells.

Phage readily form complexes with cell-free or cell-associated F-pili even at 0° and in the absence of an energy source. Knolle (1967b) has suggested that phage adsorption is diffusion controlled, since the

rate of attachment is strongly temperature-dependent. Although Valentine and Strand (1965) reported a role for divalent cations in this process, it now appears that the only ionic requirement for attachment of male-specific DNA and RNA phage is satisfied by an ionic strength of 0.08 or greater by either mono- or divalent cations (Tzagoloff & Pratt, 1964; Danziger & Paranchych, 1970a).

An extensive kinetic analysis of adsorption of R17 phage to E. coli HB11 has been conducted by Brinton and Beer (1967) who concluded that "phage particles are weakly and reversibly adsorbed to the sides of free and attached F-pili with an equilibrium constant of 5×10^9 ml/particle at 0°". Under these conditions, phage particles remained unchanged as evidenced by no loss in phage viability. This is consistent with the observations of Valentine and Strand (1965) who noted that phage particles remain infectious after binding to cell-free F-pili fragments, indicating the reversible nature of the adsorption reaction.

Several lines of evidence indicate that the A protein is involved in phage attachment to F-pili of host cells. The earliest report along this line comes from Lodish et al. (1965) who first observed that a certain group of f_2 phage mutants (temperature-sensitive and amber mutants) failed to adsorb to host bacteria. Amber mutants of this type, moreover, differed from infectious phage by being sensitive to RNase degradation (Heisenberg, 1966, 1967). No defect in the coat protein, or RNA as judged by its infectivity to spheroplasts, could be demonstrated in these mutants (Argetsinger & Gussin, 1966; Lodish et al., 1965). The most convincing evidence in support of the idea that A protein is an attachment protein comes from

R17 phage reconstitution experiments performed by Roberts and Steitz (1967). These workers noted that when small amounts of purified A protein were included in such reconstitution mixtures, the yield of infectious particles increased by several hundred-fold. Although these data strongly argue for the involvement of the A protein in phage attachment, final proof for this concept will require the direct demonstration of the attachment of isolated A protein to F-pili.

The second stage of the RNA phage infection, "phage eclipse", is a temperature-dependent reaction (Danziger, 1968) which ensues immediately after attachment of phage to actively-metabolizing host cells at 37°. The dependency of this process on a cellular energy source — possibly ATP, is indicated by studies in which ATP levels of host cells have been correlated to their ability to eclipse phage (Danziger & Paranchych, 1970b). Phage eclipse, like that of attachment, exhibits no need for divalent cations, and proceeds normally even when all divalent cations are chelated by EDTA (Paranchych, 1966). Since phage RNA becomes transiently susceptible to attack by RNase during the eclipse event (Zinder, 1963; Knolle & Kaudewitz, 1963; Valentine & Strand, 1965), it seems plausible that this reaction involves some alteration of the phage structure, whereby some component is removed from the phage particle to facilitate the injection of the phage genome into the cell. That this indeed may be the case has been inferred from the observation that phage particles eclipsed in the presence of EDTA retain their RNA but are susceptible to degradation by RNase (Silverman & Valentine, 1969). Moreover, that the pili structure (or perhaps a pili-associated enzyme) itself is responsible for phage eclipse is suggested by the isolation of bacterial "eclipse" mutants

which exhibit normal phage attachment but lack the phage eclipse function (Silverman et al., 1967a, b). The detailed molecular aspects of the F-pili mediated phage eclipse reaction, however, remain obscure and await further study.

Penetration of phage RNA, like that of eclipse, is an energy requiring reaction; it is prevented by 0.02 M KCN or NaN_3, in starved cells, or by a temperature of 0° (Ippen & Valentine, 1965; Knolle, 1967b; Brinton & Beer, 1967). In this respect, it is similar to bacterial conjugation (Curtiss and Stallions, 1967). Knolle (1967b) has reported an activation energy of 36 Kcal/mole for the "invasion" of E. coli by phage fr and that the rate of "invasion" is extremely temperature sensitive, being reduced to about 10^{-3} when the temperature is lowered from 37° to 4°. "Invasion", as defined by this author, encompasses all the early stages of the phage infective process, i.e. phage adsorption, eclipse and RNA penetration. It is interesting to note that if one corrects the activation energy for RNA phage "invasion" by subtracting that for phage eclipse (approx. 11 Kcal/mole, Danziger & Paranchych, 1970b), this value coincides with that reported for DNA phage penetration (Tzagoloff & Pratt, 1964). These authors noted a 20-fold increase in the rate of penetration of DNA phage from 15° - 45°. Thus, it appears that the energetics of infection of RNA and DNA male-specific phages may be quite similar. In contrast to the RNA phage attachment and eclipse steps, which occur in the absence of divalent cations, RNA penetration exhibits a specific requirement for these ions (Paranchych, 1966). The precise function of these ions in this process, however, is presently not understood.

Two hypotheses have been advanced to explain the seemingly

intricate involvement of F-pili in the transport of nucleic acid during male-specific phage infection and bacterial conjugation. The first, elaborated upon earlier in this chapter, is the "F-pili conduction" model of Brinton (1965). This model is based on the observations that the dense central core, seen in negatively-stained preparations of F-pili is of sufficient dimensions to accommodate a single-stranded RNA or DNA molecule. Further, the presence of F-pili correlated well to competence of cells in the mating function and in supporting infection by male-specific phages (Brinton, 1965). A strikingly different model is the "F-pili retraction" model postulated by Marvin and Hohn (1969). This model was founded on the observation that F-pili disappear completely from log-phase cultures of E. coli infected with Ff (a filamentous DNA phage). Their interpretation was that F-pili had retracted into the cell. According to this model, the adsorption of RNA phage to the side of the F-pilus or of DNA phage or female (F^-) cells to the tip of the pilus all trigger a retraction of the pilus into the cell. In support of the latter model, Ou and Anderson (1970) noted that F-pili tend to draw mating pairs together to create a more fertile union, thus indicating that the F-pilus retracts into one of either of the donor or recipient cells during conjugation. It has, however, not yet been established whether this phenomenon accompanies RNA phage penetration. Thus, whether the two models are mutually exclusive, i.e., that all nucleic acid transport via F-pili proceeds by only <u>one</u> mechanism, or not, is presently open to question. Although it has been established that only one of the three types of nucleic acid (e.g., phage RNA or DNA, host DNA) may be transferred through a given pilus at any one time (Knolle, 1967a; Ippen & Valentine, 1967; Novotny <u>et al</u>., 1968), it is quite conceivable that they occur by entirely different

mechanisms.

With the foregoing as background, an attempt has been made to clarify some of the uncertainties related to the early stages of RNA phage infection. This thesis describes experiments performed to elucidate the function of the phage R17 A protein in the phage attachment, eclipse and penetration stages. Furthermore, the fate of the phage coat protein and A protein during infection has been investigated. Lastly, attempts have been made to explain the physiological and biochemical alterations elicited in host cells and associated F-pili by phage R17 infection.

CHAPTER II

MATERIALS AND METHODS

A. Materials

1. Bacteria and Bacteriophage

(a) Bacteriophage

The RNA phage R17 originally isolated by Paranchych and Graham (1962) was used throughout the studies described in this thesis.

(b) Bacteria

The following is a list of the strains of Escherichia coli employed:

Strain	Genotype
E. coli K12	Hfr_1 met^- (λ^+)
E. coli B/r HB11	F^+ lac^+/lac^- Sm^s
E. coli AB257	Hfr met^- T_1^r
E. coli B	wild type

All strains of E. coli were maintained on hard agar plates for routine use. Permanent stocks were stored on hard agar slants in wax-sealed vials.

Aseptic technique was observed in all procedures involving the handling of phage or bacteria.

2. Bacterial Culture Media

(a) Tris (hydroxymethyl) amino methane maleic acid minimal salts (TMM) medium

The basic TMM salts solution contained the following components

in moles per liter:

Tris, 0.05; maleic acid, 0.05; NaCl, 0.043; KCl, 0.027; NH_4Cl, 0.019; Na_2HPO_4, 0.001; Na_2SO_4, 0.001

After dissolving the above in distilled water, the pH was adjusted to 7.3 with concentrated NaOH and the solution autoclaved at 110° for 15 min under a steam pressure of 20 lbs/in^2.

Complete TMM (CTMM) medium was prepared by combining the sterile components listed in the following proportions:

Basic TMM salts solution	910 ml
Eagles amino acid concentrate (100x)	10 ml
50% (w/v) glucose	10 ml
0.25% (w/v) L-methionine	10 ml
0.5 M $MgCl_2$	10 ml
20 mg % d-biotin	50 ml

(b) Various TMM-based media

(i) CTMM(-aa) medium. This medium contained all the regular components of CTMM medium with the omission of the Eagles amino acid concentrate.

(ii) Lactose TMM (LacTMM) medium. This medium, used solely for culturing E. coli HB11, contained 50% lactose in place of glucose as the carbon source.

(iii) Other variations of this culture medium are as described in the text.

(c) Trypticase soy broth (TSB)

Trypticase soy broth (Baltimore Biological Laboratories)	15 g/l
NaCl	8 g/l

Autoclaved solutions had a final pH of 7.2 - 7.3.

(d) Hard agar

Trypticase soy broth	30 g/l
Bacto-Agar (Difco Laboratories)	15 g/l

After dissolving, the solution was autoclaved and dispensed while warm into disposable Petri dishes.

(e) Top agar

Top agar was prepared as above except that the final agar concentration was 11 g/l. Sterile top agar was stored in 50 ml volumes at 4° until used. At this time, the agar was melted in a boiling water bath, dispensed in 2-ml aliquots into sterile culture tubes and maintained in the liquid state until used by incubating in a 45° water bath.

3. Bacteria and Phage Diluent

All dilution of bacteria or phage to be plated was done in a sterile solution composed of 0.9% (w/v) NaCl, 5 mM $MgCl_2$, and 5 mg % bovine serum albumin (Sigma Chemical Co.). Diluent was dispensed in 10 ml volumes into sterile dilution tubes (20 mm) and stored at room temperature until used.

4. Chemicals, Enzymes and Reagents

Unlabeled amino acids (L series), ribonucleosides, ribonucleoside mono- and triphosphates, sodium dextran sulfate 500 (NaDS), lysozyme (egg-white), pancreatic ribonuclease (5x recrystallized) and bovine serum albumin were purchased from Sigma Chemical Co. Electrophoretically purified DNase (RNase free) and snake venom phosphodiesterase were from Worthington Biochemical Corp. Polyethylene glycol 6000 (PEG) was supplied by J.T. Baker and Co. as were the Baker-Flex polyethyleneimine (PEI)-cellulose sheets employed for thin-layer chromatography.

Urea (ultra-pure) was purchased from Mann Research Laboratories. Sodium dodecyl sulfate (SDS) from Matheson, Coleman and Bell was used without further purification. Tetramethylethylenediamine (TEMED) and 2-mercaptoethanol (ME) were supplied by Eastman Organic Chemicals. The NCS reagent employed for solubilizing polyacrylamide gel slices was from Nuclear Chicago. All other reagents required for polyacrylamide gel electrophoresis were purchased from Canal Industries Corp.

Rifampin B-grade (Rifampicin) and urocanic acid were obtained from Calbiochem.

5. Radioactive Materials

Radioactive precursors for the preparation of labeled phage R17 were obrained from the following sources: ^{32}P (inorganic phosphage) - Charles Frosst; ^{14}C-amino acid mixture (100 μCi/ml in 0.1 N HCl) and 4-5 ^{3}H-leucine (sp.act. 40 Ci/mM) - New England Nuclear; 2-5-^{3}H-histidine (sp.act. 3, 5, 5.15, and 30 Ci/mM) - Schwarz Bioresearch Inc.; 2-5-^{3}H-histidine (sp.act. 45 Ci/mM) - Amersham/Searle.

B. Routine Procedures

1. Culture of Bacterial Cells

Experimental cultures were grown at 37° in a rotary-shaking water bath from a 1/100 dilution of an overnight E. coli culture. In order to achieve maximum aeration and avoid excessive breakage of F-pili, shallow cultures (generally 20% of the flask volume) were shaken at a speed of 120 rpm in baffled culture flasks (Bellco Glass Co.). Under these conditions cultures generally reached a cell density of 2×10^8 cells/ml at a time three hours after inoculation.

2. Determination of Phage Concentration and Bacterial Cell Density

(a) Bacterial cell density

The density of viable E. coli cells in TMM medium was determined from a standard curve constructed by plotting the absorbancy at 650 mμ of a 1.0 ml volume of culture vs the viable cell counts. Viable cell counts were determined by plating an appropriate dilution of the culture on hard agar plates and incubating overnight at 37°.

(b) Phage concentration

The actual number of phage particles in a phage solution was determined from optical density measurements with a Beckman spectrophotometer, using 7.66 absorbancy units/mg/ml/cm as extinction coefficient at 260 mμ (Gesteland & Boedtker, 1964). Assuming a mol.wt. of 3.6×10^6 daltons per phage particle, as reported by the foregoing workers, one absorbancy unit was calculated to contain 2.18×10^{13} phage particles.

The concentration of infectious phage (PFU/ml) in purified phage preparations was ascertained by the plaque assay described later in this chapter.

3. Phage Preparation and Purification

(a) Preparation of crude lysates

Cultures of E. coli (K12 Hfr_1 or AB257 Hfr) were grown at 37° in CTMM medium to a cell density of 4×10^8 bacteria/ml, then infected by the addition of purified phage R17 at a multiplicity of 40 PFU's/cell. After allowing several minutes for phage adsorption, incubation was continued with vigorous shaking (~150 rpm) for 3-4 hrs at which time partial cell lysis was obvious. Cell lysis was completed by adding lysozyme (66 mg/l) and chloroform (3.3 ml/l) followed by a further incubation at 37° for 30 minutes. The final yield of infectious phage in crude lysates varied from 8×10^{11} - 1.2×10^{12} PFU/milliliter.

(b) Concentration and purification of phage from crude lysates

The technique used to purify phage R17 was a modification of the liquid two-phase polymer method of Albertsson (1967). Following the addition of polyethylene glycol (PEG), 71.5 g, sodium dextran sulfate (NaDS), 2.06 g, and NaCl, 18g, per liter of crude lysate, the mixture was shaken until thoroughly homogeneous and stored at 4° for 18-24 hrs to allow phase formation. At this time, most of the top PEG phase (comprising 90-95% of the total volume) was removed by aspiration, care being taken not to disturb the interphase. The lower NaDS phase and the interphase material containing cell debris and the highly concentrated phage were then centrifuged at 480 g for 10 min to effect complete phase separation and the lower NaDS phase was then removed through a

Pasteur pipette introduced into the bottom of the centrifuge tube. Phage was removed from the contaminating cell debris by extracting the interphase material with five equal volumes of 0.15 M NaCl, buffered with 0.015 M sodium citrate, pH 7.0 (SSC) each time centrifuging out cell debris at 10,000 g for 10 minutes. To precipitate the residual NaDS, the phage extract was made 0.2 M with respect to KCl, set on ice for 1 hr and centrifuged at 10,000 g for 10 minutes. Following exhaustive dialysis against SSC (4°), the phage was pelleted by centrifugation at 70,000 g for 4 hrs in the Spinco #30 rotor and finally resuspended in 3 ml of sterile trypticase soy broth (TSB) in preparation for banding in a CsCl density gradient.

(c) CsCl banding of partially purified phage R17

As a final purification step, partially purified phage preparations were banded in CsCl density gradients. This was accomplished by adjusting the density of the phage solution to 1.4 g/cc, by the addition of solid CsCl, and centrifuging at 105,000 g for 40 hrs in the Spinco SW39 rotor. After piercing the bottom of the tube, the contents of the gradient were collected into a series of tubes and the fractions containing the band of infectious phage were pooled and dialyzed against SSC to remove CsCl. Figure 2.1 illustrates the radioactivity and infectivity profile of a phage preparation labeled with ^{14}C-amino acids. The fractions pooled in this case were fractions 13-15, since these three fractions contained more than 90% of the total infectivity in the gradient. As seen in the figure, no infectivity is associated with the lighter banding radioactive peak, which probably contains empty phage capsids.

Recovery of phage originally present in the crude lysates

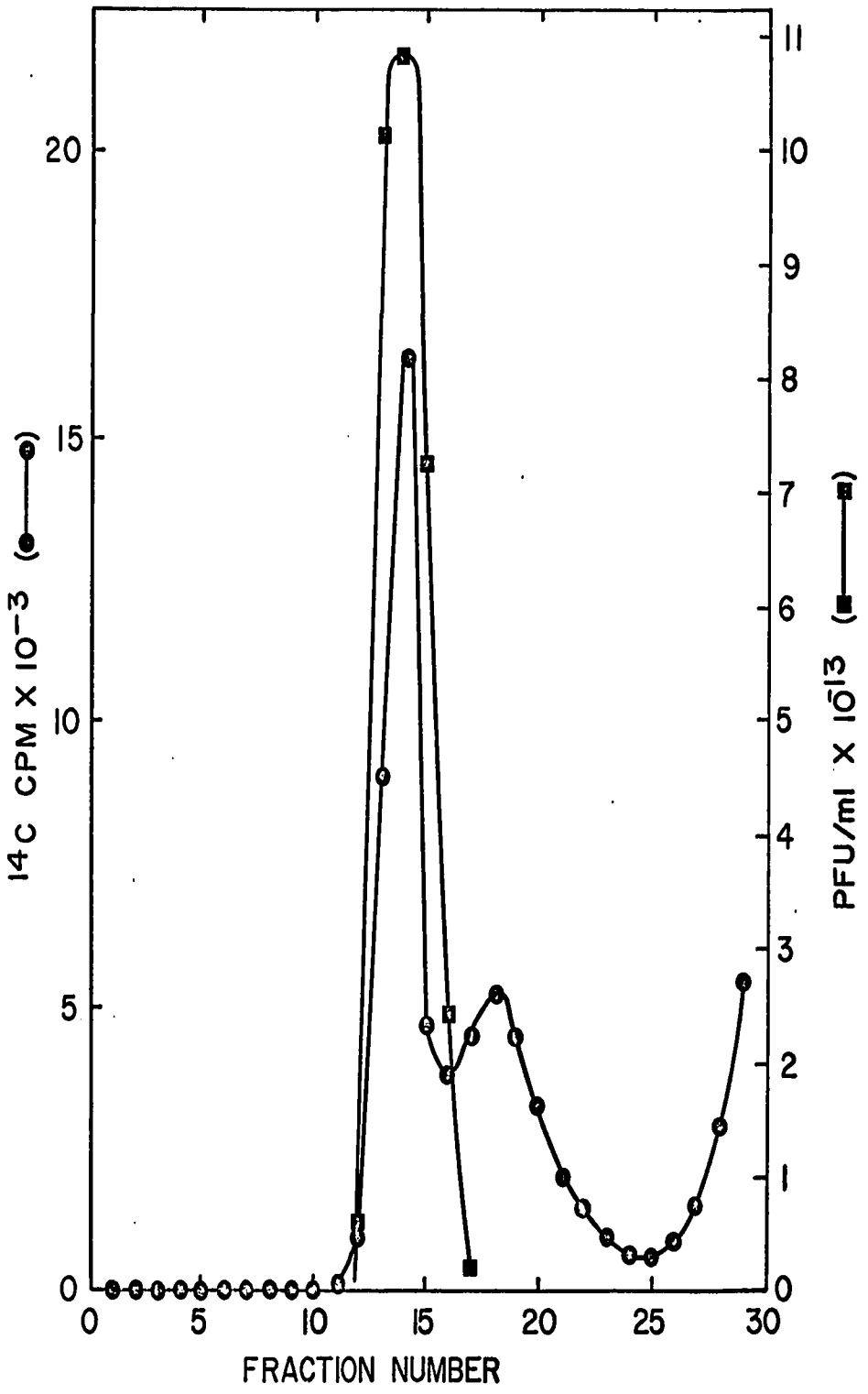

Figure 2.1. Cesium chloride density banding of ^{14}C-labeled phage R17. The experimental details are given in the text.

(measured as PFU) was normally about 90% while the particle/PFU ratio ranged from 5 to 12.

(d) Storage of purified phage stocks

Purified phage stocks were stored in concentrated solutions ($1\text{-}4 \times 10^{14}$ PFU/ml) at 4° in SSC containing 15 mg/ml TSB and a drop or two of chloroform. The latter additions were found to stabilize phage viability and prevented bacterial growth in the storage medium. Phage could be stored under these conditions for periods of time in excess of 6 months without appreciable loss of phage infectivity.

4. Radioactive Labeling of Phage R17

(a) ^{32}P-Labeling of R17 phage RNA

Cultures of E. coli (K12 or AB257), adapted to CTMM(-aa) medium by overnight culture, were grown in 100 ml of fresh medium and infected with purified phage R17 as described for the preparation of crude lysates. At 10 min post-infection, a neutralized solution of ^{32}P inorganic phosphate (10 mCi) was added and incubation continued. Purified phage obtained from such crude lysates generally had a specific radioactivity of 2×10^{-7} cpm/particle. When phage of higher specific radioactivity was desired, the phosphate concentration in the medium was lowered to 0.5 mM. This resulted in 10-20-fold increase of the radioactivity incorporated into phage. About 99% of the ^{32}P-label in purified phage was soluble in hot 5% TCA.

The CTMM(-aa) medium was the preferred medium when preparing radioactive phage since phage yields invariably were 10-20% higher than those obtained in CTMM medium.

(b) Labeling of R17 phage coat protein with ^{14}C-amino acids

E. coli K12 Hfr_1 was grown to a cell density of 3×10^8 bacteria/ml in 50 ml CTMM(-aa) medium. At this time, the culture was infected at a multiplicity of infection of 10 PFU's/cell, and 5 min later, 200 μCi of a ^{14}C-labeled amino acid mixture (100 μCi/ml), previously adjusted to pH 7.4 with 0.1 N NaOH, was added. Cell lysis and phage purification were as already described. The specific radioactivity of phage so prepared was 2.5×10^{-8} cpm/phage particle.

The ^{14}C-radioactivity associated with phage R17 prepared as described above was found predominantly in phage protein as seen by more than 95% of the radioactivity being insoluble in hot 5% TCA. Figure 2.2 shows the radioactivity profile obtained by electrophoresis of total phage protein from ^{14}C-labeled R17 in SDS-polyacrylamide gels. Estimates based on the radioactivity present in gel slices of the two discernible radioactivity peaks (A = A protein, c.p. = coat protein) reveal that only about 2% of the total radioactivity of phage labeled in this manner is contained in the phage A protein whereas 98% resides in the coat protein.

(c) Labeling of R17 phage A protein with radioactive histidine

The preparation and characterization of ^{3}H-histidine labeled phage R17 is described in a separate chapter of this thesis (see Chapter 3).

(d) Dual isotope labeling of phage R17

Phage preparations labeled with a combination of ^{32}P (inorganic phosphate) and ^{3}H- or ^{14}C-labeled amino acids were prepared as follows. One liter cultures of E. coli K12Hfr_1 were grown in CTMM(-aa)

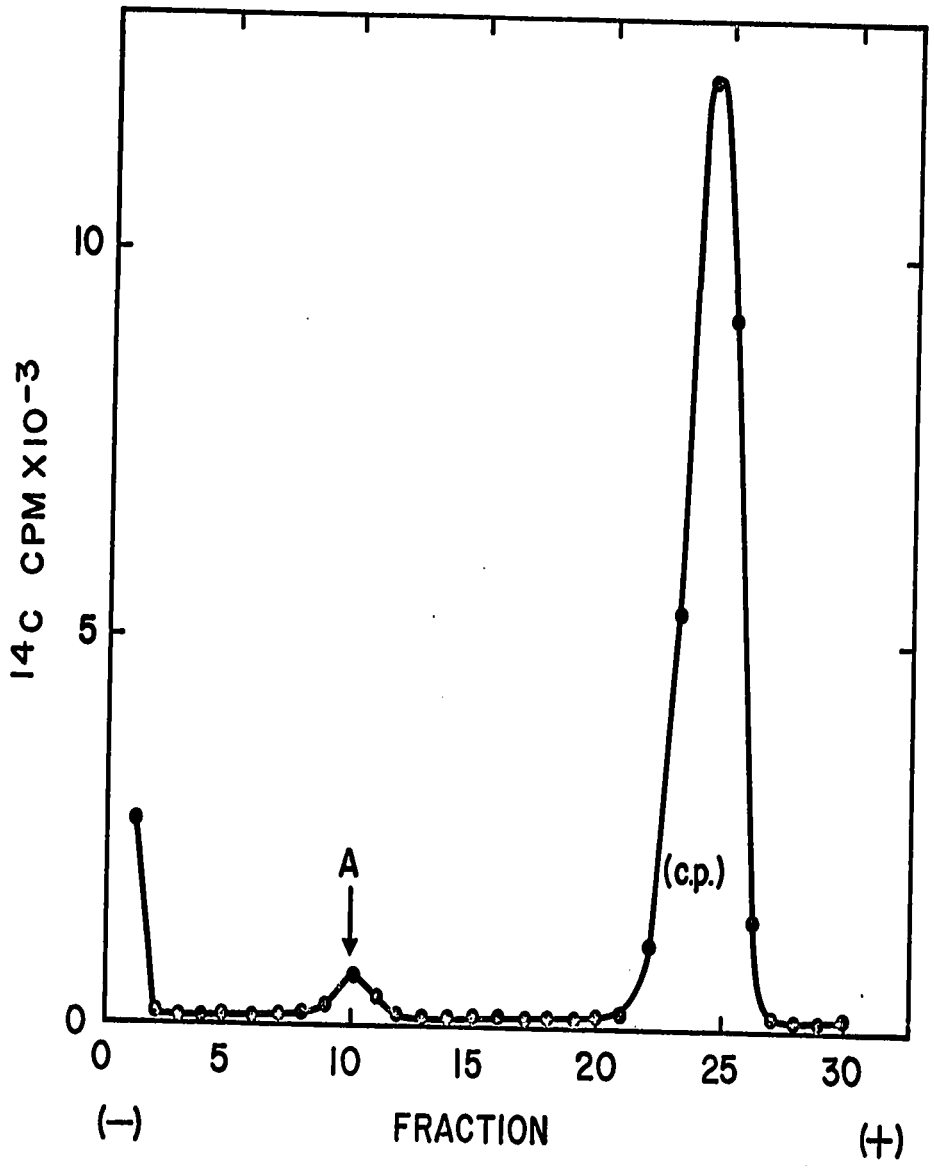

Figure 2.2. Electrophoresis of ^{14}C-amino acid labeled R17 phage protein in SDS-polyacrylamide gels.

A 0.2 ml portion of purified ^{14}C-amino acid labeled phage R17 was solubilized, electrophoresed, and prepared for radioactivity measurement as outlined in section D of this chapter. Electrophoresis was at a constant current of 5 m Amps/tube for 2.5 hours. The direction of migration in this and all subsequent gel electropherograms is toward the anode (left to right in the diagram). A = A protein. c.p. = coat protein.

medium to a cell density of 2 x 10^8 bacteria/ml, after which the culture was infected at a multiplicity of 1 PFU/cell. Appropriate amounts of ^{32}P (inorganic phosphate) and ^{3}H- or ^{14}C-labeled amino acids were added and incubation at 37° continued. Cell lysis and phage purification were as described above.

C. Phage Assays

1. Plaque Assay for Infectious Phage

Determination of infectious phage concentrations was carried out by mixing 1.0 ml of a diluted phage sample and 0.2 ml of seed culture (approx. 4 x 10^8 bacteria/ml) with 2.0 ml of liquified top agar, followed by pouring the entire contents onto hard agar in a disposable Petri dish. After allowing the top agar to harden, the plates were inverted and incubated at 37° overnight before scoring for plaques.

2. Eclipse Assay

Eclipse of infectious phage was monitored by assaying aliquots of cultures for infectious phage at various time periods after the addition of phage. Usually a culture was grown to a cell density of 2 x 10^8/ml in CTMM medium after which RNase was added to a final concentration of 100 μg/ml. Purified phage was then added to the culture at the desired PFU/cell ratio and incubation continued. The added RNase serves to prevent infection (Zinder, 1963) of host cells and thus facilitates true measurements of the viability of the phage originally added.

At designated time periods after phage addition, 0.1 ml aliquots of the sample were removed and rapidly chilled (by mixing with

10 ml of sterile saline kept on ice) to stop the eclipse process. After removal of the last sample, suitable dilutions were made and a 1.0 ml portion assayed for infectious phage by the plaque assay procedure.

3. Phage Attachment Assays

(a) Filtration assay for phage attachment

The filtration assays were performed essentially as outlined by Danziger and Paranchych (1970a). Samples (0.5 - 2.0 ml) were removed from cultures infected with radioactively labeled phage and filtered through Gelman GA-6 triacetate filters previously soaked with 10 ml of ice-cold TMM medium under a vacuum of 6 in of mercury. Filters were washed with three 5-ml portions of cold TMM medium, dried and assayed for radioactivity.

(b) Centrifugation assay for attachment

When attachment of infectious phage was to be determined, 0.3 ml aliquots of the infected culture were removed and centrifuged for 3 min at 15,000 rpm in a refrigerated Spinco Model 152 Microfuge. A portion of the supernatant was then assayed for infectious phage by the plaque assay procedure. The difference in the infectivity in a control and the supernatant was taken as a measure of the infectious phage attached.

Attachment of total phage by centrifugation was accomplished by centrifuging a 5 ml portion of a culture infected with radioactive phage in the cold at 10,000 g for 10 minutes. The supernatant was discarded and after carefully wiping the tube, the cell pellet was resuspended in 1 ml of distilled water and 0.2 ml portions transferred

to Whatman 3 MM filter discs (2.5 cm diam.) by pipette. Filters were thoroughly dried with a forced-air hair dryer before measurement of radioactivity.

4. Penetration Assay

The penetration assay was devised to determine the amount of a given phage component (RNA, A protein, coat protein) which actually enters the cell during the infective process. At predetermined time periods, suitable aliquots of a culture infected with radioactive phage R17 were removed and rapidly chilled in a methanol-dry ice bath to stop further penetration (Brinton & Beer, 1967). Extracellular phage was then removed by subjecting the infected cells to wash cycles with ice-cold basic TMM salts medium (no Mg^{++}). This was accomplished by centrifuging the cells at 10,000 g for 10 min followed by resuspending the cell pellet by repeated trituration with a 10 ml volume of fresh medium. The final cell pellet was resuspended in distilled water and 0.2 ml portions were transferred to Whatman 3 MM filter discs for radioassay.

Figure 2.3 illustrates the efficiency of this technique in removing unpenetrated phage from infected cells. In this experiment, a culture of E. coli K12 Hfr_1 was grown to a cell density of 4×10^8 cells/ml after which it was divided into two equal portions. Both cultures were then infected with ^{32}P-labeled phage at a multiplicity of 400 particles/cell; one at 37° and the other after chilling in ice. Both cultures were then incubated at the indicated temperature for 20 minutes. At the end of this time period, the culture incubated at 37° was rapidly chilled following which both were assayed for penetrated radioactivity as described in the previous paragraph. An aliquot (0.2 ml) of the resuspended cell pellet after each washing was

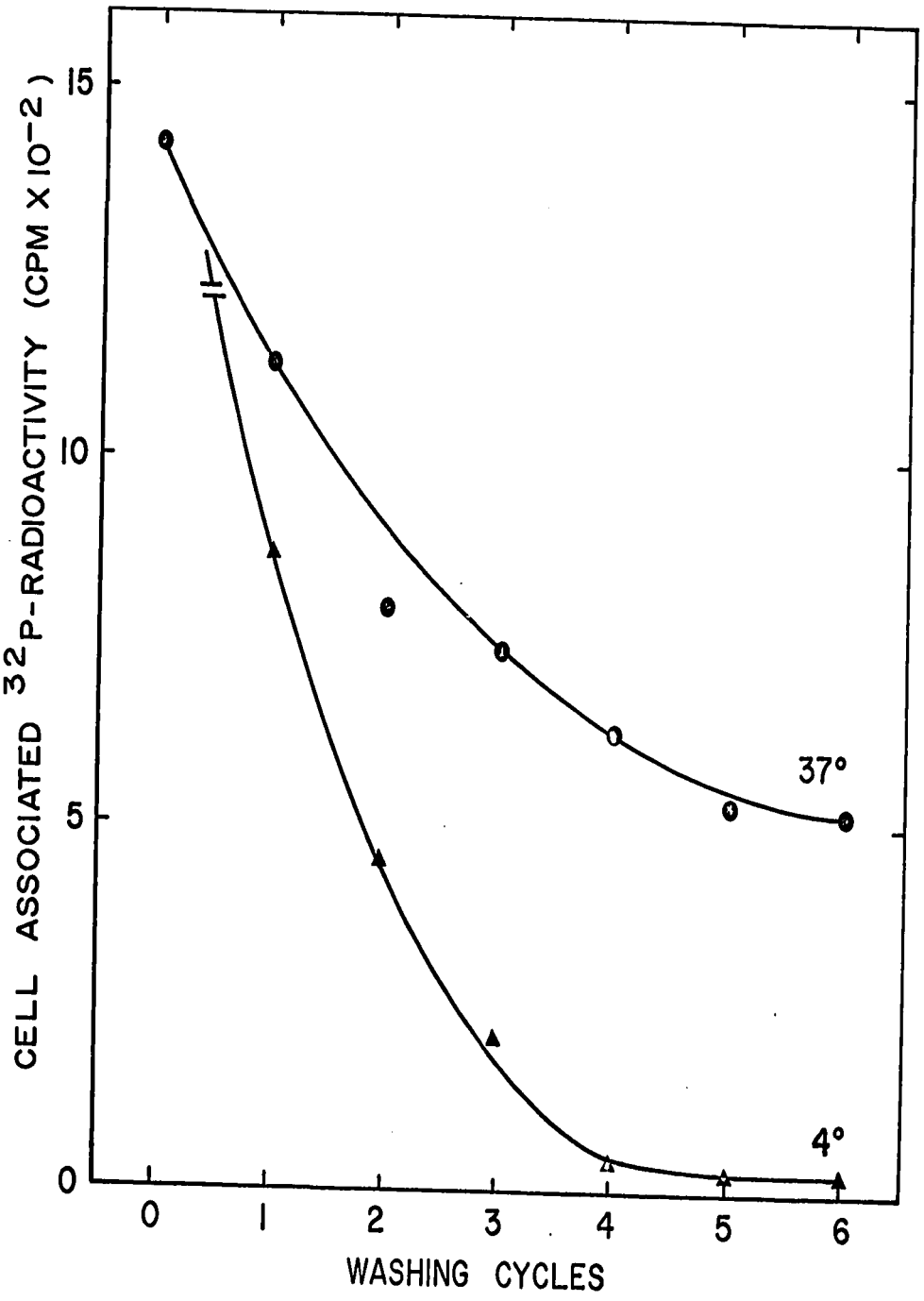

Figure 2.3. Efficiency of the penetration assay in removing extracellular phage material from infected cells.

The experimental details are given in the text. The radioactivity associated with the 4° sample prior to washing was 3200 cpm.

transferred to Whatman 3MM filter discs, dried and counted for radioactivity.

As may be noted in Figure 2.3, most of the extracellular phage is removed in the first two washes and decreasingly smaller amounts in subsequent washes, and only a negligible amount is removed in the final washing. Moreover, in agreement with Brinton and Beer (1967), it is seen that no phage RNA penetration occurs when phage is added to cells at 4°, since no appreciable radioactivity above background is found with such cells after the last several washes.

When a sonicated extract of the 6x washed cells, from the culture infected at 37°, was analyzed by centrifugation in sucrose density gradients, 96% of the ^{32}P radioactivity sedimented at a rate slower than that of free phage and the remaining 4% was skewed into heavier regions of the gradient, sedimenting faster than free phage. Thus it is clear that this assay measures specifically only phage material penetrated into host cells.

D. SDS-Polyacrylamide Gel Electrophoresis Techniques

1. Solubilization of Radioactive Phage Proteins

(a) Total phage proteins

Purified R17 phage, suspended in 0.01 M sodium phosphate buffer, pH 7.1, 2% SDS, 1% ME, was degraded by incubation at 37° for 2-5 hrs (Loh & Shatkin, 1968). Samples were then dialyzed overnight at room temperature against 3 liters of 0.01 M sodium phosphate, pH 7.1, 0.1% SDS and 0.1% ME (Summers et al., 1967).

(b) Pili-associated and penetrated phage proteins

Phage proteins associated with isolated pili or cells after

infection with radioactive phage were prepared for electrophoresis by a method similar to that of Vinuela et al. (1967). Aliquots of each were diluted to 6 ml with buffer containing 0.1 M Tris-HCl, pH 8.4, 0.01 M EDTA, 1% ME. This suspension was then extracted (3x) with a 2 ml volume of freshly distilled buffer-saturated phenol. The pooled phenol phases formed by low speed centrifugation (4500 rpm, 5 min) after each extraction step were transferred to 3/8" dialysis tubing, closed tightly and dialyzed against 2 liters of 0.1 M acetic acid, 1% ME. Two hrs later, the buffer was changed and dialysis continued until the phenol phase had decreased to about 1 ml. After opening the bags and carefully removing the top aqueous phase with a Pasteur pipette, the bags were closed tightly and dialyzed overnight against a 500 ml volume of 9.0 M urea, 0.05 M acetic acid, 1% ME. The dialysis bags were then transferred to 500 ml of 0.1 M Tris HCl, pH 8.4, 0.01 M EDTA, 8.6 M urea, 1% ME, and dialyzed for 2 hrs with vigorous stirring while streaming N_2 gas through the medium. A final step consisted of dialyzing against 1000-2000 volumes of 0.01 M sodium phosphate, pH 7.1, 0.1% SDS, 0.1% ME for 24 hrs with one change of buffer. All operations were carried out at room temperature.

2. Polyacrylamide Gel Electrophoresis

The gel solutions contained 10% (w/v) acrylamide 0.05 M sodium phosphate, pH 7.1, 0.1% (w/v) SDS, 0.27% (w/v) N,N' bis-methylene acrylamide, 0.075% (w/v) TEMED, 0.075% (w/v) ammonium persulfate (catalyst). After addition of catalyst, the gel solution was filtered through a Millipore type HA 0.65μ filter and de-aerated under vacuum. Columns were formed by pouring 1.0 ml of solution into a coated glass

tube (6.3 x 0.5 cm inner diameter), layered with 1 cm of water and allowed to polymerize at room temperature. After 20-30 min, the water layer was drawn off and the columns inserted into the assembly. Samples (0.2 ml) of the phage proteins containing suitable levels of radioactivity were mixed 1:1 with 60% glycerol and applied to the column by layering underneath the electrophoresis buffer (0.1% SDS in 0.6 M sodium phosphate, pH 7.1). Electrophoresis was carried out at room temperature under a current of 5 m Amps/tube, supplied by a Savant Regulated Power Supply (Model HV 100 TC, Savant Inst. Inc., Hicksville, N.Y.), for 2.5 - 3 hours. After electrophoresis, the gels were removed and fitted into a leucite block with calibrated (2 mm) transverse slits. Gel fractions were obtained by slicing the gel into 30 equal sections. Radioactivity in each of the fractions was determined by a method described by Fromageot and Zinder (1968). Each slide was placed into a 10 mm test tube and allowed to react with 50 µl of distilled water for 1 hr at room temperature. At this time, 0.2 ml of Nuclear Chicago Solubilizer (NCS reagent) was added after which the tubes were shaken briefly and set aside for another hour. An additional 0.3 ml of NCS reagent was then added and the tubes stored overnight in the dark. Samples were then transferred to scintillation vials by rinsing each tube with 3-5 ml portions of toluene-base scintillation fluid and counted in a liquid scintillation spectrometer.

E. Thin-Layer Chromatography Techniques

1. ^{32}P-Labeling and Preparation of Cell Extracts

A 0.2 ml volume of a stock solution of ^{32}P-sodium phosphate (2.5 mCi/ml) was added to 20 ml of CTMM medium immediately after

inoculation with an overnight culture of E. coli K12 Hfr_1. This early addition of ^{32}P-radioactivity insured its equilibration with cellular NTP pools well before phage infection. When the culture reached a density of 2×10^8 bacteria/ml, the experiment was started by withdrawing samples for optical density measurements and for extraction of cellular NTP's. Five minutes later, the culture was infected at the desired phage multiplicity and sampling continued.

Extraction of NTP's was as follows. A 0.1 ml sample of the culture to be analyzed was removed and rapidly mixed with 0.1 ml of ice-cold 2M formic acid in a Beckman microfuge tube and was kept at 4° for 30-60 minutes. Cell extracts were then centrifuged for 1 min at 15,000 rpm in a Spinco Model 152 refrigerated microfuge. The resulting supernatant was used directly for the analysis of nucleoside triphosphates.

2. Two-Dimensional Chromatography of Nucleotides on PEI-Cellulose Sheets

The two-dimensional chromatographic procedure is essentially that outlined by Randerath and Randerath (1964). A 20-50 µl sample of cell extract (or phage RNA hydrolysate), was spotted approximately 2 cm from the left and bottom side of washed Baker-Flex PEI-cellulose sheets. The diameter of the spots was limited to 5 mm by repeated spotting of 3-5 µl aliquots, followed by intermediate drying with a forced-air hair dryer. Desalting, when necessary, was accomplished by gently rinsing the sheets for 2-3 min in a shallow pan containing a methanol:water (1:1 v/v) mixture. Suitable nucleotide markers (4 µl of a 0.2% w/v standard) were then applied to facilitate location of the radioactive nucleotides after development.

Chromatography in the first dimension consisted of developing the sheets in the following LiCl solvents in sequence for the time periods indicated:

(i) 0.2 M LiCl 2 min

(ii) 1.0 M LiCl 3 min

(iii) 1.6 M LiCl ~1.5 hr

The latter period, being the period required for the latter solvent to reach the 14 cm marker, varied slightly from one chromatogram to another without affecting resolution. The sheets were then removed, air dried, and washed free of LiCl by gentle agitation in a shallow methanol bath for 15 minutes.

Development in the second dimension was effected by dipping the sheets into different formate buffers (pH 3.4) in sequence as follows:

(i) 0.5 M sodium formate (pH 3.4) 0.5 min

(ii) 2.0 M sodium formate (pH 3.4) 2.0 min

and (iii) 4.0 M sodium formate (pH 3.4) for the period of time required to move the solvent front to the 15 cm marker. After drying the sheets, marker spots were located by exposure to ultra-violet light and marked lightly by pencil. The outlined spots were then cut out and prepared for scintillation counting by placing each into a scintillation vial containing 5 ml of toluene-base fluor.

F. Electron Microscopy

1. Sample Preparation

A small aliquot (50 µl) of an appropriate culture was placed

on carbon-backed collodion films supported on 200 mesh copper grids, allowed to adsorb for 30 min and the excess removed with adsorbent paper. Negative staining of specimens was carried out with 2% (w/v) phosphotungstic acid at pH 5.7. Samples were then mounted in a Philips 300 EM electron microscope and examined at an accelerating voltage of 40 KV.

2. Enumeration of F-Pili and Length Measurements

Negatively-stained samples of E. coli HB11 were examined and scored directly for pili frequency from the electron microscope viewing screen (magnification 4690x). Pili were enumerated only where adequate cell dispersion permitted the origin of the pilus to be identified. Counts were also restricted to those cells that were uniformly stained and clearly outlined.

F-pilus lengths were measured directly from photographically enlarged electron micrographs (taken on 35 mm Kodak Fine Grain Positive Film 5023), with the use of a map odometer (Keuffel and Esser Co.). Two typical electron micrographs are shown in Plates I and II of the Appendix. Observed F-pilus lengths were corrected for obscuration by the cell using the method described by Novotny et al. (1969a).

G. Isolation of Phage RNA

^{3}H-histidine labeled R17 phage (3 mg) was resuspended in 5 ml of buffer containing 0.1 M KCl, 0.001 M EDTA, 0.001 M $MgCl_2$ and 0.01 M Tris-HCl, pH 7.2. The suspension was then made 1% (w/v) in SDS and 0.005% in NaDS, and mixed well. After incubation at 37° for 10 min and subsequent chilling in ice, a Macaloid (Union Carbide) suspension was added to give a final concentration of 0.2%. Deproteinization was

achieved by shaking on a Vortex shaker for 1 min with an equal volume of buffer-saturated phenol. The resulting emulsion was centrifuged at 4500 g for 10 min, after which the upper aqueous layer was removed, and Macaloid and phenol added as before. The whole extraction procedure was repeated three times. Residual phenol in the final aqueous phase was extracted with 6 equal portions of ether. The RNA was then precipitated by the addition of 2 vols of cold (-20°) methanol and 0.1 vol of 20% potassium acetate, and storing overnight in a freezer (-20°). The precipitated RNA was then collected by centrifugation, ether-dried, and stored under vacuum in a dessicator at -20° until used.

H. Hydrolysis of R17 Phage RNA by Snake Venom Phosphodiesterase

A 1 ml portion of partially purified snake venom phosphodiesterase (freshly dissolved in distilled water to a final concentration of 1 mg/ml) was added to ^{3}H-labeled phage RNA (13 A_{260} units), dissolved in 10 ml of buffer (0.1 M Tris-acetate, pH 9.4, 0.01 M $MgCl_2$), and incubated at 40° for 4 hours. Progress of RNA hydrolysis was followed by removing 0.2 ml aliquots of the digestion mixture at various intervals and assaying for cold TCA-insoluble radioactivity. The pH was maintained at 9.4 by the addition of 0.1 N NaOH. When hydrolysis was complete, 1.5 ml aliquots were removed, and lyophilized in preparation for thin-layer chromatography.

I. Determination of Dry Cell Weight

To determine the cell mass of normal and infected E. coli, the absorbance at 650 mμ of a growing E. coli culture was correlated with the weight change of thoroughly dried GA-6 Gelman filters before

and after filtering a given volume of culture. The filters were placed into pre-weighed scintillation counting vials and heated at 250°F in a drying oven until a constant weight was achieved. The average of the cell weights determined in this way gave a value of 0.363 (±0.007) mg/ A_{650} mμ absorbancy unit/ml.

J. Sucrose Density Gradient Sedimentation Analysis

1. Preparation of Gradients

Linear gradients, (4.5 ml) of 5 - 20% sucrose in various buffers, were prepared with the use of a two-chamber gradient making device (Buchler Inst. Corp., Fort Lee, N.J.) (Britten & Roberts, 1960). Before using, gradients were equilibrated by storing at 4° for 2-4 hours.

2. Sucrose Density Gradient Centrifugation

Aqueous samples (0.3 - 0.5 ml) were layered onto equilibrated gradients by introducing the pipette tip into the gradient meniscus and allowing the sample to flow gently onto the gradient, care being taken at this time to avoid turbulence or sample mixing. Gradient tubes were then placed into sealed rotor tubes (Spinco SW39 or SW50) and centrifuged at 4° for the desired time period in a Beckman Model L Preparative Ultracentrifuge.

3. Gradient Fractionation

Following centrifugation, the sample tubes were carefully placed into a gradient fractionating device (Buchler Inst. Corp., Fort Lee, N.J.) and secured tightly by a threaded cap screw. The bottom of the tube was then punctured and fractions collected dropwise into a series of tubes or mounted filter discs.

K. Radioassay by the Filter Disc Method

Samples (50 - 200 µl) to be assayed for TCA-insoluble radioactivity were transferred by pipette to pencil-marked 3 MM Whatman filter discs (2.1 cm diameter) impaled onto a styrofoam base with steel pins, and processed by the scheme illustrated below (Mans & Novelli, 1960). Radioactive materials from sucrose density gradient fractions were collected dropwise onto discs and thoroughly air dried before TCA treatments.

1. Hot TCA-Insoluble Products

TABLE 2.1

Preparation of Filter Discs for Radioactivity Measurements.

Step	Time	Wash Medium	Temperature
1	30 min	10% TCA	4°
2	5 min	5% TCA	4°
3	45 min	5% TCA	90°
4	15 min	5% TCA	4°
5	15 min	Ether·Ethanol (1:1)	37°
6	15 min	Ether	20°

2. Cold TCA-Insoluble Products

Treatment was similar to that shown in the above scheme with the substitution of a 15 min 5% TCA (4°) wash for Step 3.

L. Radioisotope Counting

A toluene-base scintillation phosphor, prepared by the addition of 4 g Omnifluor (New England Nuclear, Boston, Mass) per liter of scintillation-grade toluene, was used throughout for all radioassay experiments. Thoroughly dried samples on Gelman GA-6 filters, or Whatman 3MM filter discs were placed into thin-walled glass scintillation vials. Following the addition of 5 ml of scintillation mix, the vials were capped tightly and counted in a Beckman 2000 LS scintillation spectrometer.

When the radioactivity of samples containing dual isotopes was to be quantitated, the gain setting was adjusted to a value at which the overflow between counting channels was restricted to the higher energy isotope. The amount of overflow of ^{14}C and ^{32}P radioactivity into the ^{3}H channel under these conditions generally was 35-40% and 5-10%, respectively.

A counting time of 5 min was generally sufficient to reduce the counting errors due to the random process of decay to less than 1%. In cases where low levels of radioactivity were present, the samples were counted for a sufficient length of time to reduce the error to less than 5%. All sample values were corrected for background radioactivity registered in controls containing no added radioactivity.

CHAPTER III

PREPARATION AND CHARACTERIZATION OF PHAGE R17 LABELED WITH RADIOACTIVE HISTIDINE

A. Introduction

Although considerable progress has been made towards the elucidation of the structure and function of the RNA phage coat protein (Weber, 1967; Robertson et al., 1968; Spahr et al., 1969; Sugiyama & Nakada, 1970) and the RNA synthetase (see review by Stavis & August, 1970), relatively little is known about the A protein. This probably stems from the fact that phage particles contain very little A protein (only one molecule per phage particle) thus making it difficult to prepare sufficient amounts with which to carry out experiments. Fortunately this problem can be partially overcome by exploiting the fact that the A protein is the only histidine-containing polypeptide associated with wild-type phage R17 (Steitz, 1968a). This means that it should be possible to specifically label the phage A protein with radioactive histidine and thereafter examine it in a variety of ways to determine its biochemical role in the infectious process. Such experiments, however, require that the A protein be of a high degree of radiochemical purity[1], having a specific radioactivity of at least 1×10^{-8} cpm/particle. Since only five of the estimated 350 - 400 amino acids of the A protein are accounted for by histidine (Steitz, 1968b), it was evident that optimal labeling conditions would have to be employed to achieve this objective.

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[1] Percent of the phage radioactivity insoluble in hot 5% TCA.

This chapter summarizes the studies carried out to define the conditions under which optimal labeling of the phage R17 A protein occurs. The limitations and difficulties encountered in these studies are discussed.

B. Results

1. Incorporation of ^{3}H-histidine Into Phage R17

Since the specific radioactivity of purified ^{3}H-histidine labeled RNA phages is usually very low, it is important to demonstrate that the radioactivity in phage so labeled is not due to adsorption of labeled host proteins to the phage surface, or to trapping of the labeled amino acid inside the particle. This was accomplished in the following manner.

Three 100-ml cultures of *E. coli* K12 Hfr_1 (A, B and C) were grown to a cell density of 4×10^8/ml in CTMM(-aa) medium, after which cultures A and B were infected with purified phage R17 at a multiplicity of ~10 PFU/cell. ^{3}H-Histidine (sp.act. 5 Ci/mM) was then added to flasks A and C to a final concentration of 2 μCi/ml, following which the cultures were incubated at 37°. After 90 min the cells in the uninfected culture (C) were artificially lysed by the addition of lysozyme (100 μg/ml) and EDTA (10 mM) and subsequent incubation at 37° for 30 minutes. Cultures A and B were aerated until partial lysis was observed (5 hrs), at which time lysozyme (100 μg/ml) was added to each flask and incubation continued for 30 min to facilitate complete lysis. The contents of flask C (containing the artificially lysed ^{3}H-histidine labeled cells) were then mixed with the non-radioactive phage in flask B. Both phage preparations (A and B) were then purified by the two-phase

partition method and banded in CsCl as described in Chapter 2.

The specific radioactivities of the purified control phage (grown in the absence of ^{3}H-histidine and mixed with the ^{3}H-histidine labeled cell lysate) and the ^{3}H-histidine labeled phage were 7.5×10^{-11} and 2×10^{-9} cpm/particle, respectively. The control phage thus contained only about 4% as much radioactivity as the ^{3}H-histidine labeled phage. It was therefore concluded that the purification procedure removes essentially all of the ^{3}H-histidine label not incorporated into the phage particle. The level of contaminating label under these conditions, moreover, is sufficiently low so as not be interfere with proposed experiments.

Figure 3.1 shows the results of an analysis of the ^{3}H-histidine labeled phage by sedimentation in a 5-20% sucrose gradient. As may be seen, all three properties of the phage (optical density, radioactivity and infectivity) coincide in the single symmetrical peak in the gradient. Further, since more than 95% of the radioactivity in such a phage preparation remains insoluble after treatment with cold TCA, it was concluded that it was incorporated into the phage particle.

2. Characterization of the Radioactivity Incorporated Into Phage Labeled With ^{3}H-Histidine

Subsequent analysis of the purified ^{3}H-histidine labeled phage by heating with 5% TCA revealed that approximately 40% of the label incorporated into whole phage was solubilized by this treatment. This observation suggests that a significant fraction of the ^{3}H-histidine added to R17-infected cultures is somehow channeled into RNA synthesis. To gain some insight into a possible mechanism whereby the incorporation of this amino acid into protein is diverted during phage

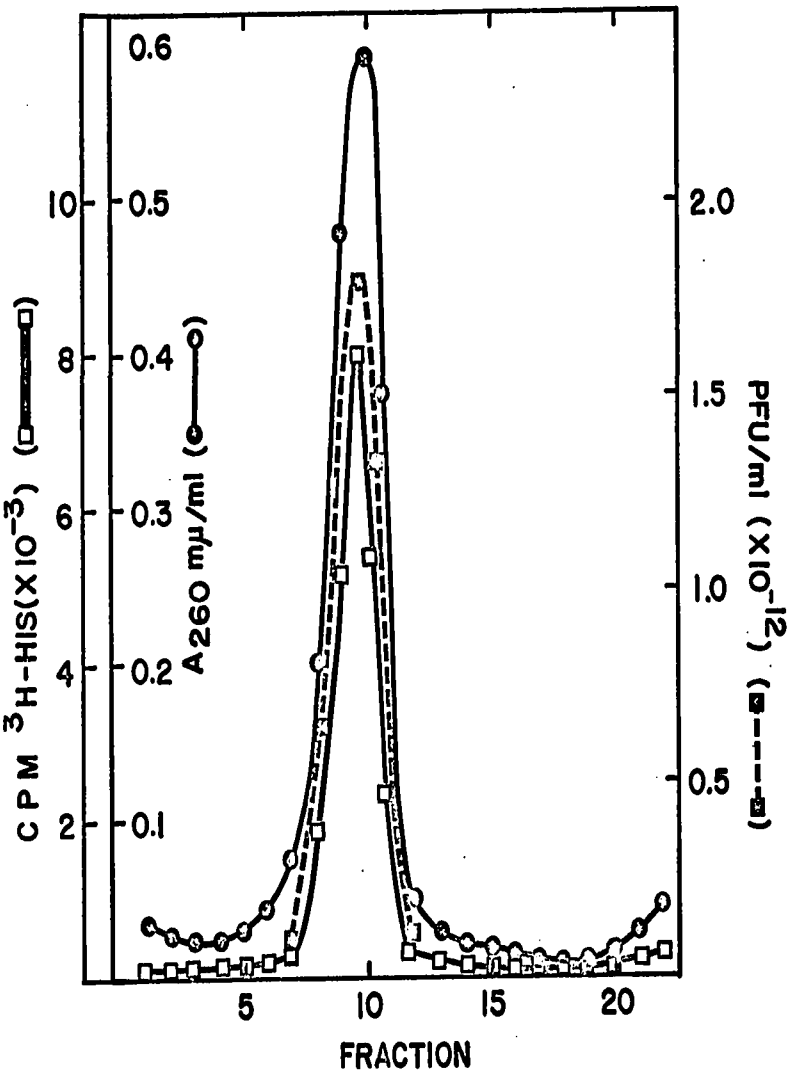


Figure 3.1. Distribution of radioactivity, optical density and phage titre of purified ^{3}H-histidine labeled phage R17 in a sucrose gradient.

A 0.1 ml portion (1.5 A_{260} units) of the purified ^{3}H-histidine labeled R17 preparation was layered onto a 4.5 ml linear (5-20%) sucrose gradient, made up in TMM medium, and centrifuged at 45,000 rpm for 45 min in the Spinco SW50 rotor. Following fractionation of the gradient (0.2 ml fractions) into tubes containing 0.8 ml distilled water, the absorbancy of each fraction was measured at 260 mμ. Portions of 0.1 ml and 0.8 ml were then assayed for infectivity by the plaque assay procedure and cold TCA-insoluble radioactivity, respectively.

infection, the distribution of the radioactivity in the four constituent nucleoside monophosphates of phage RNA was determined. This was accomplished by hydrolysis of phage RNA (extracted from ^{3}H-histidine labeled R17) with snake venom phosphodiesterase and separation of the nucleoside 5'-monophosphates (NMP's) by chromatography on PEI-cellulose thin layer sheets (see Methods & Materials). Figure 3.2 shows the resolution of the four NMP's obtained by chromatography of the phage RNA hydrolysate on PEI-cellulose thin layer sheets (Randerath & Randerath, 1964). As illustrated, the four NMP's are separated sufficiently to permit accurate quantitation of the radioactivity present in each. The results of the radioactivity analysis (Table 3.1) show that the radioactivity originally present in ^{3}H-histidine is directed specifically into purines, since it is distributed about equally between AMP and GMP; only about 3% is accounted for by CMP and UMP.

TABLE 3.1

Distribution of Radioactivity in Nucleoside 5' Monophosphates of a Snake Venom Digest of RNA Extracted From ^{3}H-Histidine Labeled R17*

Nucleotide	Radioactivity (cpm)
GMP	2781
UMP	85
AMP	3111
CMP	77

*Experimental details are given in Figure 3.2.

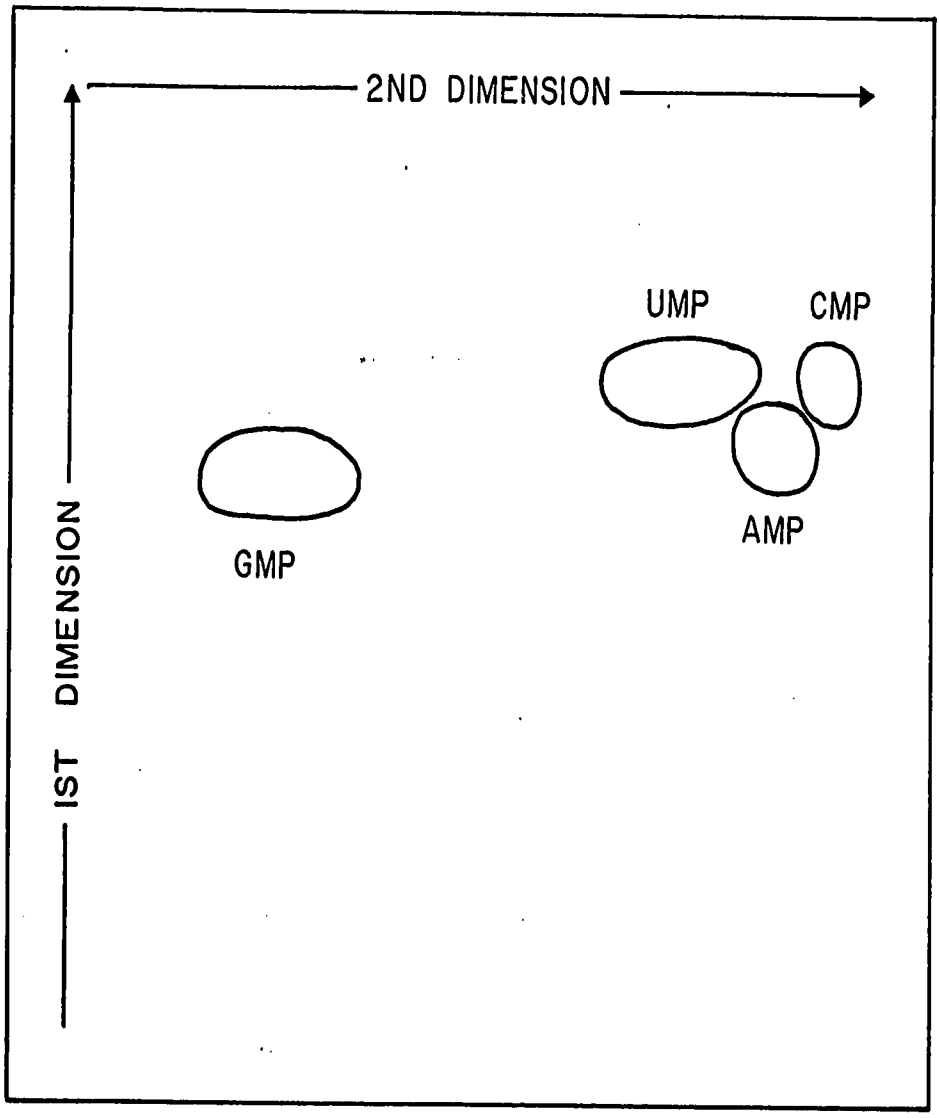


Figure 3.2. Thin-layer chromatography of nucleoside-5' monophosphates of a snake venom digest of phage RNA on Baker-Flex PEI-cellulose sheets.

Lyophilized hydrolysates were dissolved in 100 µl of distilled water and a 50 µl portion spotted onto Baker-Flex PEI-cellulose sheets. Sample application and development of the chromatograms were as outlined in the Methods section. After locating the NMP spots under ultra-violet light, the spots were cut out from the dried sheets and assayed for radioactivity.

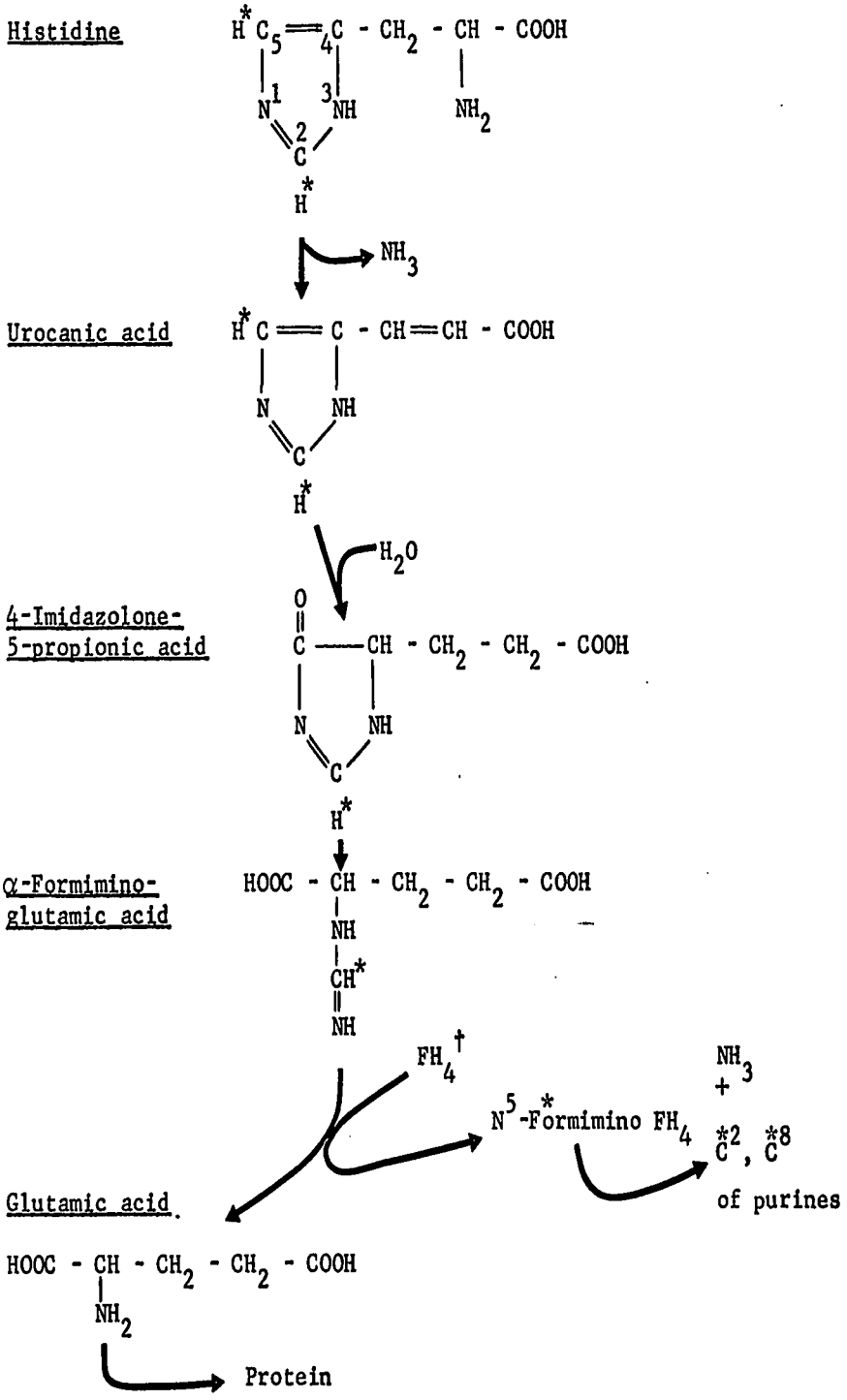
The appearance of radioactivity in phage RNA (purines) from ^{3}H-histidine can be explained in one of several ways:

1. Perhaps the ^{3}H-histidine employed in labeling phage contains some radioactive purine nucleosides or its precursors as contaminants.
2. It is possible that some of the ^{3}H-histidine added to R17-infected cultures is degraded and thence incorporated into phage RNA (and protein) in some other form.

The first suggestion seemed highly improbable in view of the product analysis data of the ^{3}H-histidine (obtained from suppliers), which showed a radiochemical purity of 95-99%.

The second possibility however, seemed very probable and hence an attempt was made to explain the results on this basis. The reactions pertinent to what appears to be the major pathway of histidine catabolism in aerobic bacteria - the urocanic acid pathway - (Barker, 1961) are depicted schematically in Figure 3.3. Examination of this scheme reveals that the end-products of histidine catabolism are glutamate and N^{5}-formiminotetrahydrofolate; both of which participate in the de novo synthesis of purines. It is also to be noted from the figure that the radioactivity (*) originally present in the 2-5-^{3}H-histidine is present only in the latter of the two end-products.

If this scheme is valid, then it may be assumed that the bulk of the radioactivity in phage RNA purines is derived from the tetrahydrofolate derivative, since the glutamate generated by the degradation of 2-5-^{3}H-histidine is unlabeled.



*Denotes position of tritium label of 2-5-^{3}H-histidine employed in these studies.

†FH_4 = tetrahydrofolate

Figure 3.3. Pathway for histidine degradation.

3. The Effect of Growth Conditions on the Incorporation of ^{3}H-Histidine Into Phage Protein

As mentioned earlier, the success of studies on the physiological role of the R17 A protein depends on the specific labeling of this phage moiety with radioactive histidine. Several experiments were thus performed with the following objectives in mind:

(a) to create growth conditions under which a major proportion of the ^{3}H-histidine entering the phage particle is incorporated into protein;

(b) to establish optimal labeling conditions (i.e., time of labeling) to obtain phage of high specific radioactivity.

It was reasoned that the former objective might be achieved either by repressing the degradation of added ^{3}H-histidine, or alternatively by dilution of its degradation products with carrier. Achievement of the second objective rested in the judicious addition of ^{3}H-histidine, of the highest specific activity available, to the infected culture at a time when maximum incorporation of this amino acid into phage A protein occurs. With this in mind, several experiments were performed. The results, summarized in Table 3.2, illustrate the effect of the growth medium and the time of labeling on the specific radioactivity and radiochemical purity of ^{3}H-histidine labeled phage obtained under a variety of conditions. In all cases, cells were grown to a cell density of 4×10^{8}/ml and infected with purified R17 at a multiplicity of 40 PFU/cell. E. coli AB257 was used as host in the latter preparations since 20-40% higher phage yields were consistently produced by this strain.

TABLE 3.2

Effect of Various Growth Conditions and Labeling Times
on the Radiochemical Purity and Specific Radioactivity of Phage R17

Expt.	*E. coli* Host	^{3}H-histidine Precursor		Final Concn. μCi/ml	Culture Medium	Labeling Period Post-Infection** (min)	Specific+ Radioactivity (cpm/particle)	Radiochemical Purity (Hot TCA-Insoluble Fraction)
		Commercial Source	Specific Activity					
1	K12 Hfr_1	S*	3 Ci/mM	20	CTMM(-aa)	6 ⟶	4.3×10^{-9}	38%
2	K12 Hfr_1	S*	5 Ci/mM	2	CTMM(-aa)	10-24	5×10^{-10}	39%
3	AB257 Hfr	S*	5.15 Ci/mM	20	CTMM(-aa)	0 ⟶	7.1×10^{-11}	65%
4	AB257 Hfr	S*	30 Ci/mM	40	†CTMM (A)	17-22	1×10^{-8}	57%
5	AB257 Hfr	‡A-S	45 Ci/mM	37.5	CTMM (A)	15-22	1.3×10^{-8}	66%

*S Schwarz Bioresearch Inc.

‡A-S Amersham/Searle

†CTMM(A) described in the text.

** Pulse was terminated by addition of unlabeled histidine to a final concentration of 10 μg/ml.

+ Based on cold TCA-insoluble radioactivity.

Firstly, it may be seen that addition of ^{3}H-histidine to a culture at the time of infection (Table 3.2, Expt.3) gives rise to a relatively high level of radiochemical purity, but yields phage of only a low specific radioactivity (7.1×10^{-11} cpm/particle). Presumably this is so because the radioactivity in the histidine pool is already severely depleted by the onset of A protein synthesis. This suggestion is in accord with the results of Nathans et al. (1969) who observed only a negligible amount of A protein synthesis before 15 min after infection. Also in agreement with their data is the observation that phage of 10 - 100 times higher specific radioactivity is obtained when ^{3}H-histidine is added at a time after infection (Expt.1 & 2 vs 3) even if only 1/10 as much label is employed (Expt.2). Thus the time of labeling is a critical factor which determines the specific radioactivity of R17 phage obtained under these conditions. This is indicated more clearly in the last two experiments of Table 3.2.

Although phage of higher specific radioactivity is obtained when ^{3}H-histidine is added after infection, a larger proportion of the incorporated radioactivity also is present in the phage RNA under these conditions (Expts.1 & 2 vs 3, Table 3.2). An attempt was therefore made to decrease the incorporation of label into phage RNA, by the addition of various compounds which prevent the incorporation of the radioactive breakdown products of 2-5-^{3}H-histidine, into phage RNA.

In the last two experiments of Table 3.2, the CTMM(A) medium consisted of the CTMM(-aa) medium supplemented with urocanic acid, adenosine, guanosine, cytidine, uridine, and 19 amino acids (no histidine), at the time of infection. Each of the compounds were added to give a final concentration of 10 μg/ml, except serine which was present

at 30 μg/ml. The effect of these additions on increasing the extent of the overall incorporation of ^{3}H-histidine into phage is evident from Expt.4 and 5 in Table 3.2. In addition, an increase in the incorporation of ^{3}H-histidine into protein is also seen. Thus, it may be seen that the addition of histidine metabolites to the culture medium is effective in decreasing the relative amount of radioactivity incorporated into phage RNA.

Other experiments showed that the addition of formate and glutamate (30 μg/ml each) to the culture medium at the time of infection produced no further decline in the non-specific labeling of phage RNA, over that seen in the last two experiments of Table 3.2. This would indicate that incorporation of extraneous radioactivity from ^{3}H-histidine probably stems from some other pathway of histidine catabolism operative in E. coli. Nevertheless, the combined measures taken in the last two phage preparations (Expt.4 & 5, Table 3.2) proved to be effective in producing phage containing a level of ^{3}H-histidine radioactivity adequate for physiological studies, and hence no further attempts were made to further optimize labeling conditions.

4. Characterization of the Radioactivity Incorporated Into Protein of ^{3}H-Histidine Labeled R17

At this point it seemed critical to determine whether the radioactivity originally present in the 2-5-^{3}H-histidine might in some way be metabolized and subsequently incorporated into some other amino acid. Therefore, protein extracted from whole phage was subjected to acid hydrolysis and the resulting amino acids were analyzed by high-voltage paper electrophoresis at pH 6.5. Under these conditions, the basic and acidic amino acids migrate toward opposite electrodes, while

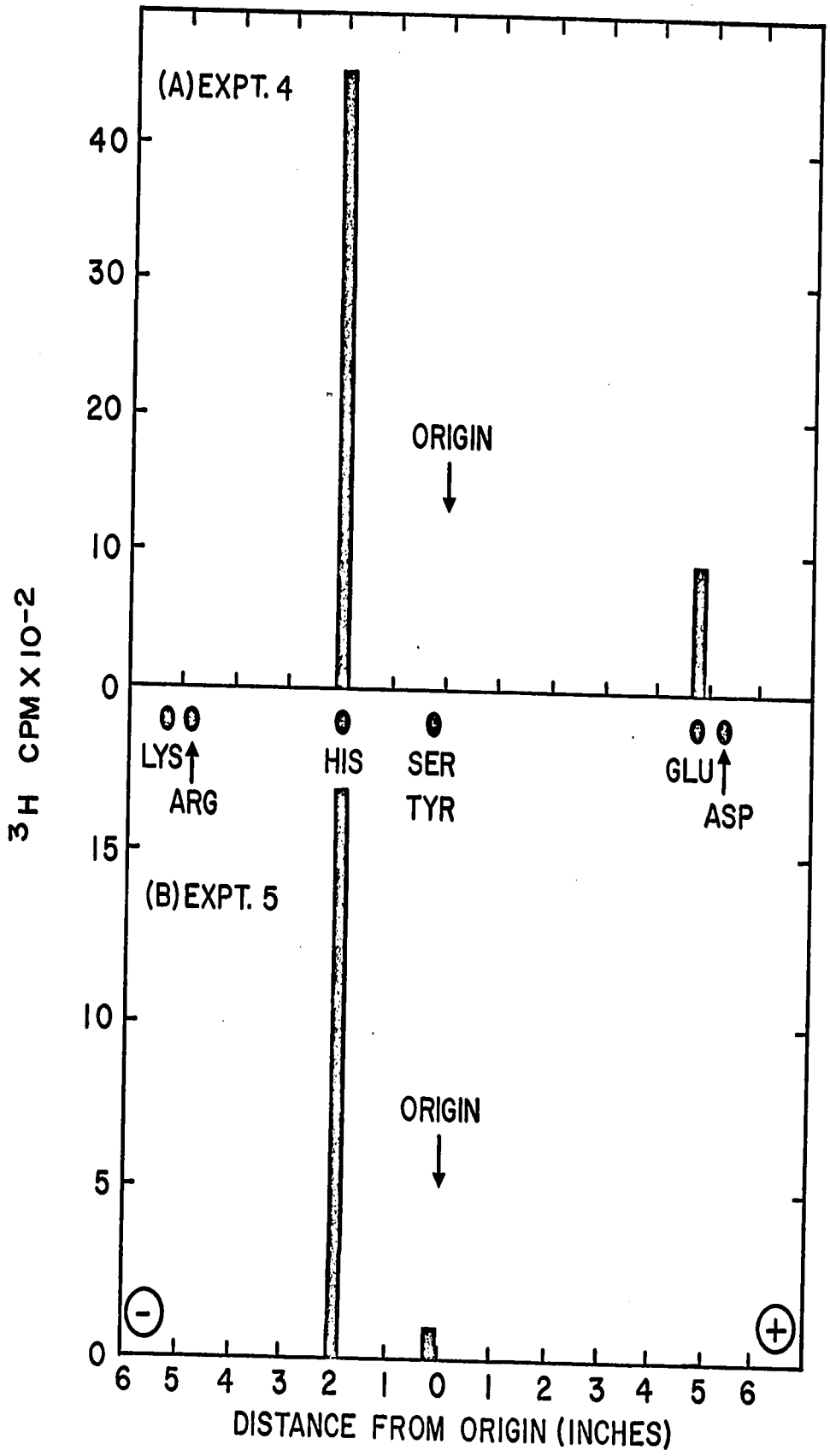
the neutral amino acids remain at the origin. Before electrophoresis, the appropriate amino acids (glutamate, aspartate, lysine, arginine, histidine, serine and tyrosine) were applied to separate lanes on the electropherogram as position markers.

Figure 3.4 A and B present the results of a study in which hydrolysates of total phage protein, extracted from the ^{3}H-histidine labeled phage shown in Table 3.2, Expt.4 and 5 respectively, were subjected to high voltage paper electrophoresis. As expected, the label of both phage hydrolysates was present predominantly in one fraction coincident with the histidine marker. A smaller fraction, tentatively identified as glutamate, accounted for roughly 18% of the radioactivity in Figure 3.4A, and approximately 5% of the total radioactivity in Figure 3.4B remained at the origin with serine and tyrosine. When a small amount of the ^{3}H-histidine used for the preparation of the phage in Expt.4 (Table 3.2), was analyzed by high voltage paper electrophoresis, the distribution of the radioactivity was identical to that shown in Figure 3.4A. The only explanation for the divergent results obtained with the two phage preparations thus is that there are differences in the purity of the ^{3}H-histidine obtained from the two commercial sources.

Electropherograms of similar studies of ^{3}H-histidine labeled R17 grown in CTMM(-aa) medium (Expt.1-3, Table 3.2) invariably contained radioactivity at the origin as well as in the Glu, Asp, Lys and Arg positions, sometimes accounting for as much as 40% of the total radioactivity. When intact phage was electrophoresed under the same conditions, all the label remained at the origin, indicating that the ^{3}H-histidine (or any other radioactivity) present is incorporated into phage and is not merely a free amino acid somehow trapped inside the particle.

Figure 3.4. High voltage paper electrophoresis of total phage protein hydrolysates of ^{3}H-histidine labeled R17 at pH 6.5.

Total phage protein was isolated from a 0.4 ml aliquot of ^{3}H-histidine labeled R17 by phenol extraction as outlined in Chapter 2 and precipitated by extraction of the phenol phase with anhydrous ether. The protein residue was taken up in 1 ml of 6 N HCl and hydrolyzed *in vacuo* by incubation at 110° for 18 hours. After evaporating to dryness, the hydrolysate was dissolved in 100 µl of distilled water and 50 µl spotted directly onto a standard sheet of Whatman 3MM electrophoresis paper. Electrophoresis was at 55 V/cm for 45 minutes. After drying, the chromatogram was cut into 1/4" transverse strips and each fraction prepared for radioactive measurement. Amino acid markers were located by spraying the appropriate strips of the chromatogram with ninhydrin. Expt.4 and Expt.5 refer to the experiments described in Table 3.2. The recovery of radioactivity from the hydrolyzed phage protein was approximately 65%.


(A) EXPT. 4
40
30
20
10
0
ORIGIN
LYS
ARG
HIS
SER
TYR
GLU
ASP
(B) EXPT. 5
15
10
5
0
ORIGIN
⊖
⊕
6 5 4 3 2 1 0 1 2 3 4 5 6
DISTANCE FROM ORIGIN (INCHES)
^3H CPM X 10^{-2}

Although it is safe to conclude that all the ^{3}H-histidine label of the total phage protein samples analyzed in Figure 3.4 is derived solely from the A protein, the source of the other amino acids (presumably glutamate and neutral amino acids) was uncertain at this time. This is true because the samples analyzed were total phage protein: a composite of A protein and coat protein. It thus became necessary to resolve the two structural phage proteins in order to compare the distribution of the label between the two species. This analysis is described in what follows.

5. SDS-Polyacrylamide Gel Electrophoresis of Protein From ^{3}H-Histidine Labeled R17

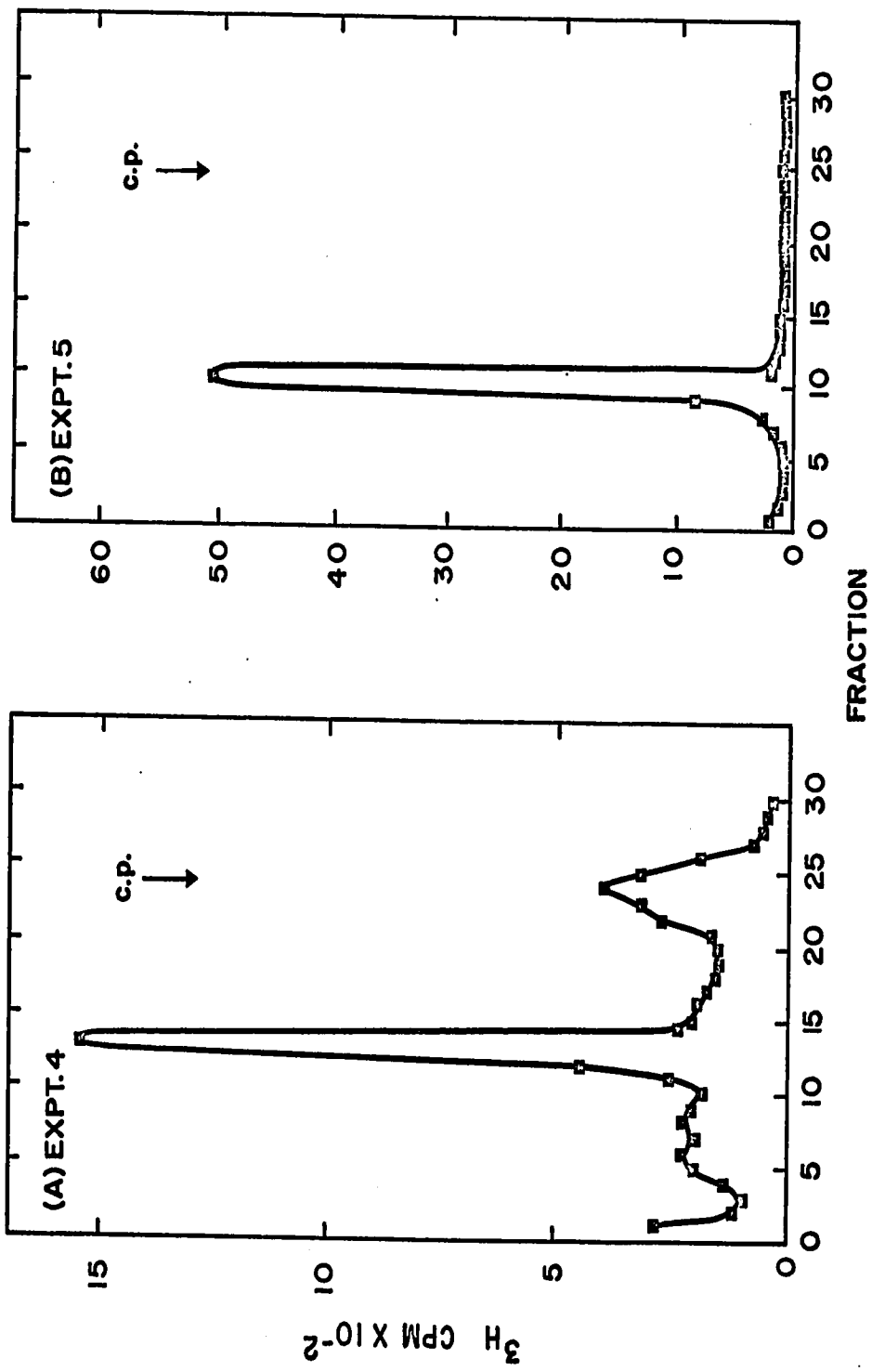
Electrophoresis of total phage protein from ^{3}H-histidine labeled phage (Expt.4 & 5, Table 3.2) in SDS-polyacrylamide gels is shown in Figure 3.5. Examination of the figure confirms the prediction that the label in such phage particles is incorporated largely into A protein. It may further be noted that the distribution of the radioactive level between the two phage proteins (Figure 3.5A) is approximately equal to that between the radioactivity migrating with histidine and glutamate in Figure 3.4A. It is noteworthy that protein of ^{3}H-histidine labeled phage in Figure 3.5B exhibited negligible amounts of radioactivity in positions other than that occupied by the A protein. It was therefore concluded that it is the sole carrier of radioactivity in phage so prepared.

C. Discussion

The specific labeling of the R17 A protein by the addition of radioactive histidine to R17-infected cells has proven to be more complex

Figure 3.5. SDS-polyacrylamide gel electrophoresis of total phage protein from ^{3}H-histidine labeled R17.

Total phage protein from ^{3}H-histidine labeled R17 (150 μg) was solubilized, electrophoresed and analyzed for radioactivity as described under Materials and Methods. Electrophoresis was at 5 mAmps/tube for 2.5 hours. The marker arrow (c.p.) indicates the position of coat protein, from phage uniformly labeled with ^{14}C-amino acids, electrophoresed under identical conditions. Expt.4 and Expt.5 refer to the experiments described in Table 3.2.


(A) EXPT. 4
(B) EXPT. 5
c.p.
c.p.
^3H CPM X 10^{-2}
FRACTION
0
5
10
15
20
25
30
10
20
30
40
50
60

than was originally anticipated. Experiments described in this chapter demonstrate that the culture medium and the time of labeling are of critical importance in achieving this objective.

When low amounts of ^{3}H-histidine (2 μCi/ml) are added to cultures at the time of infection, anomalous incorporation of radioactivity into RNA was minimal (Table 3.2, Expt.3), but the specific radioactivity of the resultant phage was inadequate to permit meaningful studies on the A protein to be conducted. The addition of higher levels of ^{3}H-histidine (10-20 μCi/ml) at various times after infection, on the other hand, led to an increased labeling of the RNA moiety of the phage (Expt.1 & 2, Table 3.2). Analysis of a snake venom hydrolysate of such labeled phage RNA by chromatography on Baker-Flex PEI-cellulose sheets (Figure 3.2) indicated that some of the label originally present in ^{3}H-histidine was selectively incorporated into the purine nucleotides, AMP and GMP (Table 3.1). An explanation for this is obtained from the scheme in Figure 3.2 which shows that the catabolic end-products of histidine metabolism by the urocanate pathway are glutamate and N^{5}-formimino FH_4: the latter of which is a precursor for de novo purine biosynthesis. Further, the finding that, under certain conditions, up to 40% of the radioactivity present in phage protein (isolated from ^{3}H-histidine labeled phage) is accounted for by amino acids other than histidine, and that the addition of various compounds to dilute the hypothetical breakdown products of histidine tends to minimize the incorporation of radioactivity into RNA, supported this interpretation. The incorporation of radioactive impurities present in some commercial preparations of ^{3}H-histidine was also inferred from the radioactivity profiles obtained by electrophoresis of total phage proteins in SDS-polyacrylamide gels (Figure 3.5A).

A labeling technique which involves dilution of intermediate breakdown products of histidine with large doses of carrier, and the use of high specific activity ^{3}H-histidine (45 Ci/mM) of high radiochemical quality (Amersham/Searle) during a critical period of the infectious cycle (15-22 min after infection), has proved to be effective in obtaining ^{3}H-histidine labeled phage A protein with a high degree of radiochemical purity. Specific radioactivity of such phage (1.3×10^{-8} cpm/particle) has permitted studies on the physiological role of A protein in phage infection to be conducted. The results of such studies are described in forthcoming chapters.

CHAPTER IV

PHAGE HETEROGENEITY AND HOST PHAGE INTERACTIONS

A. Introduction

Purified preparations of male-specific E. coli RNA phages invariably contain a large proportion (80-95%) of noninfectious particles (Loeb & Zinder, 1961; Paranchych & Graham, 1962). Separation of infectious from noninfectious particles, however, has not yet been achieved since both phage species possess identical sedimentation and banding characteristics. Moreover, there is presently only fragmentary evidence as to the nature of the defect in the noninfectious particles. Thus one can speculate that the defect arises from an inability of certain particles to attach to the host bacterium or that defective particles fail to inject their RNA into the host cell. The defect may also reside in the phage RNA itself, arising either from base substitutions in the RNA, or actual scissions of one or more phosphodiester bonds. Lastly, the possibility exists that a given fraction of phage in any population is devoid of A protein resulting from some inconsistency in the maturation process. The investigations described in this chapter are aimed at distinguishing between the foregoing possibilities.

B. Results

1. Demonstration of R17 Phage Heterogeneity

One means of determining whether the defect in noninfectious phage particles is related to a failure to adsorb to sensitive bacteria is the simple comparison of the attachment capability of a total phage population with that of the infectious phage in the same preparation.

Experiments of this type were carried out at 4° to prevent anomalous effects of phage eclipse and to insure the establishment of an equilibrated system. Attachment of total phage to E. coli HB11 was measured by using purified ^{32}P-labeled R17 phage and a procedure involving filtration through cellulose acetate membrane filters. Attachment of infectious phage to host cells, on the other hand, was monitored by a combination of centrifugation and plaque assay procedures. Both methods are described in detail in Chapter II.

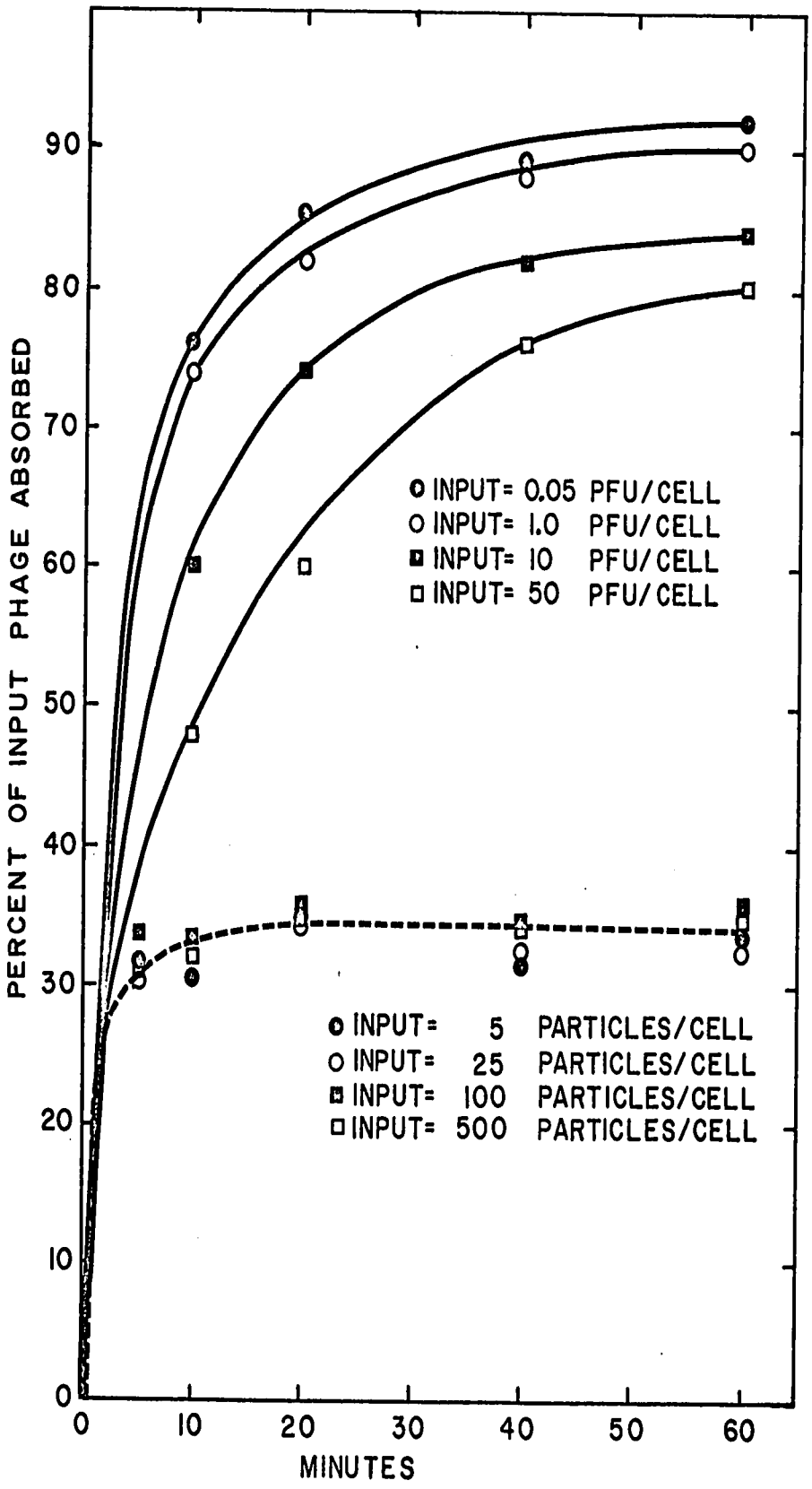
The bottom curve in Figure 4.1 (broken line), which represents that portion of the overall phage population adsorbed to E. coli HB11 at 4°, shows that phage attachment reaches a maximum between 10-20 min after phage addition to the culture and represents between 30-35% of the input phage. This fraction is constant over a wide range of multiplicities. It is noteworthy that identical results were obtained whether the radioactive label is in the phage RNA, or in the coat protein. These results are in complete agreement with those of Brinton and Beer (1967) who reported that the maximum fraction of ^{32}P-labeled R17 phage which attaches to E. coli HB11 at 0° is 0.31.

From the foregoing, it was concluded that (a) the phage attachment sites of F-pili are not yet saturated at an input concentration of 500 particles per cell, and (b) that the extent of phage attachment over the range examined is linear and independent of the multiplicity. An extension of this study revealed, moreover, that saturation occurred only when multiplicities of over 1000 particles/cell were used, and that a single cell could adsorb a maximum of about 300 phage particles.

The solid lines in Figure 4.1 represent the attachment of

Figure 4.1. Attachment of infectious and total phage to E. coli HB11 at 4°.

A culture of E. coli HB11, grown to a density of 5×10^8 bacteria/ml at 37°, was chilled on ice and divided into 10-ml aliquots. After adding the indicated amount of purified ^{32}P-labeled phage R17, each culture was maintained at 4° and gently shaken at regular intervals. Periodically, aliquots were removed and assayed for absorbed phage as described in Materials and Methods (Chapter II).


PERCENT OF INPUT PHAGE ABSORBED
MINUTES
0
10
20
30
40
50
60
70
80
90
INPUT= 0.05 PFU/CELL
INPUT= 1.0 PFU/CELL
INPUT= 10 PFU/CELL
INPUT= 50 PFU/CELL
INPUT= 5 PARTICLES/CELL
INPUT= 25 PARTICLES/CELL
INPUT= 100 PARTICLES/CELL
INPUT= 500 PARTICLES/CELL

only the infectious particles in the phage preparation. As shown here, a notably greater proportion of this species is adsorbed to bacteria, being in excess of 90% of the input at lower multiplicities. In contrast to total phage, it is evident that both the rate and the level of attachment of infectious phage (PFU) are dependent on the phage/cell input ratio.

Although the reason for the notably dissimilar attachment of total vs infectious phage is presently not clear, it may be interpreted in terms of inherent differences in their affinity for phage attachment sites on F-pili. For example, as suggested by the figure, an equilibrium is formed as early as 5 min after the addition of phage, after which there is no increase in the total number of phage particles attached to cells. At the same time, however, there is an increase in the attachment of infectious phage. This implies that at equilibrium there is an exchange between attached and unattached phage, during which the proportion of infectious phage attaching to the F-pilus increases because of their greater affinity for the F-pilus. Appealing as this idea may be, it is evident that further work is required before this or related interpretations can be accepted with any degree of certainty.

The observation that 80-90% of infectious phage can adsorb to host bacteria, whereas no more than 1/3 of the total phage population can do so, strongly supports the contention that R17 phage particles of purified preparations are heterogenous with respect to their interaction with F-pili.

2. Reversibility of the Host-Phage Interaction at 4°

Although the heterogeneity of phage in purified preparations of R17 is clearly demonstrated in the preceding section, the study

provides no direct evidence as to whether phage particles adsorb to host cells in a reversible or irreversible fashion. A critical study was therefore carried out to determine what fraction of the phage population is deficient with regard to attachment, and the extent to which such defective particles are able to interact with host bacteria. It was reasoned, for example, that if phage attachment to F-pili is irreversible, then all free phage remaining after a single attachment would be expected to be defective in attachment. If, on the contrary, attachment is governed by equilibrium considerations and hence is reversible, a significant fraction of the free phage remaining after a single attachment presumably would still be capable of adsorbing to host bacteria. Accordingly, an experiment was designed to show:

(a) whether phage attachment to host cells is reversible, and

(b) if so, what is the attached/unattached ratio of competent phage, i.e., those capable of adsorbing, in equilibrated host-phage mixtures.

A strain of E. coli (K12 Hfr_1), which is piliated to a greater extent than E. coli HB11 and shows slightly higher adsorption values, was used as host in this experiment. Firstly, 5 ml of chilled culture (5×10^8 cells/ml) was added to 5 ml of ^{32}P-labeled R17 phage (2.5×10^{11} particles) resulting in a final cell density of 2.5×10^8/ml and a phage multiplicity of 500 particles/cell. The suspension was gently shaken at 4° for 20 min, after which the cells were removed by centrifugation and the supernatant assayed for remaining ^{32}P-radioactive phage. The whole procedure was repeated three more times, each time using a 5 ml suspension of fresh cells and 5 ml of the supernatant from the previous attachment step.

The rationale for this experiment was the following. If phage attachment to sensitive cells in an irreversible process, then virtually all the phage competent in attachment would adsorb out during the first incubation and little or no phage removal would occur with subsequent suspensions of fresh cells. If, on the other hand, this host-phage interaction is a reversible one, a portion of the phage remaining after each adsorption step would adsorb during each successive treatment with bacteria.

The results of the experiment are shown in Table 4.1. It is evident from column 3 of the table that some phage was adsorbed out during each of the four incubations, although the amount of adsorbable phage remaining after the third incubation was negligible. It was therefore concluded that the host-phage interaction at 4° is a reversible one.

Since essentially all the competent phage was removed from the phage preparation by four successive treatments with bacteria, it was possible to calculate the actual amount of competent and defective particles in (a) the original phage preparation, as well as (b) each of the phage supernatants. The results of these calculations are listed in columns 4, 5 and 6 of Table 4.1. As shown, the first incubation resulted in the attachment of approximately 2/3 of the total competent phage in the preparation to the cells. Of the competent particles remaining after the first incubation, approximately 2/3 was again adsorbed out during the second cell suspension. Following this, the amount of competent phage was so depleted that reliable measurements were not possible.

Further, it is noteworthy that although there was a progressive decrease in the amount of competent phage in each cell suspension,

TABLE 4.1

Adsorption of ^{32}P-Labeled Phage from a Phage Suspension by Successive Additions of *E. coli* K12 Hfr_1

Cell Suspension Number*	Input (Part./cell)	Percent of Input Phage Adsorbed	Phage Adsorbed (Part./cell)	Unattached Competent Phage (Part./cell)	Defective Phage (Part./cell)	Attached Phage / Unattached Competent Phage
1	500	42	210	136	154	1.66
2	145	30.4	43.5	25.0	77	1.74
3	51	18.9	9.7	2.8	38.5	3.46
4	21	6.6	1.4	0	19.6	-

A culture of *E. coli* K12 Hfr_1 was grown in CTMM medium to a density of 5×10^8 bacteria/ml, then chilled to 4°. Five ml of the culture was added to 5 ml purified P^{32}-labeled R17 phage suspended in CTMM at a concentration of 2.5×10^{11}/ml. The mixture was gently shaken at 4° for 20 min, then centrifuged at 10,000 g for 10 minutes. The supernatant solution was assayed for radioactivity, then mixed in a 1:1 ratio with a fresh portion of the bacterial culture. After again shaking the sample for 20 min at 4°, the bacteria were removed by centrifuging at 10,000 g for 10 min and the supernatant solution was assayed for its content of radioactivity. In all, the procedure was repeated four times. The values given are the means of two experiments.

*Cell density in each case was 2.5×10^8 cells/ml.

the amount of defective phage remained fairly constant. Since each suspension of phage with fresh cells represented a 1:2 dilution of the phage solution, the concentration of defective particles should have been halved by each successive step. That this, in fact, was the case is shown in column 6 of Table 4.1.

On the basis of the foregoing considerations, it was concluded that approximately 30% of the phage in a purified preparation is defective in the attachment function, while the remaining 70% attach to host bacteria in a reversible manner, the ratio of attached/unattached competent phage being about 1.7 under these conditions.

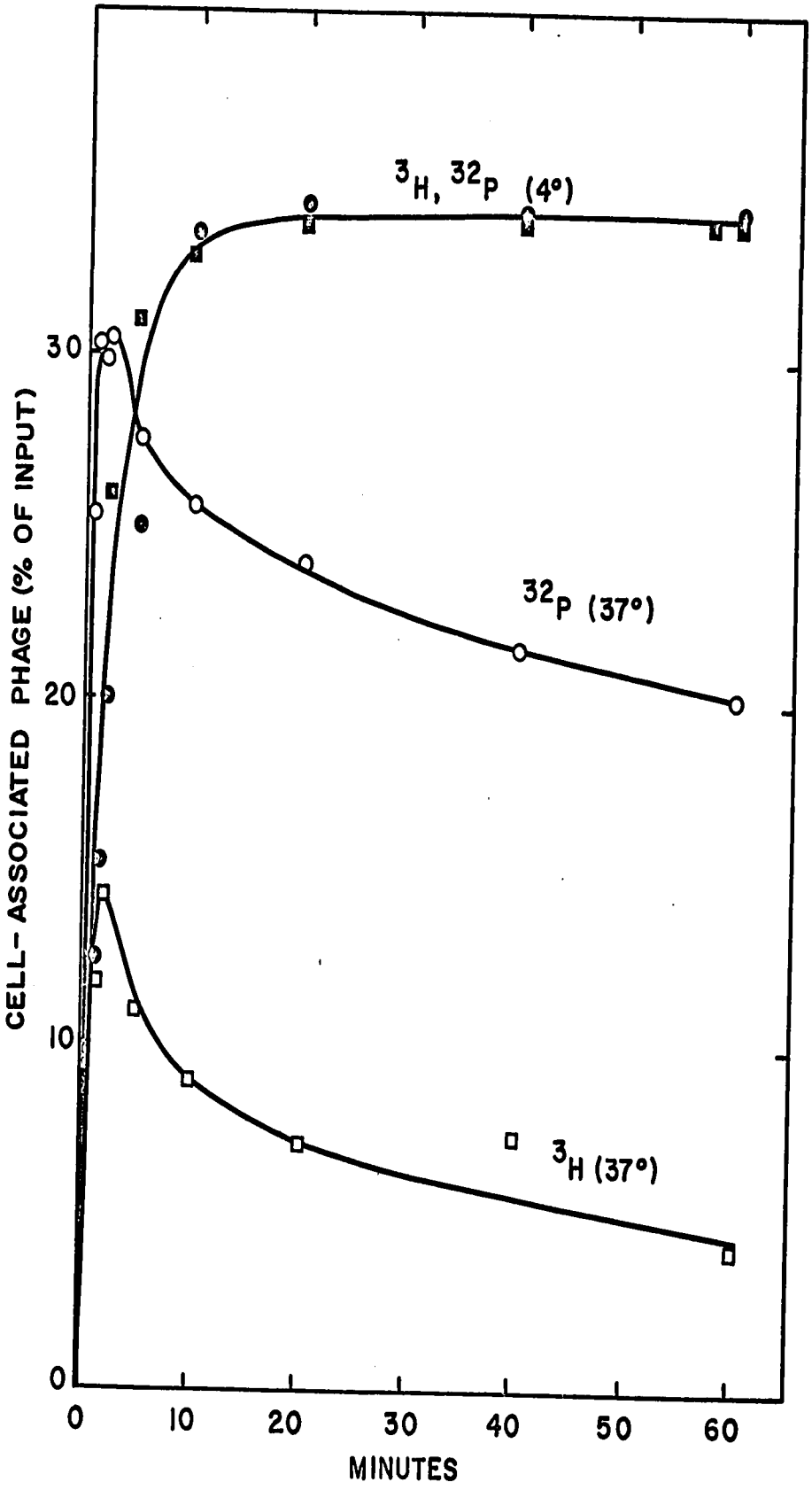
3. Host-Phage Interaction at 37°

To determine what effects phage eclipse might have on adsorbed particles, analogous experiments were subsequently carried out at 37°. In this study, a purified preparation of doubly-labeled phage R17, in which the RNA was labeled with ^{32}P and the coat protein with ^{3}H-leucine, was employed. The phage preparation was incubated with cultures of *E. coli* HB11 at 4° and 37°, and aliquots were removed at appropriate time intervals and assayed for radioactivity by the filtration assay.

It is evident (Figure 4.2) that the pattern of attachment of phage to bacteria is substantially different at 37° than at 4°. At the latter temperature, both coat protein and RNA radioactivity attach to the same extent, and remain at this level for the duration of the experiment. At 37°, the rate of attachment of phage to bacteria is increased, and attachment is followed by considerable elution of radioactivity from the cells. In the case of the coat protein (^{3}H), elution is so rapid that the amount of cell-associated radioactivity never reaches the level it is capable of reaching at 4°. In fact, the maximum amount of cell-

Figure 4.2. Attachment of ^{32}P-^{3}H-labeled R17 phage to *E. coli* HB11 at 37°.

A culture of *E. coli* HB11 was grown to a cell density of 5×10^8/ml in Lac TMM medium, divided into two equal portions, and one chilled to 4° on ice. Both cultures were then infected with purified ^{32}P-^{3}H-labeled R17 phage at a multiplicity of 100 particles/cell and incubated at the respective temperatures for 60 minutes. During this time, 1.0 ml aliquots were periodically removed and assayed for cell-associated radioactivity by the filtration assay.


^{3}H, ^{32}P (4°)
^{32}P (37°)
^{3}H (37°)
CELL-ASSOCIATED PHAGE (% OF INPUT)
0
10
20
30
0
10
20
30
40
50
60
MINUTES

associated coat protein detected was only about 14% of the input at 2 min after infection.

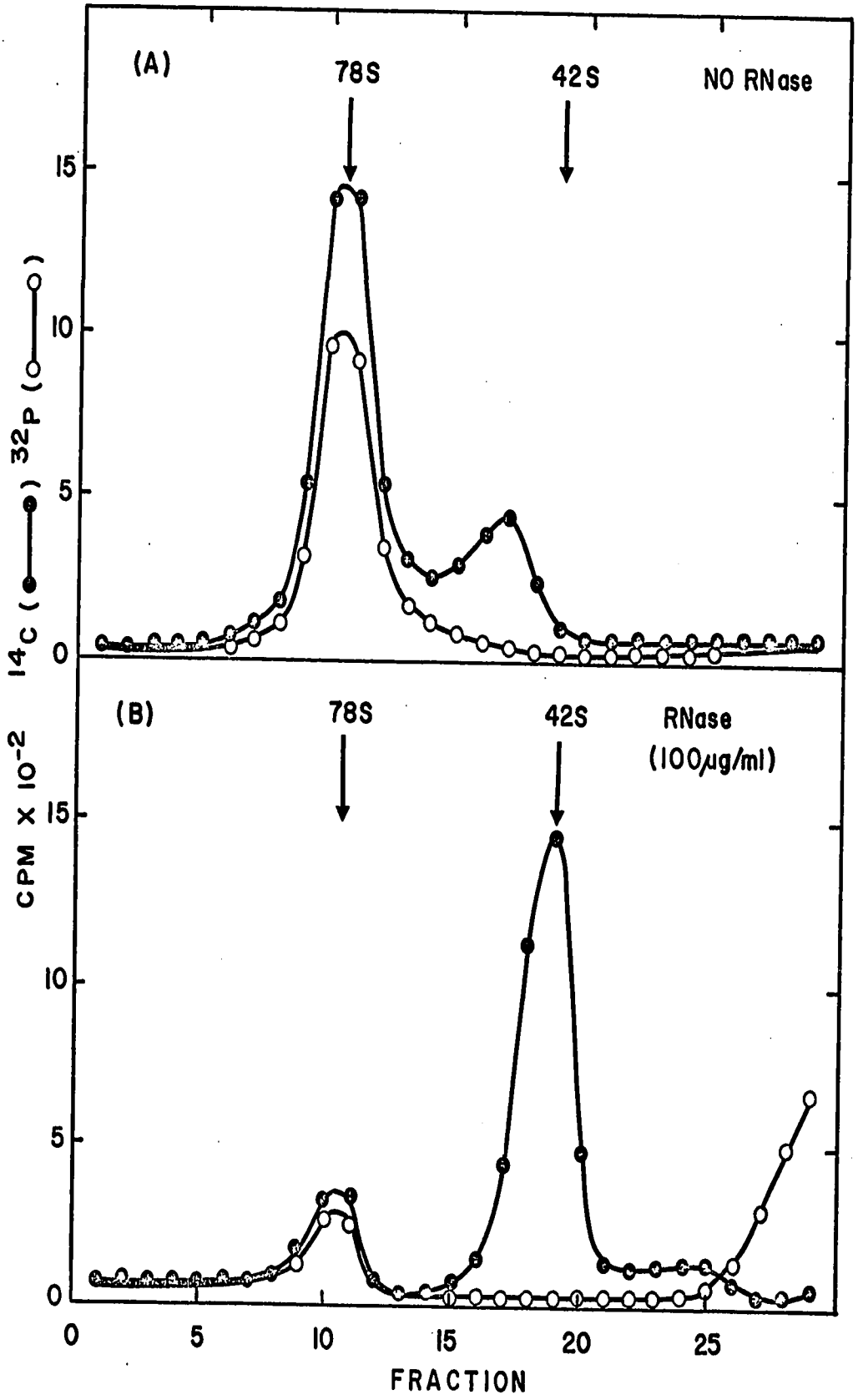
The amount of phage RNA (^{32}P) becoming cell-associated is considerably greater than that of coat protein (^{3}H). This is so because some of the RNA is injected into the cell during infection. It is evident, however, that not all of the phage particles which interact with cells succeed in injecting their RNA since some of the RNA associated with cells at 2 min after infection becomes dissociated at later times.

The physical nature of phage particles which have undergone the type of adsorption and elution shown in Figure 4.2 was next examined by a variation of the foregoing experiment. This involved incubation of doubly-labeled phage (^{14}C-coat protein, ^{32}P-RNA) with *E. coli* HB11 at 37° for 60 min, followed by analysis of the products by sedimentation in sucrose gradients.

The results of the experiment (Figure 4.3A) show that about 70% of the phage sediment as intact 78S particles, while about 30% sediment as a slower fraction. Whereas the 78S particles appear to contain a full complement of RNA, particles in the slower-sedimenting region contain varying amounts of RNA. This is consistent with the observation in Figure 4.2 that some phage RNA, as well as coat protein, becomes dissociated from the bacteria during the experimental procedure. Although it is not possible to measure precisely that portion of the total phage population which releases its entire RNA complement, estimates from the slow-sedimenting fraction indicate the amount is of the order of 10%, a value corresponding to the known content of infectious phage in purified preparations. This class of phage, which injects all or part of its RNA and contains the infectious phage, will be designated

Figure 4.3. Sedimentation analysis of R17-infected cultures of E. coli HB11.

A culture of E. coli HB11 was grown to a cell density of 5×10^8 bacteria/ml and then divided into two equal portions. One portion was made to contain 100 μg/ml RNase, after which both cultures were infected with ^{14}C-^{32}P-labeled R17 phage (physical particle/cell ratio = 100) and incubated at 37° for 60 minutes. The cultures were subsequently chilled, and aliquots of 0.3 ml were subjected to sedimentation in linear (5-20%) sucrose density gradients (SW39 rotor; 2 hrs at 35,000 rpm). The contents of each tube were then dropped out into 10-drop fractions, and each fraction was assayed for ^{14}C and ^{32}P radioactivity.


(A)
78S
42S
NO RNase
15
10
5
0
^{14}C (●—●) ^{32}P (○—○)
(B)
78S
42S
RNase
(100µg/ml)
CPM X 10^{-2}
15
10
5
0
0
5
10
15
20
25
FRACTION

as Class I particles for purposes of discussion later.

Figure 4.3B illustrates the results of an accompanying experiment, which was identical to the previous one, except that the culture contained 100 μg/ml RNase. Unexpectedly, about 90% of the phage populations was converted into empty 42S capsids by this treatment. It is important to note that when a phage preparation was incubated with RNase alone under the same conditions, no such net conversion of 78S to 42S particles occurred. This signifies that some process during interaction of phage with F-pili of host cells at 37° is responsible for the sensitization of phage to RNase.

The results thus show that although 30% of the phage population cannot be shown to attach to host bacteria by the methods used earlier in the investigation (Table 4.1), some form of interaction must in fact occur. Although the duration or the strength of this reaction is insufficient to cause the dissociation of phage RNA from coat protein, it is sufficient to render the particles vulnerable to RNase which, if present, results in extensive degradation to the phage genome. This second class of phage particles which interact with host cells at 37°, but do not inject their RNA, will be referred to as Class II particles.

It is of interest to consider the nature of the residual 78S phage in Figure 4.3B. The phage in this fraction of the gradient, which comprised about 10% of the total phage population, is completely resistant to RNase and does not inject any of its RNA into host cells. Such phage, moreover, was found to be noninfectious and completely incapable of adsorbing to host cells. This suggests that yet another class of phage particles (denoted as Class III particles) exists in wild-type preparations of phage R17. Since this class of phage particles exhibits

no detectable interaction with host cells whatsoever at 4° or 37°, it may be suggested that they lack some phage component, possibly A protein.

It is important to emphasize at this point, that the foregoing classification scheme for R17 phage particles is arbitrarily based on their interaction with host bacteria at 37° (conditions optimal for infection) and does not take into consideration, their attachment characteristics at 4°. Summarizing briefly, Class I particles are those which inject all or part of their RNA into host cells upon interaction at 37°, their empty or partially empty capsids being released into the medium (Figure 4.3A). Class II particles also interact with host cells under the same conditions, but no RNA injection occurs. After interaction, these particles may be degraded to empty capsids by treatment with RNase (Figure 4.3B). Lastly, Class III particles exhibit no interaction with host bacteria nor are they sensitized to RNase when incubated at 37°. Needless to say, they do not inject their RNA (Figure 4.3B).

4. Distribution of A Protein in Wild-Type Phage R17

Roberts and Steitz (1967) have reported that addition of A protein to reconstitution mixtures of phage R17 results in a several hundred-fold increase in the infectivity of the particles produced. It has been inferred from these studies that the A protein promotes the attachment of phage to host cells. Thus, it seemed possible that the failure of Class III phage particles to interact with host cells might be related to the absence of the A protein.

Several prerequisites must be met before this hypothesis can be subjected to direct investigation. Firstly, in looking for specific attachment of phage to host bacteria (assuming that this property is

conferred to phage by the presence of the A protein, Steitz, 1968b), it is imperative to introduce some radioactive marker specifically into A protein, so that its function in attachment may be monitored independently. Having such a phage preparation, it should be possible, by the above criterion, to attach 100% of the label to host cells.

A second requirement is the presence of phage with a general radioactive label, either in the coat protein or RNA moiety. Here all phage particles alike would be uniformly labeled, regardless of whether the A protein is present or not. With such a phage preparation, any radioactivity remaining in the supernatant, after titration of the competent phage with host cells, might be attributed to phage particles lacking A protein. Thus a differential adsorption of radioactivity from the two phage preparations described might be predicted.

The following experiment was performed to test the above hypothesis. A mixture of two purified R17 phage preparations (1:1 on the basis of cpm), one labeled in the coat protein with a mixture of ^{14}C-amino acids (Figure 2.2) and another in the A protein with ^{3}H-histidine (see Figure 3.5B) was added to a log-phase culture of E. coli K12 Hfr_1, at a multiplicity of 1000 particles/cell, and the mixture incubated at 4° for 10 min to allow adsorption of phage to bacteria. The bacteria were then collected by centrifugation, after which small aliquots of the supernatant solution (containing unadsorbed phage) were analyzed for the distribution of ^{14}C and ^{3}H radioactivity. The unadsorbed phage was then added to fresh bacteria and the entire procedure repeated four more times.

The results of the experiment are shown in Table 4.2. It may be seen that the ratio of $^{14}C/^{3}H$ radioactivity in the supernatant increased markedly with each adsorption step, indicating a preferential

TABLE 4.2

Comparison of Coat Protein and A Protein Radioactivities in Adsorbed and Unadsorbed Phage R17 Particles Following Serial Treatments with *E. coli* K12 Hfr_1

Number of Attachments	Ratio of $^{14}C/^{3}H$ Radioactivities in the Supernatant Solution*	Ratio of $^{14}C/^{3}H$ Radioactivities in the Cell Pellet*
0	1.3	-
1	2.7	0.8
2	5.7	0.8
3	7.3	0.8
4	8.1	1.2
5	20.0	0.8

*The values given are means of triplicate measurements.

A culture of *E. coli* K12 Hfr_1 was grown in CTMM medium to a density of 5×10^8 bacteria/ml and then chilled to 4°. An aliquot was centrifuged for 5 min at 10,000 g to collect the cells, after which the pellet was gently resuspended in fresh TMM at a density of 5×10^9 bacteria/ml. To this bacterial suspension was added a mixture (1:1 on the basis of radioactiviey; 10:1 on the basis of ^{3}H-labeled physical particles/^{14}C-labeled physical particles) of two purified R17 phage preparations at a multiplicity of 1000 physical particles per bacterium. The phage-cell suspension was then incubated for 10 min at 4° to allow adsorption, after which the bacteria were collected by centrifugation. Small aliquots of the supernatant solution and of the resuspended cell pellet were spotted on filter discs, and assayed for cold TCA- insoluble radioactivity. The remaining supernatant solution was mixed with a fresh bacterial pellet to give a final cell density of 5×10^9 bacteria/ml and the phage attachment procedure was repeated. After again determining the distribution of cold TCA-insoluble ^{14}C and ^{3}H radioactivity in the supernatant and pellet fractions, the entire procedure was repeated three more times. The amount of radioactivity present in the initial attachment mixture was: ^{14}C, 21,000 rpm; ^{3}H, 16,000 cpm.

removal of A protein-containing phage. Since phage attachment is reversible, it is not surprising that several adsorption steps were needed to achieve almost quantitative removal of adsorbable phage. Adsorption appeared complete after five treatments of the phage mixture with fresh cells, since additional treatment resulted in no further removal of phage. The last column of Table 4.3 shows that the ratio of $^{14}C/^{3}H$ radioactivity in the cell pellet (attached phage) remained relatively constant, as had been expected. This is so because all phage, competent in attachment, presumably contain A protein and coat protein alike.

To further examine the nature of the defective phage particles remaining in the final phage suspension, aliquots of the phage mixture (before and after five successive treatments with bacteria) were analyzed by sedimentation in sucrose density gradients. It may be seen that although the initial phage mixture (Figure 4.4A) contained approximately equal amounts of ^{14}C- and ^{3}H-radioactivity, the final mixture (Figure 4.4B) was almost completely lacking in ^{3}H-radioactivity, but still retained about 10% of the ^{14}C-label.

The foregoing observations are entirely consistent with the earlier findings (Figure 4.3) that approximately 10% of the phage population consists of particles which are unable to interact with F-pili (Class III particles). The reason for the inability of such particles to interact with host cells thus may be ascribed to their lack of A protein.

It is interesting to point out that although Class III particles and A cistron amber mutants both lack A protein, they differ considerably. The latter are highly sensitive to RNase, whereas the

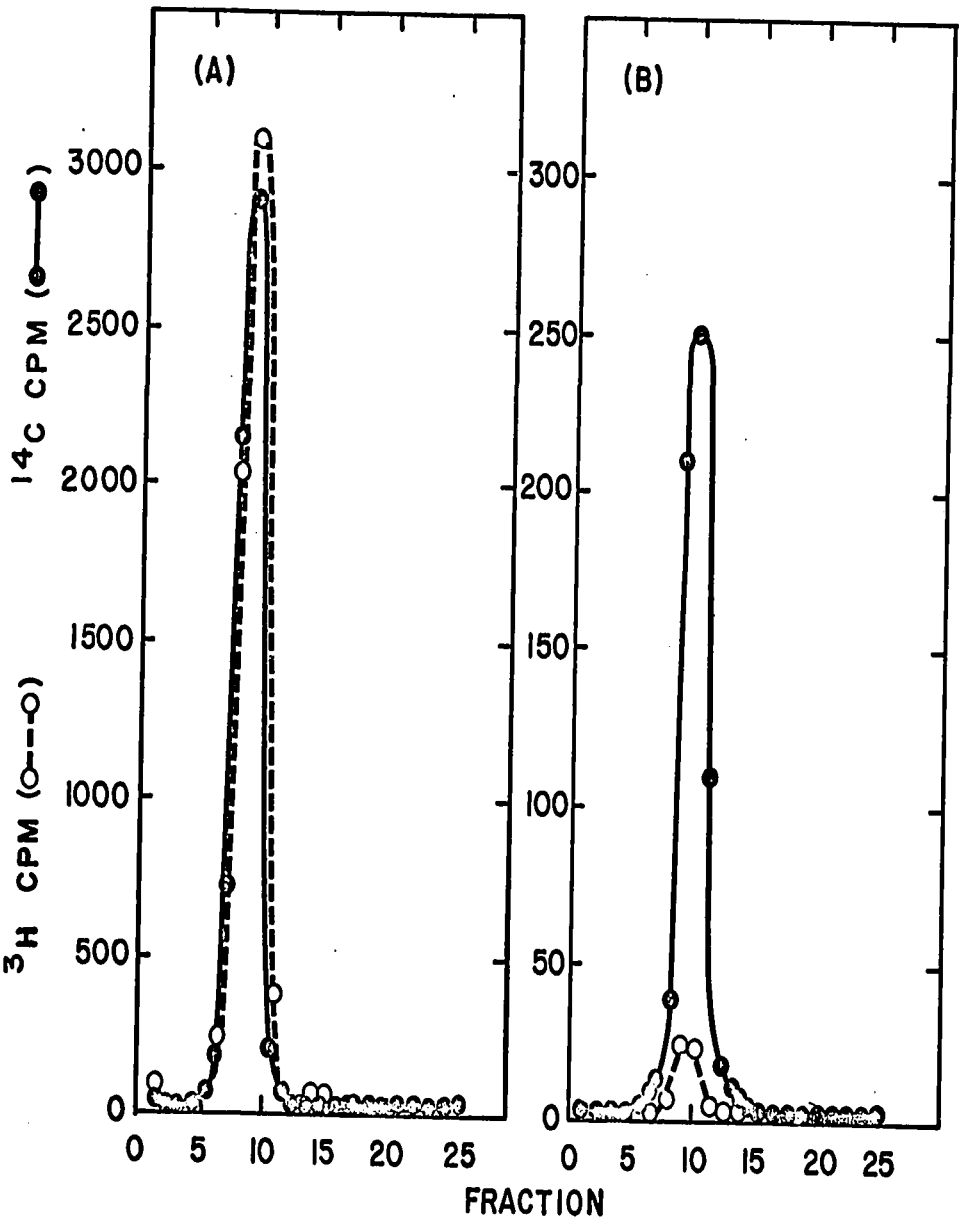


Figure 4.4. Sucrose density gradient sedimentation profiles of a ^{14}C-amino acid/^{3}H-histidine-labeled R17 phage mixture before and after five successive adsorptions to E. coli K12 Hfr_1.

The details of the experiment are given in Table 4.3. One-tenth ml aliquots of the appropriate phage solution were layered onto linear (5-20%) sucrose gradients made up in TMM. Centrifugation was for 75 min at 40,000 rpm in the Spinco SW50 rotor. Fractions of 10 drops each were collected directly onto Whatman 3MM filter discs, and assayed for cold TCA-insoluble radioactivity.

(A) Phage mixture prior to treatment with bacteria.
(B) Phage mixture following five successive treatments with E. coli K12 Hfr_1.

former are completely resistant to the enzyme. It is conceivable, therefore, that the position normally occupied by the A protein has been replaced in the Class III particles by additional coat protein subunits, or by some other type of host protein.

Since about 90% of the phage particles of purified phage preparations are adsorbed out by successive treatments with fresh host cells (Figure 4.4B), it may be inferred that the Class I and II particles comprising this fraction contain A protein. Several other lines of evidence also lend support to this suggestion. Firstly, about 90% of the phage particles of wild-type phage preparations adsorb to nitrocellulose filters, whereas defective particles lacking A protein fail to do so (Lodish et al., 1965; Heisenberg & Blessing, 1965). Secondly, direct evidence to show that Class II particles contain A protein is given in the following chapter (Figure 5.4B), where it will also be shown that the A protein of such particles is released upon interaction with host cells at 37°.

Thus, the only distinguisable feature between Class I and Class II particles, seen thus far, is the failure of the Class II particles to inject their RNA into host cells at 37° (Figure 4.3A). Why this is so remains a mystery, although it might be explained on the basis that Class II particles have a lesser affinity for host F-pili than Class I particles. To test this possibility, an experiment was carried out to determine whether the Class I particles are adsorbed preferentially over Class II particles during successive treatments of a phage preparation with fresh aliquots of cells. A phage preparation was allowed to adsorb, successively, to two aliquots of cells at 4°, after which the centrifuged phage-cell complexes were resuspended in fresh medium and incubated at 37° for 60 min to allow eclipse of the

adsorbed phage to occur. The complexes were then analyzed for Class I and Class II particles by sedimentation in sucrose gradients, and the ratio of the two phage classes in each sample determined. It was reasoned that if Class I particles are adsorbed out preferentially, the ratio of Class I/Class II particles should be greater in the first host-phage mixture than the second, since most of the Class I particles would be adsorbed out during the first treatment. In fact, the sucrose gradient profiles showed the ratio of Class I/Class II particles to be identical in each of the two attachment mixtures, indicating that there is no difference in the affinity of Class I and Class II particles for host cells. This result is somewhat surprising, since infectious phage, which are included in the Class I particles, attach preferentially at 4° (Figure 4.1). However, considering that only 5-10% of the total phage population is infectious, it is clear that this amount of phage would not significantly affect the results, since it is within the limits of experimental error. It is thus evident that the inability of Class II particles to inject their RNA into host cells cannot be explained on the basis of their relative affinity for F-pili. Alternative possibilities to explain this defect in Class II particles are discussed in a later chapter of this thesis.

C. Discussion

Several interesting features about the nature of phage attachment to host cells have emerged from the studies described in this chapter. A comparison of the kinetics of attachment of total phage with that of the infectious phage of purified R17 preparations to host cells at 4° indicates that they are heterogeneous in this respect

(Figure 4.1). Although only about 1/3 of the total phage becomes attached when mixed with host cells, at 4°, between 80-90% of the infectious phage are adsorbed out under these conditions. Investigations in which phage preparations were treated repeatedly with fresh host cells at 4°, revealed that up to 90% of the total phage were competent in the attachment function (Figure 4.4B). This led to the conclusion that phage attachment, at 4°, is reversible having an attachment ratio of 1.7 (Table 4.1).

The idea that the high ratio of noninfectious/infectious phage in purified preparations arises from some defect in the phage particle itself has received some support from studies on the interaction of phage with host cells at 37°. Investigations of this type indicate that the particles in R17 phage preparations may be divided into three classes on the basis of their interaction with host cells at 37°. The pertinent features of these three phage classes are summarized in Table 4.3.

TABLE 4.3

Comparison of the Three Classes of Phage Particles of Purified Phage R17 Preparations

Phage Class	Percent of Phage Population	A Protein	RNase Sensitivity*	RNA Injection	Attachment
I	~30%	+	+	+	+
II	~60%	+	+	-	+
III	~10%	-	-	-	-

*After incubation with host cells at 37°.

It may be seen from the above table that only those phage particles which contain A protein (Class I and Class II particles), adsorb to host cells. The reason for the failure of Class II particles to inject their RNA, however, is presently not clear. Several mechanisms may be envisaged to explain this phenomenon. The first mechanism might be that there are "preferred regions" of the F-pilus, on which phage are able to inject their RNA, as has been suggested by Valentine et al. (1969). According to this scheme all phage particles containing A protein (90% of phage population, Figure 4.4B) are identical before attachment, the factor determining whether a phage particle is of the Class I or Class II type being the position at which the phage particle attaches on the F-pilus. A second mechanism might be that the A protein is somehow involved in RNA injection and that the configuration of the A protein in Class II particles is unfavorable for RNA injection. The observation in the following chapter that both phage classes lose their A protein upon interaction with host cells at 37° is consistent with this notion. The finding that the noninfectious Class III phage particles, which are incapable of interacting with host cells at either 4° or 37°, lack A protein, suggests further that the A protein plays a key role in the early stages of infection.

Lastly, the examination of the host-phage interaction at 37° (Figure 4.2) has provided an insight into several interesting aspects of the infectious process which has hitherto remained poorly understood. For example, it is shown that the initial rapid interaction of phage with host bacteria is followed by a dissociation of the phage-cell complex, and that the phage capsid does not dissociate into coat protein subunits, but remains intact as an empty or partially empty capsid.

CHAPTER V

THE F-PILI MEDIATED ECLIPSE OF PHAGE R17

A. Introduction

It has been known for several years that infection of *E. coli* by male-specific RNA phages is prevented when a small amount of RNase is included in the culture medium (Zinder, 1963; Knolle & Kaudewitz, 1963). Since phage particles themselves are not destroyed by RNase, this has been interpreted to mean that the phage genome becomes exposed to the medium during some step of the infectious process (Valentine & Strand, 1965). Numerous studies now suggest that this RNase-sensitive step occurs on the F-pilus, presumably at or near the adsorption site of the RNA phage (Valentine & Wedel, 1965; Paranchych, 1966; Silverman & Valentine, 1969; Danziger & Paranchych, 1970b).

In this laboratory, the role of F-pili in RNA phage infection has been divided into three steps for experimental purposes. Chronologically they are:

(1) attachment of phage to the F-pilus,

(2) eclipse of phage, and

(3) penetration of the phage genome into the cell.

The second step, phage eclipse, as applied to the study of R17 infection, is defined as "that interaction of phage with F-pili which results in a sensitization of the phage RNA to RNase". This definition has proved to be convenient in that it allows eclipse of noninfectious, as well as infectious phage to be measured (see p.66).

Phage adsorbed to cell-associated F-pili proceed into eclipse by a temperature and metabolic energy-dependent process (Knolle, 1967b;

Danziger & Paranchych, 1970b). Although F-pili in the free state retain their capacity to adsorb phage, the ability of F-pili to eclipse phage is entirely abolished upon separation from the cell (Valentine & Strand, 1965; Danziger & Paranchych, 1970a) and cannot be restored even by addition of high energy compounds (Danziger & Paranchych, 1970b). Furthermore, the observation that addition of ATPase to infected cultures in no way hinders phage eclipse has led to the suggestion that a co-operative reaction between the host cell, the F-pilus and the cell wall may be required for phage eclipse to occur (Danziger & Paranchych, 1970b). A key role for the cell and the F-pilus in the eclipse step is indicated by the isolation of bacterial "pili" mutants which adsorb phage normally but are unable to carry out the eclipse reaction (Silverman et al., 1967a, b, 1968).

Despite our present knowledge about the factors that govern phage eclipse, there is presently little or no information regarding the precise mechanism whereby the phage genome is exposed to the surrounding medium during phage eclipse. This chapter describes experiments carried out with a view to answering some of the questions raised by this phenomenon.

B. Results

1. Kinetics of Phage Eclipse

To determine whether phage eclipse is restricted temporally to a short time period immediately after phage addition or whether, on the other hand, it proceeds at a constant rate over an extended period of time, an experiment was conducted in which eclipse of phage infectivity (PFU) was monitored as a function of time. The results of this study are presented in Figure 5.1.

It may be seen that under conditions of high phage input, i.e., 50 PFU/cell, the eclipsing of phage occurred only during the first 5 min of the infectious period, during which approximately 40% of the input phage was eclipsed. Thereafter, even though the culture still contained about 30PFU/cell of uneclipsed phage, no further eclipsing was detected. When lower multiplicities of infection were used, the pattern consisted of an initial rapid burst of eclipsing during the first 5 min, followed by a slower rate between 5 and 20 minutes. This second, slower, phase of phage eclipsing most probably represents eclipsing of residual phage by cells which were not exposed to phage during the earlier period. At high phage inputs, a more complete exposure of susceptible cells to phage occurs during the early period, thereby giving rise to a more synchronous pattern of eclipsing. On the basis of the foregoing considerations, it was concluded that the bulk of eclipsing activity is completed during the first 5 min of the infectious cycle, and that host cells, once having participated in the eclipse reaction, are incapable of eclipsing additional phage.

Experiments of the type described above were subsequently carried out with a wide range of phage input concentrations in order to determine the maximum amount of phage which can be eclipsed by a single cell. The results of the experiment were then plotted as the relationship between the phage input/cell and the number of PFU/cell eclipsed, and are shown in Figure 5.2. It is evident that the reaction obeys typical first-order saturation kinetics, and determination of the ordinate intercept in the reciprocal plot (Figure 5.2.inset) yielded a value of 250 as the maximum number of PFU that can be eclipsed by a single cell. Since it is known that cultures of E. coli K12 Hfr_1

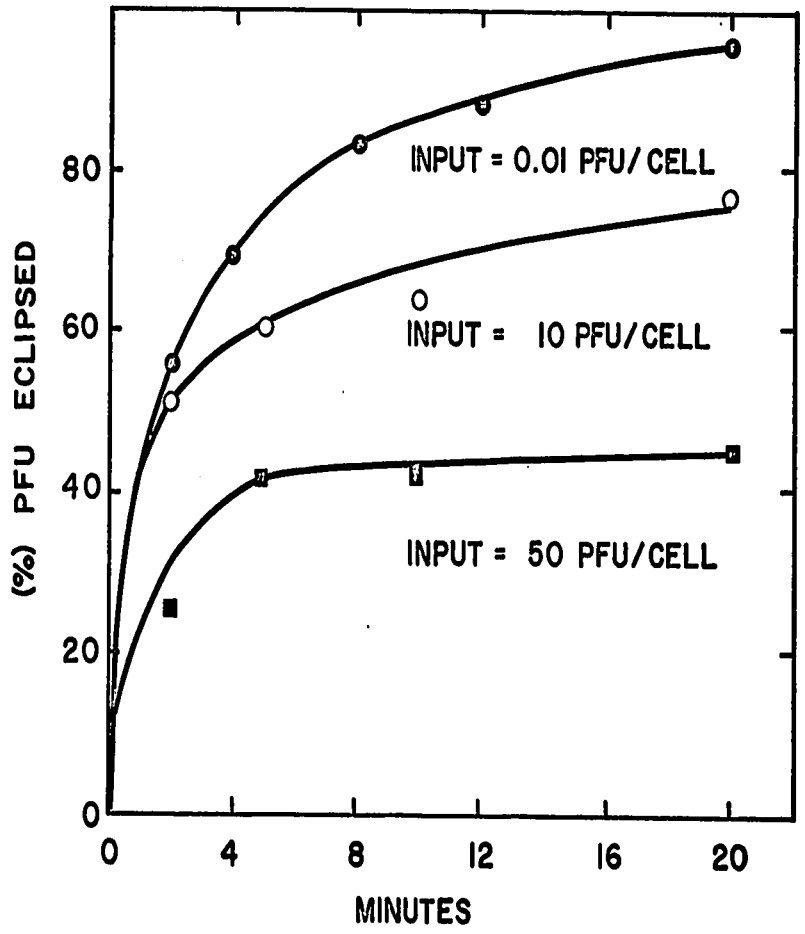


Figure 5.1. Kinetics of eclipse of phage R17 infectivity by E. coli K12 Hfr_1.

A culture of E. coli K12 Hfr_1 was grown in CTMM medium to a cell density of 2 x 10^8/ml, after which RNase was added to give a final concentration of 100 μg/ml. The culture was divided into 5 ml aliquots and phage R17 was added to each culture at the multiplicities indicated in the figure. At intervals after phage addition, aliquots were removed, diluted into chilled diluent and assayed for infectious phage by the plaque assay technique.

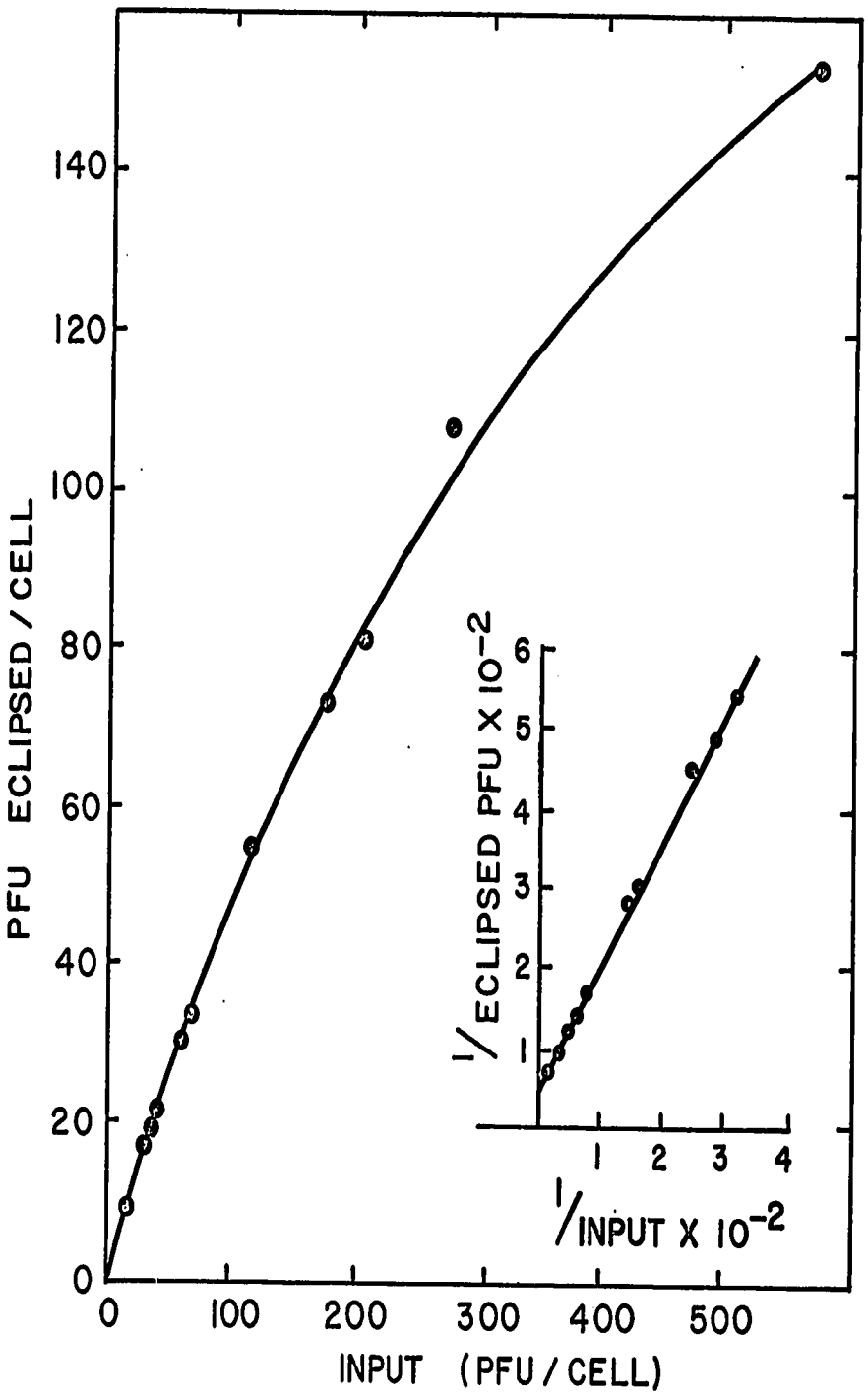


Figure 5.2. Eclipse of phage R17 infectivity by E. coli K12 Hfr_1 at various multiplicities of infection.

A culture of E. coli K12 Hfr_1 (2×10^8 bacteria/ml) was made to contain RNase to a final concentration of 100 μg/ml. Five minutes later aliquots of the culture were transferred to flasks containing the phage multiplicity indicated in the figure, incubated for 20 min, and assayed for eclipse by the eclipse assay.

contain, on the average, about one pilus per cell (Brinton & Beer, 1967; S. Ainsworth, personal communication), it is clear that single pili are capable of eclipsing a large number of phage particles during their brief burst of activity.

It is to be noted that the eclipse experiments in this section deal with eclipse of only infectious phage, and should not be confused with the eclipse of Class I or Class II phage particles, which was described in the previous chapter. Remembering also that infectious phage are included among Class I particles, and that they are distinguished from noninfectious Class I and Class II particles by their preferential attachment to host cells at 4° (Figure 4.1), it is of interest to note that the maximum amount of infectious phage which can be eclipsed under conditions of infinite phage concentration is of the same order as that needed to completely cover the average F-pilus (i.e., about 300 particles), as seen from electron microscope and phage attachment studies. It would thus appear that host cells are capable of eclipsing virtually all the phage particles which they can attach.

2. Release of A Protein From Phage Particles During the Eclipse Reaction

It has been reported that RNA phage amber mutants of the A-cistron type, grown under non-permissive conditions, are incapable of adsorbing to host cells and are sensitive to degradation by RNase (Horiuchi et al., 1965; Lodish et al., 1966; Argetsinger & Gussin, 1966). Steitz (1968a) has related these observations to the absence of A protein from such mutant phage particles. The question was thus entertained as to whether the rapid elution of RNase-sensitive phage particles from cells during eclipse (Figure 4.2), by analogy might be

attributed to some reaction of the A protein. In particular, it was of interest to know whether the A protein is displaced from the particle prior to release of the eclipsed phage into the medium. It is logical to expect that the phage eclipse reaction, being an energy-requiring process (Danziger & Paranchych, 1970b), might induce a conformational change in the phage structure so as to "eject" or otherwise loosen the A protein from the phage capsid. Such a process would presumably facilitate the exit of the RNA from the phage capsid.

The fate of the R17 A protein during eclipse may be deduced by reacting ^{3}H-histidine labeled phage with host cells at 37°, and analyzing the incubation products in sucrose gradients. Under these conditions, the appearance of the ^{3}H-histidine label with the 42S particles generated by phage eclipse (Figure 4.3B), could be interpreted as a retention of A protein by phage capsids; its appearance near the top of sucrose gradients, on the other hand, would indicate its release into the medium. Lastly, the absence of the ^{3}H-histidine label from either of the above gradient fractions would imply a transfer of the phage A protein to the host bacterium.

Based on the foregoing criteria, studies were carried out to differentiate between these alternatives. In the first experiment, a cell pellet obtained from a 50 ml culture of E. coli K12 Hfr_1 (5×10^8 bacteria/ml) was gently resuspended in 2.5 ml of fresh cold CTMM medium containing a ^{3}H-histidine (1×10^{13} particles) and ^{14}C-amino acid labeled (2×10^{13} particles) R17 phage mixture. It will be remembered that these two radioactive markers are localized in the phage A protein and coat protein respectively (see Figures 3.5 and 2.2). This resulted in a multiplicity of infection of 600 particles/cell and a ^{3}H:^{14}C cpm

ratio of 1. After dividing the culture into two equal portions, one was made 100 μg/ml in RNase, and both cultures were then incubated at 37°. Samples were removed after incubation for 5, 20 and 60 min, after which they were analyzed by sedimentation in sucrose gradients.

The radioactivity profiles of the eluted phage particles of the samples removed at 5 and 20 min (not shown here) were virtually identical to those incubated for 60 minutes. This confirms the earlier contention (Figure 5.1) that phage eclipse is complete by five minutes after infection. Figure 5.3 shows the radioactivity profile of the ^{3}H:^{14}C phage mixture eluted from the infected culture, containing no added RNase. In agreement with the study in the preceding chapter (Figure 4.3A), about 30% of the ^{14}C-coat protein radioactivity sedimented as empty or partially empty capsids (see 42S marker) after this treatment. By contrast, ^{3}H-histidine labeled phage produced only a small amount of slower sedimenting radioactive material under these conditions, signifying a separation of the phage A protein and coat protein during infection. More striking, however, is the observation that of the two labels originally present in a 1:1 ratio, a much greater portion of the ^{3}H-histidine label is lost from the 78S fraction. Thus it appears that a considerable fraction of the phage particles in the 78S region have lost their A protein, but apparently have retained their RNA. This is shown more clearly in Figure 5.4, where it may be seen that 50% of the ^{14}C (coat protein)-labeled phage is converted to empty capsids upon the addition of RNase (Figure 5.4). This shift in ^{14}C-radioactivity from 78S to 42S particles is not evident in the case of ^{3}H-radioactivity. On this basis, it was concluded that phage eclipse, as defined in the introduction of this chapter, involves the displacement of the A protein from the phage capsid. This release of A protein does

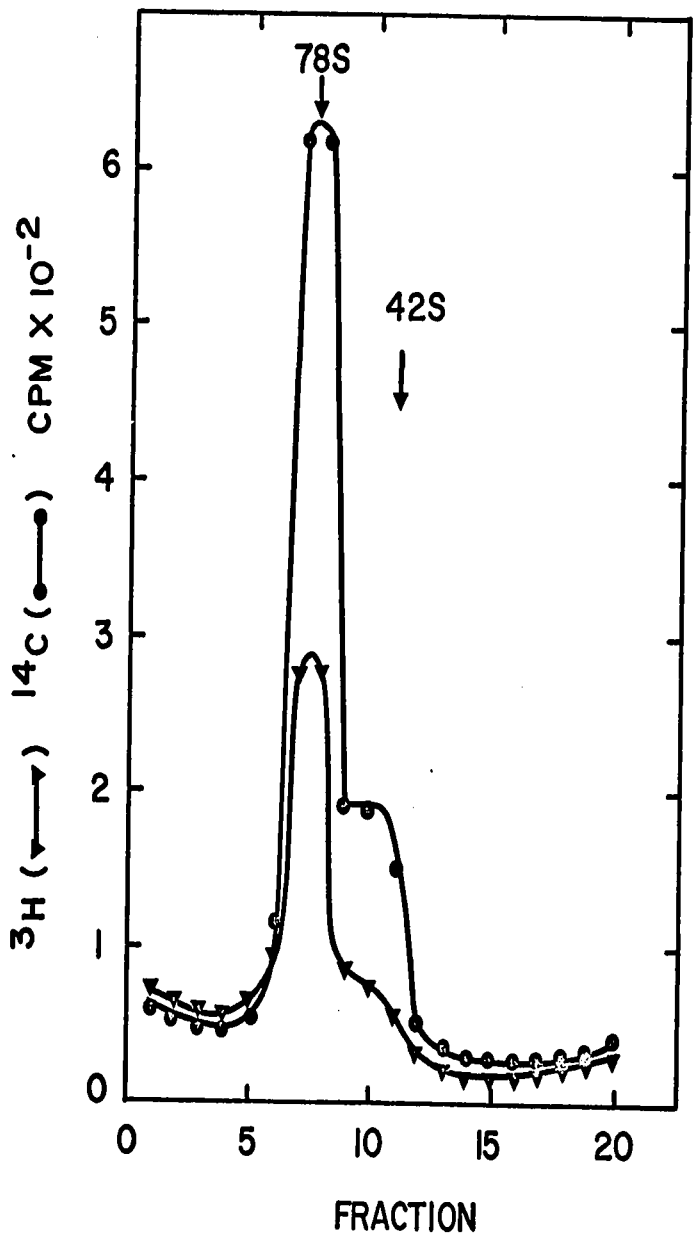


Figure 5.3. Sucrose density gradient sedimentation of eclipsed phage in a ^{14}C-amino acid/^{3}H-histidine labeled mixture of R17 phage.

The details of this experiment are given in the text. A 0.3 ml aliquot of the phage mixture incubated with E. coli K12 Hfr_1 (60 min at 37°), was layered onto a 4.5 ml linear (5-20%) sucrose gradient in CTMM medium. Centrifugation was for 45 min at 45,000 rpm in the Spinco SW50 rotor. Fractions (15 drops) were collected and assayed for hot TCA-insoluble radioactivity as described under Materials and Methods.

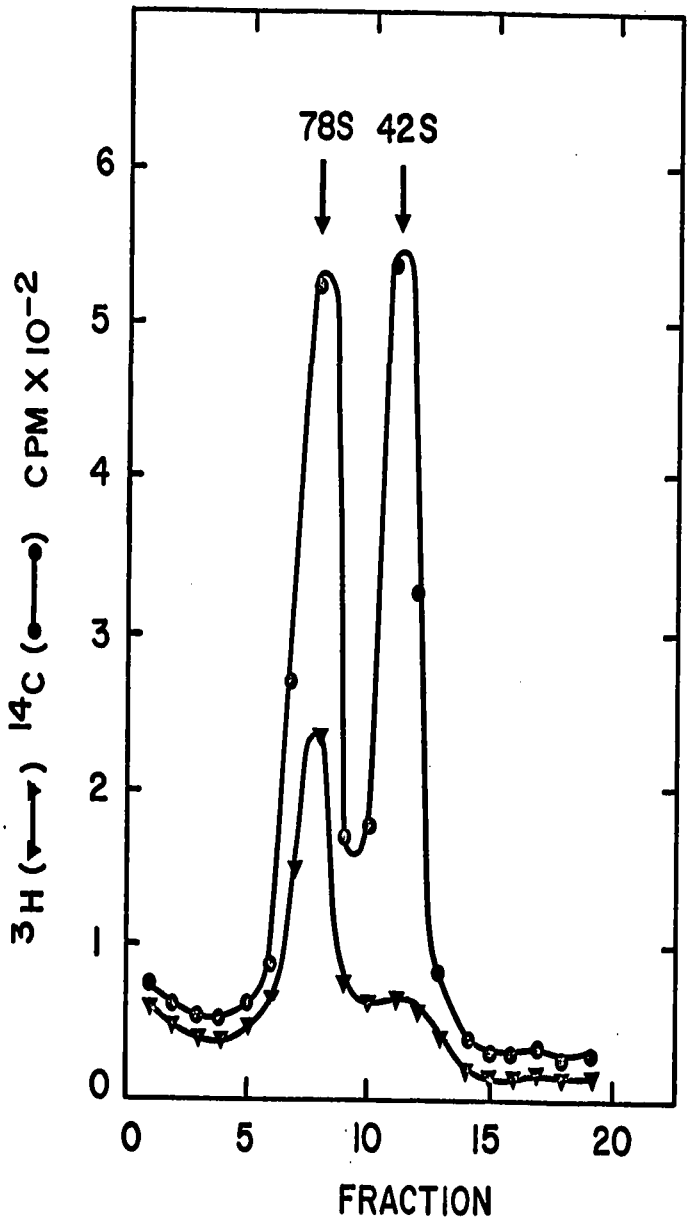


Figure 5.4. Sucrose density gradient radioactivity profile of a ^{14}C-amino acid/^3H-histidine labeled R17 phage mixture after eclipse at 37° in the presence of RNase.

The experimental details are identical to those given for Figure 5.3 except that RNase was added to a final concentration of 100 μg/ml before incubation.

not appear to be dependent on RNA injection, moreover, since most eclipsed phage particles sediment as intact phage (Figure 5.3), and appear to have retained all their RNA.

Lastly, the complete absence of ^{3}H-histidine radioactivity near the top of the gradients (fractions 19 and 20) of Figure 5.3 and 5.4 shows that the A protein is not released into the medium indicating that it most probably attached to cell-associated F-pili. Further evidence to support this contention is provided in Chapter 6 of this thesis.

The implication that the A protein becomes bound to host cells during phage eclipse may explain why, in the above experiment (Figure 5.4), only about 50% (300 phage particles) of the 600 added per bacterium are eclipsed, instead of 90% as was seen when 100 phage particles/cell were added in an earlier experiment (Figure 4.3B). It is logical for instance, that if the A protein remains on the F-pilus after phage eclipse, it would prevent subsequent attachment and eclipse of phage at that particular site, and thus limit the number of phage particles eclipsed by a single cell to the number of phage attachment sites. However attractive this suggestion may be, it is clear that further study will be needed to settle this point.

C. Summary and Discussion

The results of the experiments in this chapter may be summarized as follows:

(1) The interaction of phage R17 with F-piliated cells at 37° triggers a short burst of eclipsing activity during the first 5 min, after which they are incapable of eclipsing additional phage.

(2) A single host cell is capable of eclipsing a large number of phage particles during this 5 min period of eclipsing, the maximum number being of the order of 250 PFU.

(3) The A protein is liberated from phage particles during the eclipse reaction and is apparently transferred to the F-pilus. The release of RNA from the particle is not a requisite for the eclipse reaction since a large fraction of eclipsed phage particles retain their RNA.

The investigations described in this chapter point to a central role for the phage A protein in the phage eclipse reaction. It has been shown, for example, that only those phage particles containing A protein attach to host cells (Figure 4.4B), and that phage particles lacking A protein fail to interact with host cells at 37°. Moreover, the observation that the A protein is released from the phage particle during eclipse (Figure 5.3), and becomes associated with host cells (as inferred by its absence in the medium), suggests that the A protein may be instrumental in transferring phage RNA from the particle to the F-pilus. Once having attached to the F-pilus, the A protein may conceivably serve further to transport the phage RNA into the cell in the form of an A protein-RNA complex. In support of this concept, Oriel (1969) has demonstrated that a portion of the phage RNA and A protein of MS2 phage sediment together as a complex, after thermal and alkaline degradation of the intact phage.

The strength of the association between the A protein and RNA in Class II phage particles presumably is insufficient to facilitate

the transfer of phage RNA out of the phage capsid, since Class II particles lose only their A protein during eclipse (Figure 5.3). In this regard, it is of interest to note that the properties of eclipsed Class II particles bear a close resemblance to those of RNA phage amber mutants of the A cistron. Both species lack A protein and are unable to adsorb to host bacteria (Lodish et al., 1965). In the case of eclipsed Class II particles, this feature presumably results from the release of the A protein from the phage. Further similarities between these two phage types are that they sediment as intact 78S particles in sucrose gradients (Figure 5.3; Argetsinger & Gussin, 1966) and that both are degraded by RNase (Figure 5.4; Heisenberg, 1966a, b).

Several predictions may be made on the basis of the idea that phage RNA is complexed to A protein. The first is that such a complex exists only in Class I particles, because only this class of particles loses A protein and RNA alike upon interaction with host cells at 37° (Figure 4.3A and 5.3). Another prediction is that the A protein penetrates into the bacterium in equimolar amounts with phage RNA during infection. Investigations to test these predictions are described in the following chapter.

CHAPTER VI

THE FATE OF R17 PHAGE A PROTEIN DURING INFECTION

A. Introduction

The fate of the two major phage components (RNA and coat protein) during infection of male strains of E. coli by RNA phages is now reasonably well understood. Following the attachment of phage to the F-pili of host bacteria, the phage RNA penetrates into the cell, presumably via the F-pilus (Brinton, 1965; Valentine & Wedel, 1965), and rapidly becomes associated with polysome fractions (Godson & Sinsheimer, 1966; Erikson & Franklin, 1966; Godson, 1968). A series of complex events is then set in motion leading to the rapid replication of phage (for reviews see Weissman, 1967; August et al., 1970; Stavis & August, 1970). The phage coat protein, on the other hand, remains outside the cell (Edgell & Ginoza, 1965), being released into the medium as a structurally intact capsid after interaction of phage with sensitive cells at 37° (Danziger, 1968; Silverman & Valentine, 1969; Paranchych et al., 1970).

Although the A protein of phage R17 has been shown to be an essential component of infectious phage (Roberts & Steitz, 1967), presumably functioning to promote adsorption of phage to host cells, no corresponding investigations on the fate of the A protein during infection have been reported. Since earlier observations (Chapter 5) had shown that the A protein is released from the phage particle during the eclipse reaction, and transferred to the host cell, it seemed of considerable importance to obtain more detailed information regarding its fate in the infectious process.

Studies were therefore undertaken to resolve the following questions:

1. Is the A protein of eclipsed phage transferred to F-pili during infection or does it penetrate into the host bacteria?
2. If the latter is so, what is the quantitative relationship between penetrated A protein and RNA?
3. Whatever the course taken by the A protein during phage infection, does it undergo any detectable alteration during the infective process?

The resulting experiments showed that the A protein becomes associated with F-pili after being released from phage particles and that some of it actually penetrates into the host cell. Of special interest were the observations that approximately equimolar amounts of A protein and phage RNA penetrate the host cell, and that the A protein is fragmented into several polypeptides prior to its entry into the bacterium.

B. Results

1. What is the Destination of the A Protein Following Phage Eclipse?

To determine whether the cellular destination of the A protein following phage eclipse is the F-pilus, the bacterial cell, or both, ^{3}H-histidine labeled phage was allowed to react with host bacteria at 37°, after which the culture was sheared to separate F-pili from bacteria, and analyzed for cell-associated and F-pili associated radioactivity. The detailed experimental protocol was as follows.

A culture of *E. coli* K12 Hfr_1 was grown to a cell density of 5×10^8 bacteria/ml in CTMM medium and infected with ^{3}H-histidine

labeled phage at a multiplicity of 450 particles/cell. The infected culture was incubated at 37° for 20 min, after which further penetration was prevented by rapidly chilling the culture. Cells and attached phage were collected by centrifugation (10,000 g, 10 min) and, after discarding the supernatant, the cell pellet was resuspended in 0.5 ml Buffer B (0.01 M Tris-HCl, pH 7.8, 0.06 M KCl, 0.01 M $MgCl_2$, 0.006 M ME). The bacteria were then depiliated by forcing the suspension through a 26 (3/8") gauge hypodermic needle (five times under moderate pressure), after which the cells were separated from F-pili by centrifugation. Residual F-pili were removed from the bacteria by washing the cells, with 5 ml volumes of Buffer B (as described for the penetration assay). The two samples (cells and F-pili), both contained in a 0.5 ml volume of Buffer B, were then sonicated for 1.5 min with a temperature-regulated Biosonik probe (Bronwill Scientific, Rochester, N.Y.) to release any phage material penetrated into cells and to disrupt phage-pili complexes. Finally, a 0.4 ml portion of each sample was subjected to sucrose density gradient centrifugation, and the fractions obtained therefrom were analyzed for radioactivity.

The results of the experiment are illustrated in Figures 6.1 and 6.2. Examination of these two figures reveals that significant amounts of radioactivity were present in both gradients, indicating that the A protein was associated both with host bacteria and F-pili isolated from the infected culture. Furthermore, marked differences are apparent in the sedimentation characteristics of the A protein in the two gradients which may reflect differences in the state of the A protein present. For example, the bulk of the cell-associated radioactivity appears to sediment as free A protein (see markers, Figure 6.1).

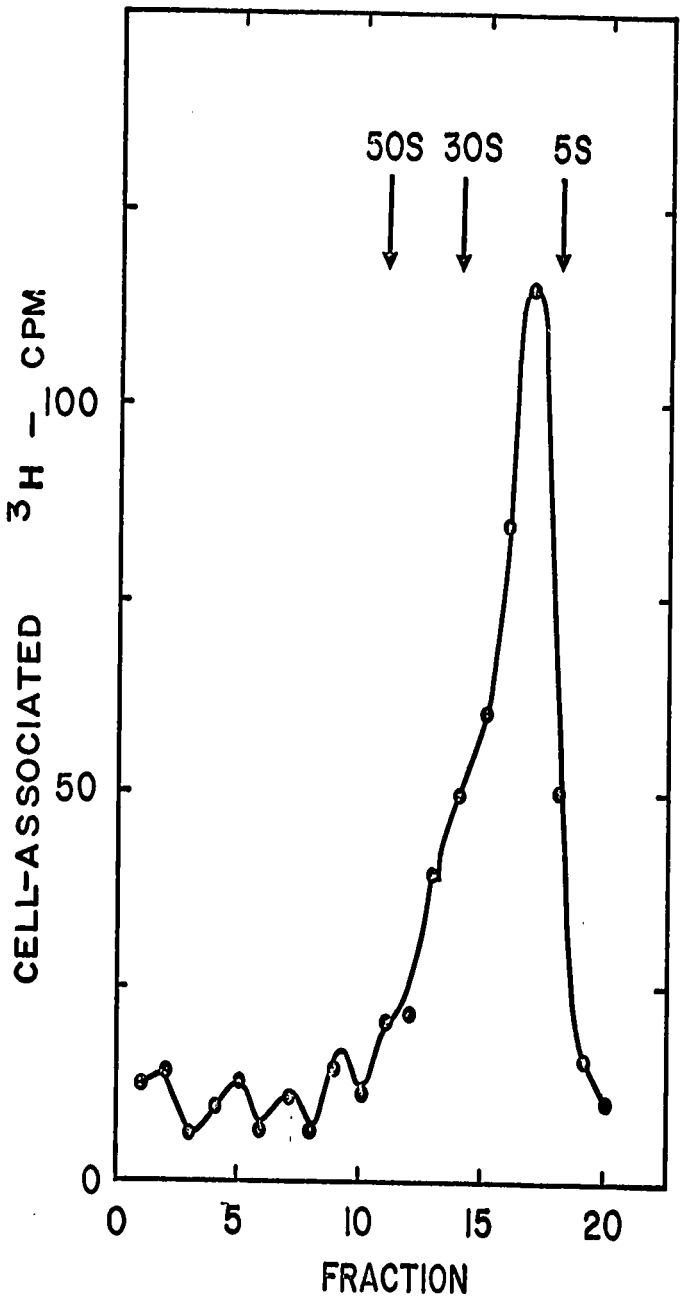


Figure 6.1. Sucrose gradient sedimentation profile of radioactivity associated with cell extracts of (^{3}H-histidine) R17-infected E. coli K12 Hfr$_1$.

The experimental details are as outlined in the text. Centrifugation was for 45 min at 45,000 rpm in the Spinco SW50 rotor. Fractions (15 drops) were collected onto filter discs and analyzed for hot TCA-insoluble radioactivity as described in Chapter 2. The marker arrows indicate the peak positions of ribosomal subunits and transfer RNA (from A_{260} mμ profiles) obtained by centrifuging a sonicated cell extract of E. coli K12 Hfr$_1$ under identical conditions.

The radioactivity associated with F-pili (Figure 6.2), on the other hand, sediments as two peaks; one corresponding to free phage (see marker arrows), and a heavier fraction probably comprised of A protein complexed to F-pili fragments (Wendt et al., 1966). It should be noted that only a small fraction of the A protein radioactivity associated with F-pili was released by the sonication treatment (Figure 6.2). This may be taken to mean that the A protein was bound very tightly to the F-pili.

When an experiment identical to the one described in Figure 6.2 was carried out with phage labeled with ^{14}C-amino acids in the coat protein, only a trace of radioactivity was found in the cell fraction. Isolated F-pili, however, contained large amounts of coat protein radioactivity. This suggests that a considerable portion of the pili-associated A protein may still be associated with intact phage (see marker arrow, Figure 6.2).

Although the results from the foregoing experiments do not permit accurate quantitation of the A protein present in host cells and with F-pili, it was calculated that 28 phage equivalents of A protein had penetrated into the cell. In an analogous experiment in which ^{32}P-labeled phage was used, it was found that the same experimental conditions resulted in the penetration of about 36 phage equivalents of RNA per cell. Taking into account the experimental errors present in such measurements, these two values (i.e., 28 phage equivalents of A protein vs 36 phage equivalents of RNA) may be considered to be approximately equal. Hence, the simplest interpretation of these results is that equimolar amounts of A protein and phage RNA penetrate the host during infection. This, in turn, leads to the suggestion that these two macromolecules may be transported into the cell in the form of a complex.

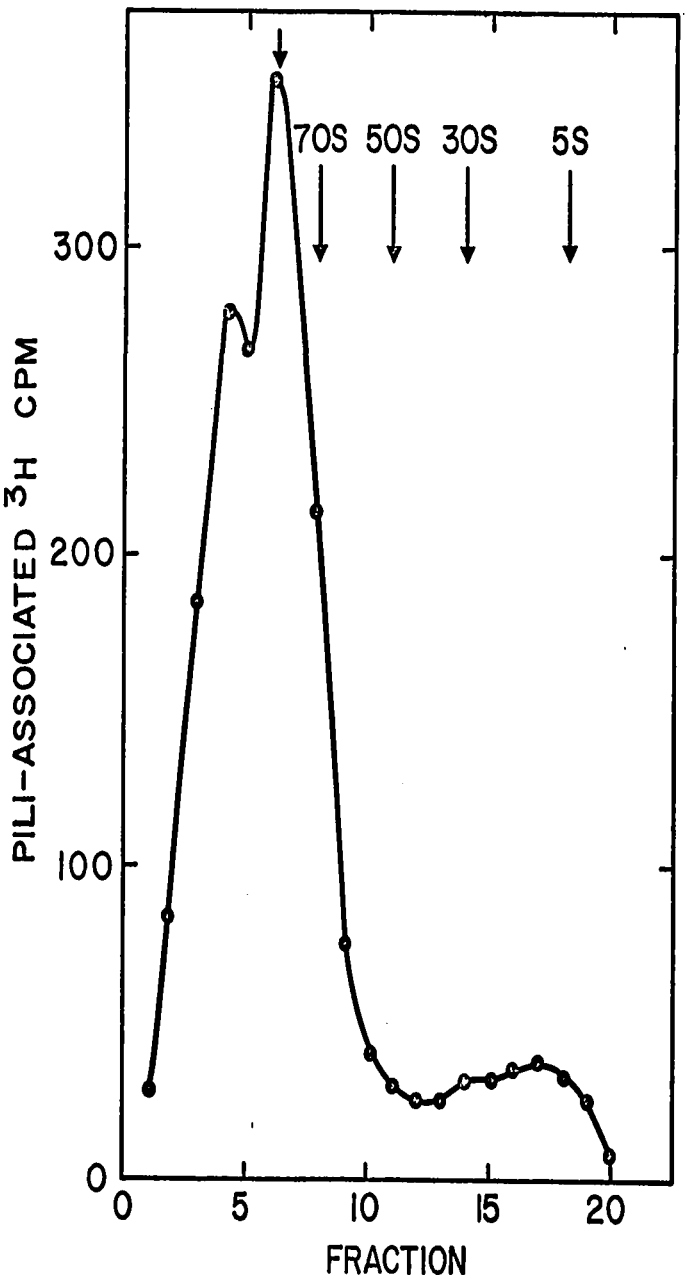


Figure 6.2. Sucrose gradient sedimentation profile of radioactivity associated with F-pili isolated from (^{3}H-histidine) R17-infected E. coli K12 Hfr$_{1}$.

The experimental details are given in the text. The conditions for centrifugation and radioactivity analysis are as described in Figure 6.1.

2. The Quantitative Relationship Between Penetrated A Protein and Phage RNA

If, as suggested by the data in the foregoing section, phage RNA penetrates into host cells as a complex with A protein, one would anticipate that their penetration characteristics would be very similar. An experiment to compare the kinetics of penetration of these two phage components was therefore performed, and the results are shown in Figure 6.3 and in Table 6.1. Firstly, it is to be noted in Figure 6.3 that the pattern of penetration of both phage components is very similar, there being an initial rapid penetration, followed by a second phase in which penetration occurs at a lower linear rate. Secondly, Table 6.1 shows that the number of phage equivalents of each phage component found in the cell at various times after infection was approximately equal throughout the experiment. The most straightforward explanation of these findings is that the two phage components penetrate into the host cell together, possibly as a complex.

3. Molecular Characteristics of Penetrated A Protein

Having established that the A protein penetrates into host cells during infection, it was decided to examine the physical properties of the penetrated A protein, to determine whether it undergoes any detectable alteration during the infective process. This problem was approached with the use of SDS-polyacrylamide gel electrophoresis, a technique by which polypeptides are separated on the basis of molecular weight. SDS, an anionic detergent, readily solubilizes proteins and disrupts their secondary structure yielding their lowest molecular form (Maizel, 1966). It was thus reasoned that if any cleavage of A protein occurs during infection, it should be detectable by this technique.

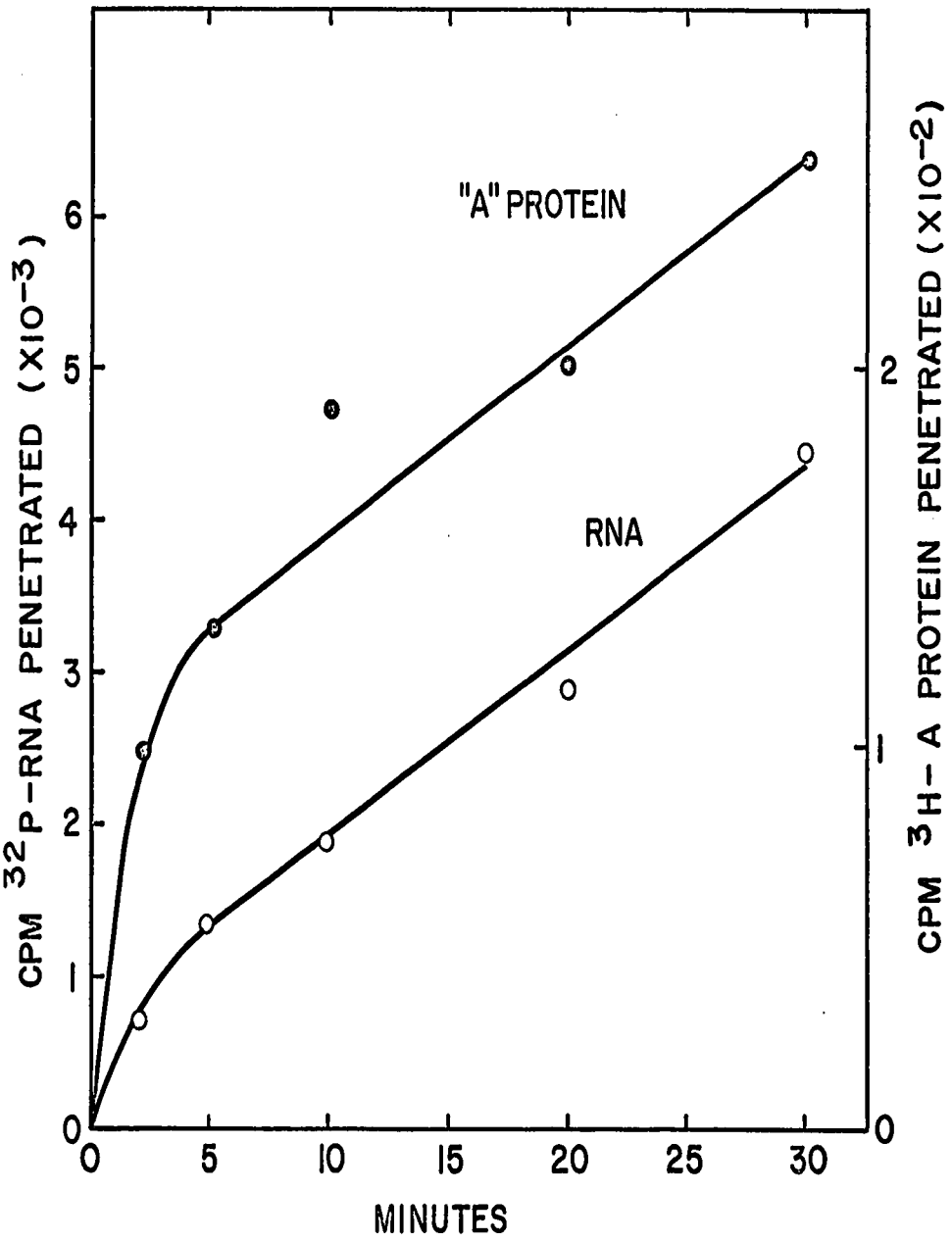


Figure 6.3. Penetration of R17 phage RNA and A protein into host bacteria.

A culture of E. coli K12 Hfr_1 was grown to a cell density of 5 x 10^8 cells/ml, then infected with a mixture of ^{3}H-histidine and ^{32}P-labeled phage (2:1 on the basis of physical particles) at a multiplicity of 300 particles/cell. The culture was incubated at 37° for 30 min, during which time aliquots were periodically removed, chilled rapidly, and washed as described for the penetration assay in Chapter 2. The resuspended cells were applied to Whatman 3MM filter discs and assayed for cold TCA-insoluble radioactivity (^{32}P-RNA) and hot TCA-insoluble radioactivity (^{3}H-A protein).

TABLE 6.1

Penetration of R17 A Protein and RNA During Infection of *E. coli* K12 Hfr_1*

Time (min) Post Infection	Phage Equivalents Penetrated/Cell	
	A Protein	RNA
2	9.3	5.3
5	12	9.9
10	17.5	14
20	19	22
30	23	33

*Calculated from the data in Figure 6.3.

Figure 6.4 illustrates an electropherogram of protein extracted from E. coli, previously infected with ^{3}H-histidine labeled phage. It should be emphasized that the cells were thoroughly washed (to remove all extracellular phage) prior to extraction of protein to ensure that the radioactivity present in the cells would be derived only from penetrated A protein. As may be seen, the penetrated A protein migrated as three electrophoretic bands, only one of which corresponds to intact A protein (peak I). Estimates of the molecular weight of the smaller components, by the method of Shapiro et al. (1967), (based on a reported value of 38,000 for native A protein, Steitz, 1968b, and 15,000 for phage coat protein, Enger & Kaesberg, 1965), yielded values of 24,000 and 15,000 respectively, for peaks II and III.

The general broadness of the bands and the trailing of ^{3}H-histidine radioactivity into the gel from the origin (left side of Figure 6.4) can be explained by the extremely high protein concentration applied to the gel column. This is so because the protein sample applied to the gel column contains all of the unlabeled host protein, in addition to the ^{3}H-histidine labeled A protein. Although the protein load applied per gel column sometimes was the equivalent of 5×10^{9} bacteria, the observed bands are authentic products of the A protein produced at some stage of the infective process. This was shown by a control experiment in which ^{3}H-histidine labeled phage was incubated with host cells for 20 min at 4°, then treated in the same manner as the phage-cell complex of the 37° experiment. The results of the experiment, illustrated in Figure 6.5, showed that only intact A protein was present under these conditions. This observation thus indicates that the phage A protein

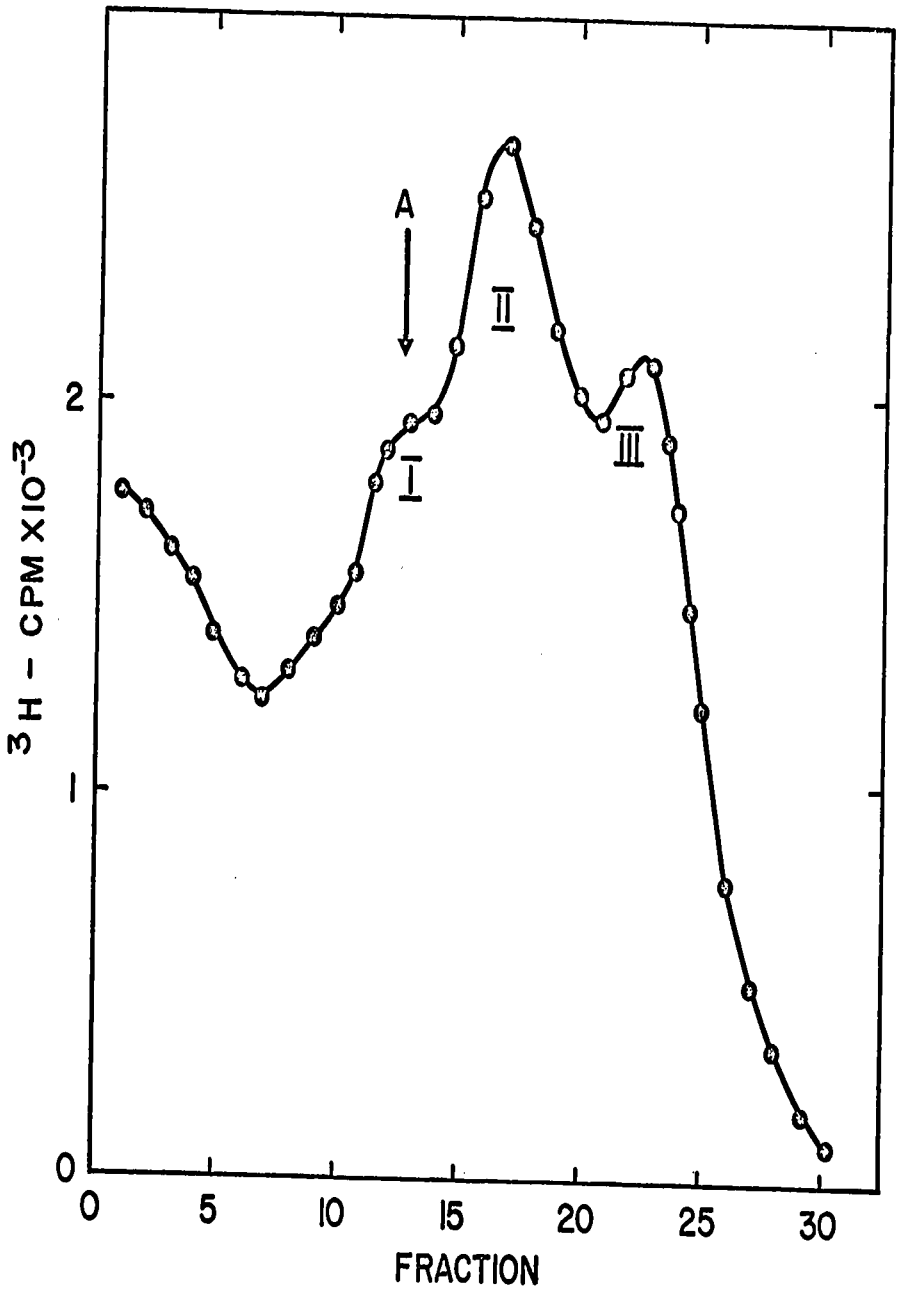


Figure 6.4. SDS-polyacrylamide gel electrophoresis of protein of E. coli infected with ^{3}H-histidine labeled R17.

A culture of E. coli AB257 was grown to a density of 4.5×10^8 bacteria/ml in CTMM(-aa) medium and infected with ^{3}H-histidine labeled phage at a multiplicity of 400 particles/cell. Incubation at 37° was continued for 20 min, after which the culture was rapidly chilled and washed as described for the penetration assay. Extraction of protein and the conditions for gel electrophoresis were as described in Chapter 2. Electrophoresis was at 5 mAmps/tube for 2.5 hours. A = A protein.

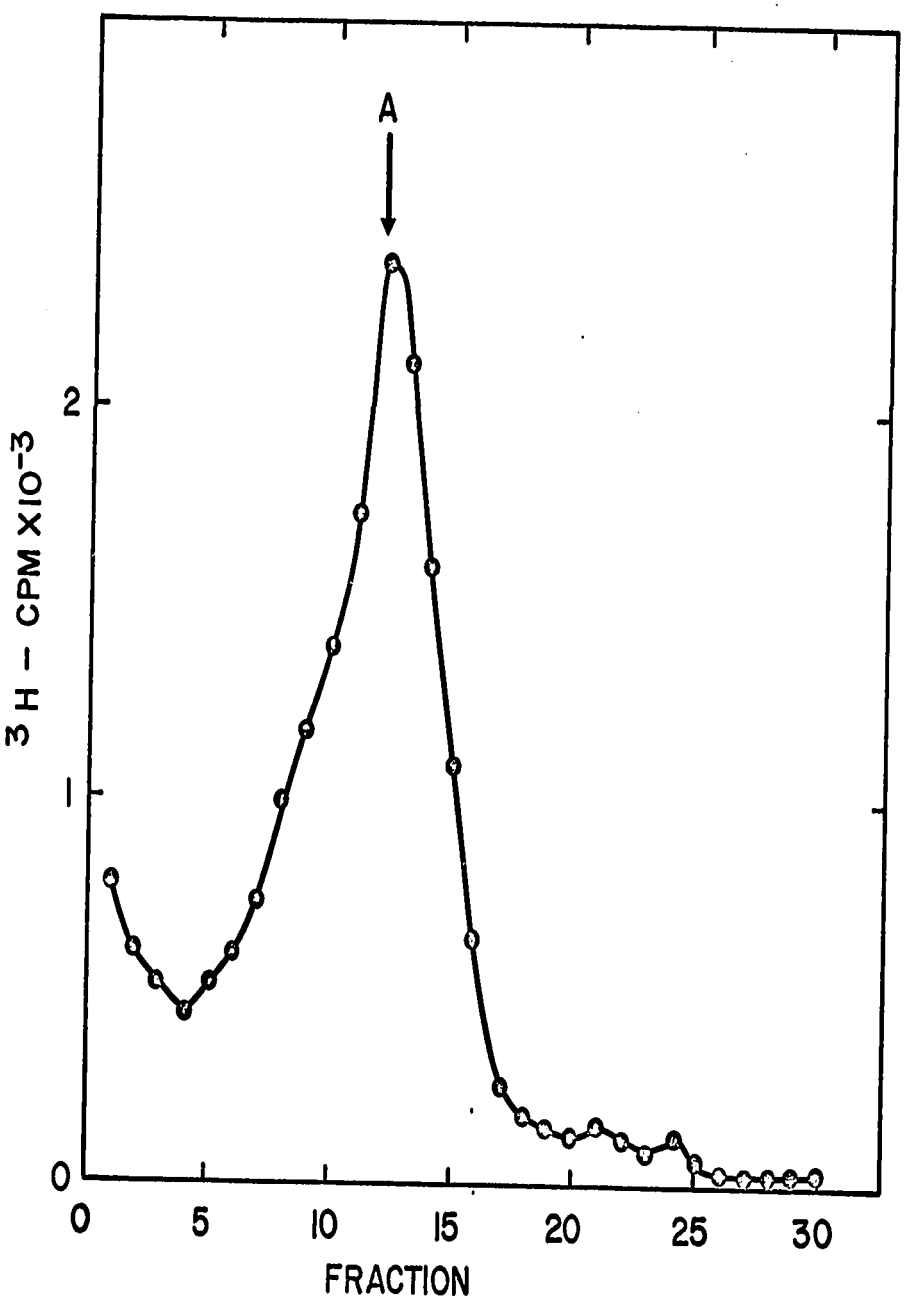


Figure 6.5. SDS-polyacrylamide gel electrophoresis of protein extracted from E. coli cells mixed with ^{3}H-histidine labeled phage at 4°.

A cell pellet obtained from 100 ml culture of E. coli AB257 (5×10^8 bacteria/ml) was resuspended in 6.0 ml CTMM medium after which ^{3}H-histidine labeled R17 (2×10^{12} particles) were added. Following incubation for 20 min at 4° to allow phage attachment, bacteria and attached phage were separated by centrifugation (10,000 g, 10 min). The protein in the cell-phage pellet was then extracted and solubilized, after which a small sample (150 µl) was electrophoresed in an SDS-polyacrylamide gel column. Electrophoresis conditions were identical to those in Figure 6.4. A = A protein.

remains unaltered by attachment of phage to host bacteria at 4°. The fact that phage viability also is unaffected by interaction with host cells at 0° (Valentine & Strand, 1965; Brinton & Beer, 1967) is consistent with this finding.

4. The Physical State of A Protein Associated With F-Pili of R17-Infected E. coli

It was next decided to determine where on the host cell the A protein is cleaved. One possibility was that the A protein penetrates into the cell as an intact molecule and that it is subsequently degraded by intracellular proteases. Another possibility was that cleavage of A protein occurs on the F-pilus prior to penetrating into the cell. To distinguish between these alternatives, an electrophoretic analysis was conducted to determine the molecular state of ^{3}H-histidine labeled A protein associated with F-pili of infected cells.

The results of this experiment, illustrated in Figure 6.6, are very similar to those obtained with cell extracts of infected E. coli (Figure 6.4). The notably better resolution obtained with the radioactive material associated with F-pili (Figure 6.6), probably reflects the smaller amounts of material applied to the gel column in this experiment. It is worth noting also that a fourth component (peak IV, Figure 6.6), estimated to have a molecular weight of less than 10,000 was detected with the F-pili fraction, but not with the cell extract (Figure 6.4). It is possible that peak IV represents yet another breakdown product of A protein which is not allowed to penetrate into the host cell.

Although the results shown in Figure 6.4 and 6.6 should be interpreted with caution, since the number of components observed in the

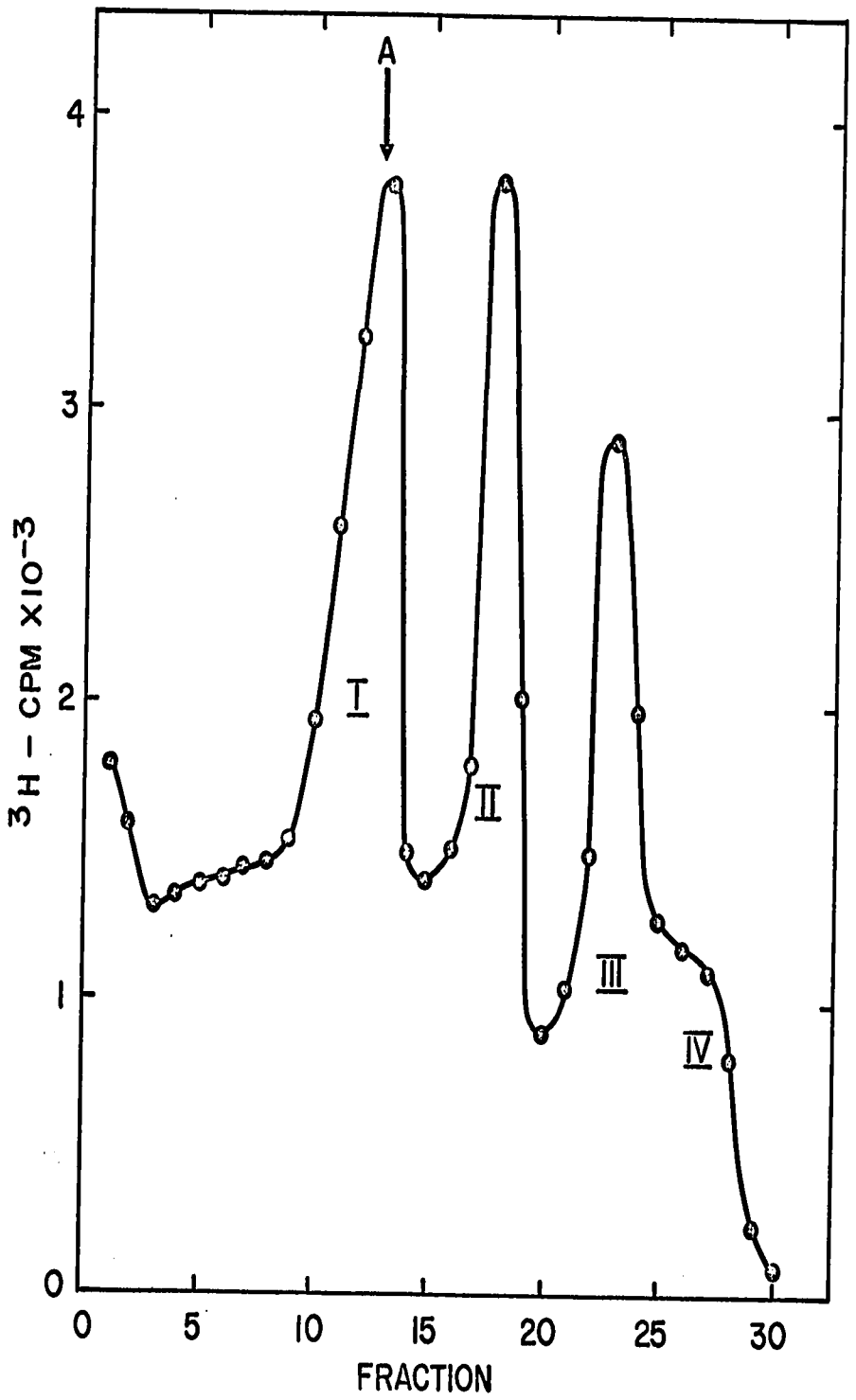


Figure 6.6. SDS-polyacrylamide gel electrophoresis of protein associated with F-pili of (^{3}H-histidine) R17-infected E. coli.

The experimental conditions were identical to those in Figure 6.4. Isolation of F-pili from infected cells was as described for Figure 6.2. Extraction of protein and electrophoresis conditions are given in Chapter 2. A = A protein.

gel column does not necessarily indicate the true number of fragments produced from A protein (i.e., fragments lacking a histidine residue would not be detected), it is worth noting that the cumulative molecular weights calculated for peaks II and III account for the total molecular weight (38,000) of intact A protein.

Finally, it is interesting to compare the relative distribution of the ^{3}H-histidine radioactivity present in peaks I, II and III of Figure 6.4 and 6.5. It is seen that significantly greater amounts of peak I (intact A protein) relative to peaks II and III and are found with F-pili (Figure 6.6) as compared to that found penetrated into host cells. A large fraction of the A protein associated with F-pili is thus probably still part of intact phage. In contrast, most of the radioactivity present inside the cell (Figure 6.4) is found in peak II, the largest fragment penetrating the cell. The amount of intact A protein penetrating into host cells appears to be quite small, if one considers that the radioactivity of peak II (Figure 6.4) is skewed towards the origin.

The results of this study show therefore that the A protein is cleaved into three fragments while on the F-pilus, and thereafter at least two of the fragments enter the host cell, along with the phage RNA. Whereas the biological significance and the biochemical mechanisms by which they occur are presently not understood, it seems likely that F-pili are involved in this reaction.

C. Summary and Discussion

The results of the investigations described in this chapter may be summarized as follows.

1. The A protein, upon being released from phage R17 particles becomes tightly associated with F-pili. It is then cleaved into three smaller protein fragments having molecular weights of approximately 24,000, 15,000 and less than 10,000 daltons. Some component of F-pili is presumably responsible for catalyzing this fragmentation of the A protein.

2. A significant amount of the two larger A protein fragments penetrate into the host cells with approximately stoichiometric amounts of phage RNA. The smallest of the A protein fragments appears to be excluded from the host cell.

The foregoing observations indicate clearly that the interaction between F-pili and A protein during phage infection is considerably more complex than was originally anticipated. The finding that approximately equimolar amounts of A protein and phage RNA penetrate the host cell strongly suggests that these two phage components enter the cell by a common pathway - possibly via the hollow core of the F-pilus. However, whether the A protein fragments penetrate with phage RNA as a complex, or whether these phage components enter the cell independently is presently not clear.

CHAPTER VII

R17 INFECTION AND THE ROLE OF F-PILI

A. Introduction

Although it is now well established that F-pili play a crucial role in the extracellular events leading to infection by male-specific phages, little is known about the mechanism whereby these structures mediate the penetration of the viral genome into host cells. Brinton (1965) first proposed the "F-pili conduction" model to account for the involvement of F-pili in male-specific phage infection and bacterial conjugation. According to this model, the nucleic acid of the infecting phage is conducted through the central core of the F-pilus. Although the physical presence of phage nucleic acid in F-pili has yet to be demonstrated, parts of this model have received substantial experimental support (Ippen & Valentine, 1966, 1967; Knolle, 1967a; Novotny et al., 1968, 1969a, b; Silverman & Valentine, 1969).

A somewhat different model to explain F-pili function has been proposed by Marvin and Hohn (1969). This hypothesis, the "F-pili retraction" model, is based on the findings that infection of log-phase cultures of E. coli with Ff phage (a filamentous DNA phage) leads to the disappearance of F-pili as determined by electron microscopy and by their attachment capacity for RNA phage. Marvin and Hohn thus postulated that the adsorption of RNA phage to the side, or DNA phage to the tip of the F-pilus triggers a retraction of this appendage into the cell.

The "F-pili retraction" hypothesis of Marvin and Hohn, is quite attractive when viewed in light of the observations described in the foregoing chapters (see Figure 5.1 and Figure 6.3). Although it is

now clear that entire RNA phage particles could not be pulled into the cell in a manner analagous to that proposed for male-specific DNA phages (Trenkner et al., 1967; Marvin & Hohn, 1969), one can envision a mechanism (illustrated in Figure 7.1) whereby phage attachment to the F-pilus via the A protein might trigger a partial retraction of the F-pilus, resulting in the release of the A protein-RNA complex from the coat protein, and its subsequent transport into the cell on the exterior surface of the F-pilus. This situation would explain the extreme RNase-sensitivity of the phage RNA during the infective process. In fact, this concept was sufficiently appealing that a series of experiments were carried out to test the feasibility of this model for R17 phage infection. The results of these investigations are described in what follows.

B. Results

1. Energetics of Phage R17 Infection

To determine the feasibility of the F-pili retraction model for RNA phage infection, a study was first carried out on the level of high-energy compounds in host cells before and after infection. The rationale for this study was based on the consideration that F-pili retraction, if occurring, should result in the utilization of considerable amounts of energy within the cell. Moreover, it would be expected that the kinetics of this energy loss would parallel very closely the unusual kinetics of the phage eclipsing reaction (Figure 5.1). In fact, numerous arguments have already been presented by workers in this field for the involvement of a cellular energy source in male-specific phage infection (Valentine & Wedel, 1965; Brinton & Beer, 1967; Knolle, 1967b;

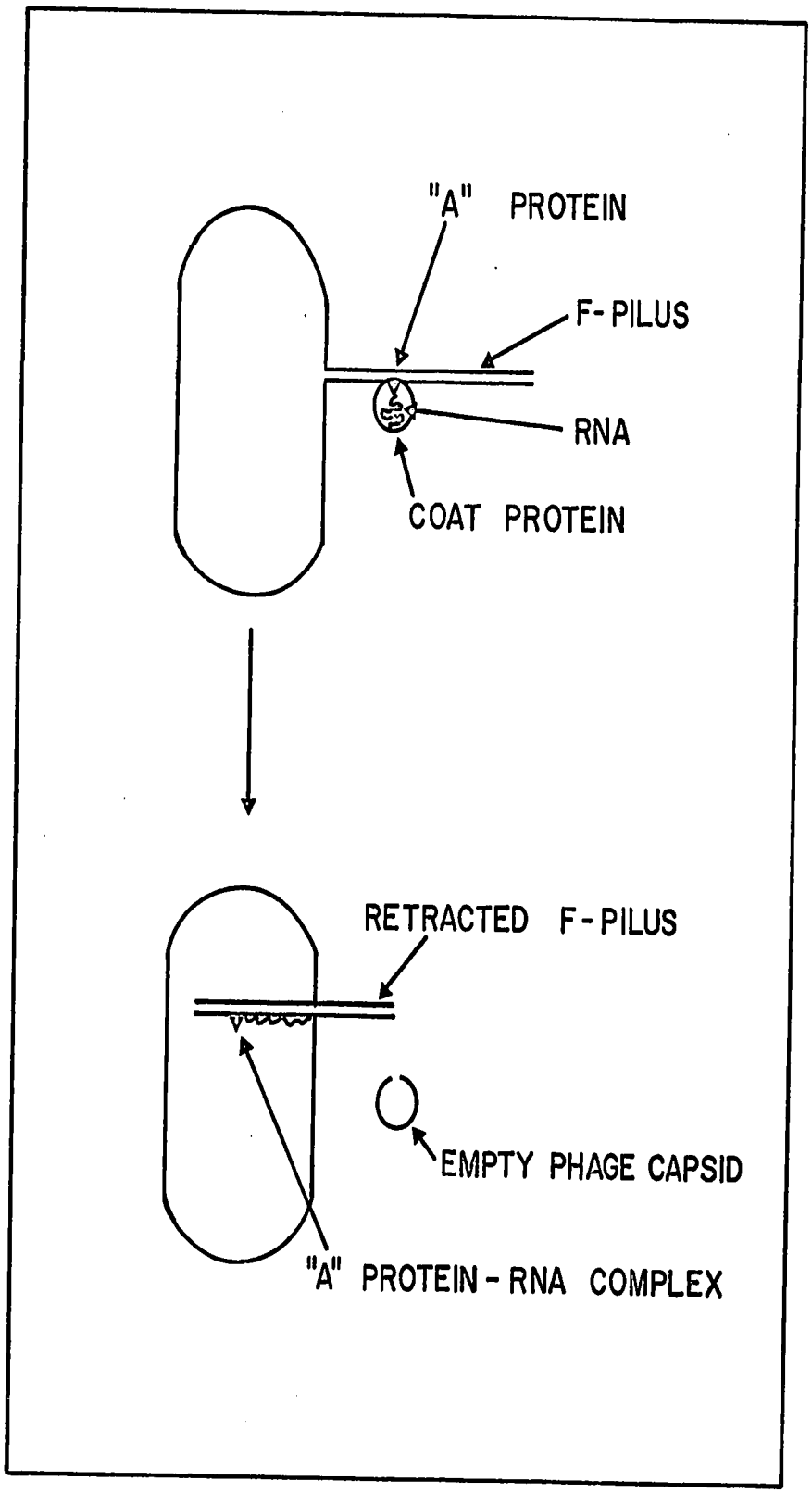


Figure 7.1. Schematic representation of a model which attempts to explain the mechanism of R17 phage infection of host bacteria.

Yamazaki, 1969; Danziger & Paranchych, 1970b). Since most of these conclusions, however, are based on indirect studies under conditions where energy production in host cells is abolished, or limited by starvation, it seems important to clarify this point by demonstrating an actual utilization of energy during the normal course of infection. Investigations were therefore performed in which the NTP levels were determined in cultures at various times before and after addition of phage. This was accomplished by a combination of ^{32}P-labeling and thin-layer chromatography techniques (see Materials and Methods).

The results of an experiment in which the effect of phage infection on cellular ATP levels was examined is illustrated in Figure 7.2. It may be seen that infection with less than 1 PFU/cell caused little change in the ATP level of the culture, whereas higher multiplicities produced a substantial reduction in the concentration of this compound in the cell. It is noteworthy that this decrease in ATP concentration occurred immediately after addition of phage to the culture and was essentially complete by 5 min after infection. In this respect, this reaction is identical to the eclipse reaction (see Figure 5.1) which also was shown to be complete by 5 min after infection.

Another interesting feature of this experiment is the effect of the phage multiplicity on the cell's ability to restore the ATP concentration to the pre-infection steady-state level. When relatively low multiplicities of infection were used, the ATP concentration was restored to its original level shortly after reaching its lowest point. At higher multiplicities, where larger reductions in ATP occurred, the cells were incapable of restoring the cellular ATP concentration to its original level. Under these conditions, ATP presumably is consumed at

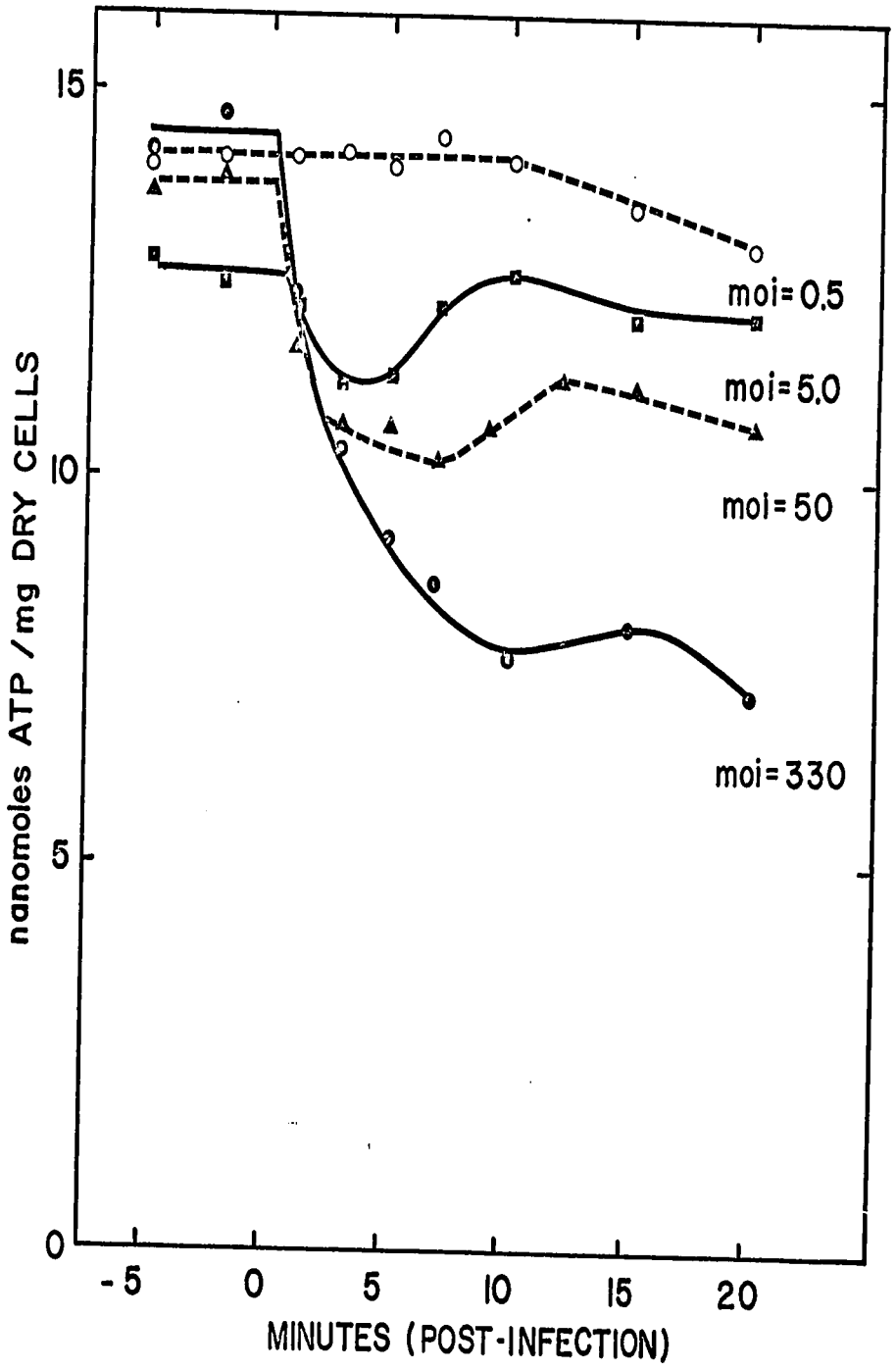


Figure 7.2. The effect of R17 phage infection on ATP levels of E. coli K12 Hfr_1.

A culture of E. coli K12 Hfr_1 was grown at 37° in the presence of ^{32}P (5×10^7 cpm/ml, added at the time of inoculation) to a cell density of 3×10^8 cells/ml. The culture was divided into four 5-ml portions and R17 phage added at the multiplicies of infection (moi) indicated. Samples were periodically withdrawn from each culture and analyzed for ATP by chromatography on PEI-cellulose thin layer sheets. The detailed procedure is outlined in Chapter 2.

a faster rate than it is synthesized by the cell. One conclusion that is apparent from the above study is that cellular ATP is utilized during phage infection by some energy-requiring process.

To determine whether the observed energy utilization is limited only to ATP, an identical analysis of GTP, UTP and CTP levels in R17-infected E. coli was also performed. The latter two NTP's were measured together because of their incomplete resolution by the chromatographic solvent system used. Table 7.1 compares the levels of these NTP's at zero and five min after infection, showing the decrease (in percent) of the appropriate NTP at various multiplicities. Although the levels of these compounds present in cells before infection varied somewhat, it may be seen that the percent decrease caused by R17 phage infection was similar for all NTP's examined.

It is important to note that the observed decrease in cellular NTP's reflects an actual utilization of high-energy triphosphates and not merely leakage from the cell; this is so because the measurements were made on whole cultures rather than on cells separated from the culture medium. Although it is not known whether the observed decrease in NTP's represents a direct utilization of high energy phosphate from these compounds, or from some high-energy intermediate coupled to them, it is clear that large amounts of cellular energy are consumed during phage infection, and that the pattern of this utilization is consistent with that which would be expected if F-pili retraction were occurring.

2. A Direct Test of the "F-Pili Retraction" Model by Electron Microscope and Phage Attachment Studies

In view of the possibility that the cellular energy consumed during phage infection might be utilized by some process other than

TABLE 7.1

The Effect of R17 Infection on Host Cell Levels of Nucleoside Triphosphates[a]

Nucleoside Triphosphate Measured	Phage Input (PFU/cell)	Nanomoles per mg Dry Cells at Time of Infection	Nanomoles per mg Dry Cells at 5 min After Infection	Per cent Decrease
ATP	0.5	14.2	14.2	0
ATP	5.0	12.7	11.2	11.8
ATP	50	13.8	10.3	25.5
ATP	330	14.4	9.2	36.1
GTP	0.5	6.1	5.9	3.2
GTP	5.0	5.8	5.1	12.0
GTP	50	5.2	3.8	26.9
GTP	250	5.2	3.0	42.3
CTP + UTP	0.5	11.8	11.3	4.1
CTP + UTP	5.0	13.6	12.0	11.8
CTP + UTP	50	11.0	7.4	32.8
CTP + UTP	330	13.0	9.5	27.0

[a]Cultures of *E. coli* K12 Hfr_1 were grown at 37° in the presence of ^{32}P (5×10^7 cpm/ml added at time of inoculation) to a density of 3×10^8 cells/ml, then infected with R17 phage at the indicated multiplicities of infection. Aliquots were removed from each culture at various intervals before and after infection, diluted into an equal volume of ice-cold 2M formic acid, then assayed for nucleoside triphosphates as described under Materials and Methods (Chapter 2).

F-pili retraction, it was decided to perform a detailed electron microscope analysis of cell-associated F-pili in cultures of E. coli before and after infection with phage R17. The rationale for this experiment was that if F-pili retraction occurs during infection, cell-associated F-pili in infected cultures should be noticeably shorter than those in uninfected cultures. Also, the fraction of cells devoid of F-pili should be greater if F-pili retract completely into the cell. The following experiment was carried out to test these possibilities.

A culture of E. coli HB11 (a strain producing only F-pili) was infected with phage R17 at a multiplicity of 500 particles/cell, and aliquots removed at 2, 5 and 20 min after infection were placed into chilled tubes containing formaldehyde (final concentration 3.7%). This treatment fixes the cell and prevents outgrowth of F-pili. A small aliquot which had been removed from the culture before infection and treated in a similar manner, served as a control. After preparing specimens of each time sample for electron microscopy as described in Chapter 2, a large number of cells from each sample was examined in the electron microscope for F-pilus lengths and frequency. The results of this experiment are summarized in Table 7.2. Plates I and II of the Appendix illustrate two representative electron micrographs from this study.

Looking first at the average F-pilus length, the data in the last column of Table 7.2 indicates that the length of F-pili decreased by approximately 30% in the first 2 min after infection, and thereafter remained constant. The fraction of the bacterial population containing F-pili (column 2), however, did not change in the first 2 minutes. Furthermore, the average number of F-pili per cell (column 3) remained

constant throughout the experimental period showing that few, if any, F-pili disappear completely from the cell. Two conclusions that may be drawn from the data in Table 7.2 thus are (a) that F-pili shorten at some time during the first 2 min after infection and (b) that a complete retraction of F-pili is ruled out.

TABLE 7.2

Effect of R17 Phage Infection on the Frequency and Length of F-Pili of E. coli HB11*

Minutes After Infection at 37°	Fraction of Bacterial Population With Pili	Average No. of Pili Per Piliated Bacterium	Average Pilus Length in Microns (μ)
0	0.54 (800)	1.60 (800)	1.69 (281)
2	0.55 (200)	1.62 (200)	1.19 (248)
3	0.42 (200)	1.56 (200)	1.28 (210)
20	0.43 (200)	1.58 (200)	1.21 (191)

*Numbers in brackets represent number of measurements made. The experimental details are as outlined in Figure 7.3.

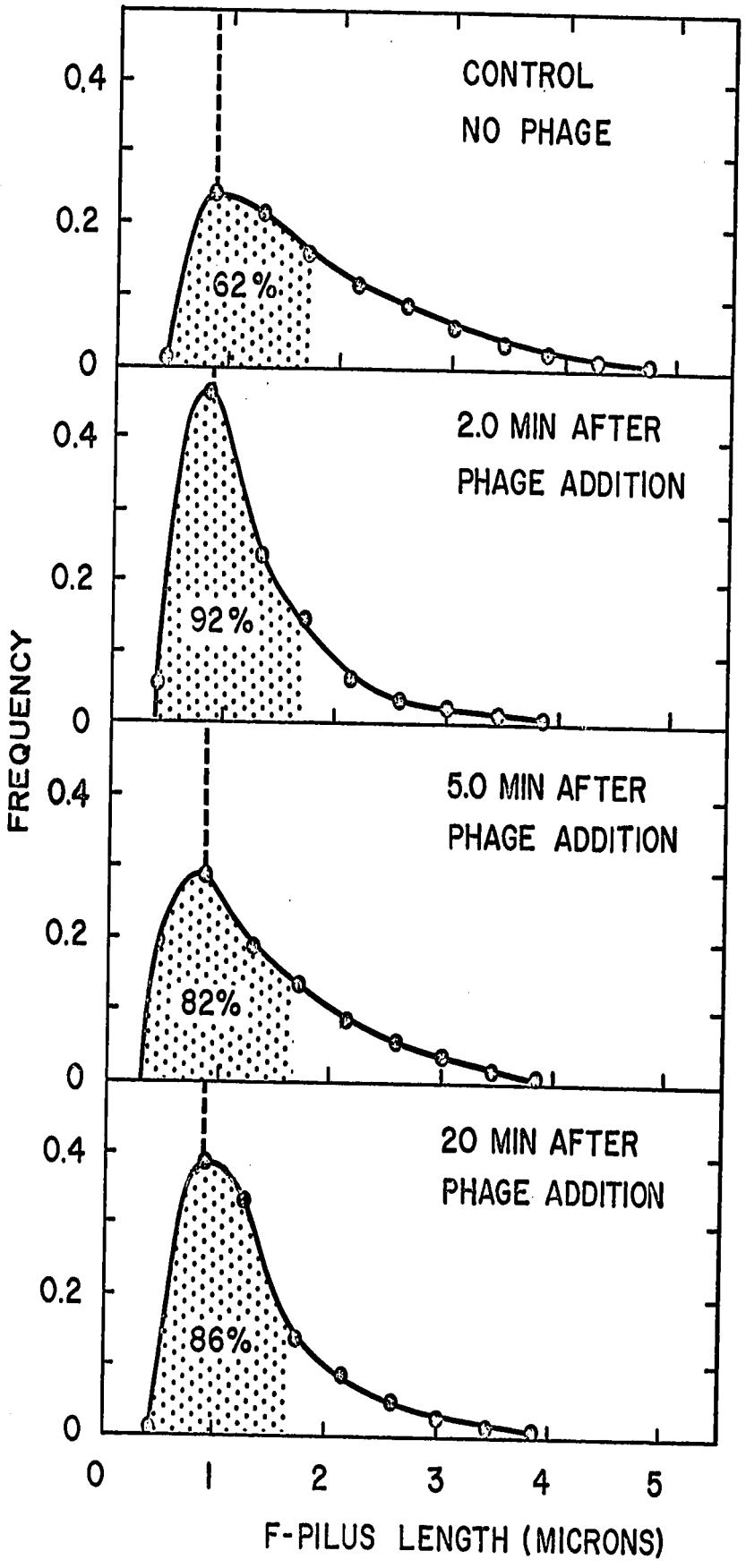
To provide a more meaningful representation of the changes occurring in the length of cell-associated F-pili during infection, the data employed for the calculation of average F-pili length in Table 7.2 was plotted in terms of the frequency distribution of F-pilus lengths. The resulting profiles are depicted in Figure 7.3. It may be seen that

cell-associated F-pili in the uninfected culture varied in length from 0.5 - 6.0 μ, and that 62% are 0.5 - 1.7 μ in length. The percentage of F-pili in this length interval (see hatched areas in the profiles, Figure 7.3) was arbitrarily chosen for comparative purposes. After phage infection, a pronounced change occurs in the frequency distribution of F-pilus lengths. As indicated in the figure, the frequency of shorter F-pili increased markedly by 2 min after infection since 92% of the cell-associated F-pili were 0.5 - 1.7 μ in length at this time. Again it may be seen that this shortening is complete by 2 min after infection, since no change in the percent of F-pili in this length interval is apparent at later times. Thus it is clear that shortening of F-pili is restricted to a short time period immediately after infection.

Although the electron microscope data (Table 7.2, Figure 7.3) suggest the possibility that a partial retraction of F-pili may occur during infection, these observations must be interpreted with caution. It is to be noted for example that no change occurred in the modal length of F-pili during the course of the experimental period (see broken vertical line, Figure 7.3). Since a decrease in the modal length would be expected if retraction were occurring, the data in Figure 7.3 can also be interpreted in terms of breakage of longer F-pili. This possibility cannot be excluded since only cell-associated F-pili were measured in the electron microscope. In addition, Novotny et al. (1969a) have shown that F-pili which break from cells under normal culture conditions adsorb to the cell surface and to the sides of attached F-pili, raising the possibility that phage infection causes a shedding of readsorbed F-pili. Clearly, both the foregoing events would be misconstrued as a shortening of F-pili in the electron microscope.

Figure 7.3. Frequency distribution of E. coli HB11 F-pilus lengths as a function of time after infection.

A culture of E. coli HB11 was grown at 37° to a density of 2 x 10^8 cells/ml and a portion infected with R17 phage at a multiplicity of 500 particles/cell. The uninfected portion of the culture (zero-time control) was treated with the same amount of phage after first being chilled on ice, in an Erlenmeyer flask containing formaldehyde (final concentration 3.7%). Formaldehyde fixes the cell and prevents outgrowth of F-pili (Novotny et al., 1969a). Incubation of the infected culture was continued for 20 min during which time aliquots were periodically removed, chilled and fixed with formaldehyde as above. Preparation of samples for examination with the electron microscope, and measurement of F-pilus lengths were as described under Materials and Methods (Chapter 2).


CONTROL
NO PHAGE
62%
2.0 MIN AFTER
PHAGE ADDITION
92%
5.0 MIN AFTER
PHAGE ADDITION
82%
20 MIN AFTER
PHAGE ADDITION
86%
FREQUENCY
0.4
0.2
0
0 1 2 3 4 5
F-PILUS LENGTH (MICRONS)

Several predictions may be made if the observed shortening of F-pili results from either a fragmentation or an elution of F-pili from infected cells. Firstly, an increase in the amount of free F-pili in the culture medium should be observed. This prediction may readily be tested by measuring the phage attachment capacity of the supernatant of infected cells. Secondly, although a simultaneous decrease in the phage attachment capacity of infected cells would be anticipated, no decrease in the overall phage attachment capacity of the whole culture should occur. If, on the other hand, F-pili shorten by a retraction mechanism, somewhat different results would be anticipated. Under these conditions no change in the amount of free F-pili in the supernatant would be expected and the phage attachment capacity of the whole culture should decrease. An experiment was therefore conducted to differentiate between these alternatives.

A culture of E. coli HB11 was grown to a cell density of 4 x 10^8 bacteria/ml, after which cells were depiliated by blending the culture at 2300 rpm for 1 minute. The cells were collected by centrifugation and washed twice with fresh medium (4°) to remove broken F-pili. The resuspended cells were then incubated for 20 min at 37° to permit outgrowth of new F-pili, and then Rifampicin (50 μg/ml) was added to the culture. After removing a portion for a control, the remaining culture was infected with purified ^{14}C-(coat protein) labeled phage R17 (200 particles/cell). Rifampicin, which has no effect on phage attachment, eclipse or RNA penetration, was added to prevent cell growth during the experimental period (Wehrli et al., 1968), and did not change the overall results. At various time periods after infection, aliquots

were removed, chilled rapidly in ice, and a saturating amount of ^{14}C-labeled phage added. After allowing 20 min at 4° for attachment, the relative amount of F-pili associated with

(a) the bacterial supernatant

(b) the infected bacteria

(c) the whole culture

of each of the time samples was determined by their ability to attach phage. The results of this study are illustrated in Figure 7.4. As may be seen in the figure, only a small amount of phage was attached by the supernatant fraction of the uninfected culture (0 time). Following infection, however, this amount increased rapidly at early times, showing that F-pili material was being released into the supernatant. It may also be seen that the amount of F-pili associated with bacteria decreased during the experimental period.

Concerning the nature of the F-pili released into the medium during infection, it will be remembered that the culture was sheared and thoroughly depiliated prior to infection, and then incubated for 20 min to allow regeneration of F-pili. Novotny et al. (1969a) have shown that under these conditions the amount of readsorbed F-pili in the culture is minimal. Bearing this in mind, the material released from the infected cells probably represents a fragmentation of attached F-pili, rather than an elution of readsorbed F-pili. It is of interest to note that this release of F-pili, like that of phage eclipse (see Figure 5.1) and ATP consumption (Figure 7.2), was complete by 5 min after infection, suggesting that these processes may be related.

It will be noted that phage addition to the culture resulted in a net increase in its phage attachment capacity (see whole culture,

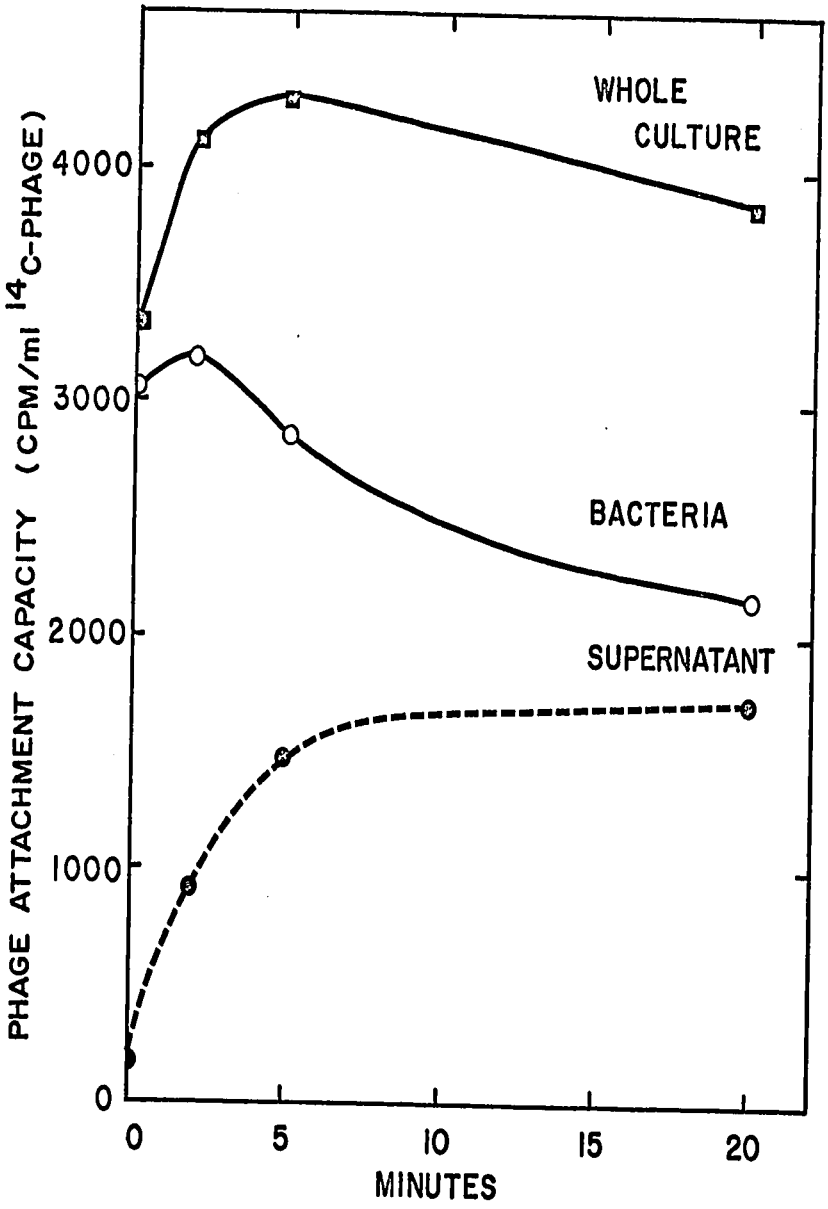


Figure 7.4. Phage attachment capacity of cultures of E. coli HB11 as a function of time after infection with R17 phage.

The experimental details are given in the text. A 5 ml aliquot of each time sample was centrifuged (10,000 g, 10 min) and the phage attachment capacity of a 2.5 ml aliquot of the supernatant determined by the filtration assay. The phage attached by the bacteria was determined by measuring the ^{14}C-radioactivity in a small aliquot of the resuspended cell pellet on Whatman 3MM filter discs. The phage attachment capacity for the whole culture was determined by adding the values obtained for the bacteria and the supernatant. Identical values were obtained by filtering portions of the whole culture.

Figure 7.4). This result cannot be explained by an outgrowth of F-pili, since it was previously shown that cell-associated F-pili shorten immediately after infection (Table 7.2, Figure 7.3). An alternate possibility may be that phage infection causes a disaggregation of F-pili, thereby exposing additional phage attachment sites. Support for this idea may be obtained from Plate II in the Appendix, where it is seen that several F-pili originating in the same cell are entwined, and that phage infection could cause their separation, thereby increasing the number of exposed phage attachment sites in the system. Whatever the reason for the increase in the phage attachment capability in the whole culture, it is clear that the apparent shortening of F-pili seen by electron microscopy is most probably due to a release of F-pili into the medium rather than an actual retraction of F-pili. It would thus appear that the F-pili retraction hypothesis presented in Figure 7.1 does not apply in the case of RNA phage infection.

C. Summary and Discussion

The results of the experiments described in this chapter may be summarized as follows.

1. Infection of E. coli with phage R17 is accompanied by a utilization of cellular nucleoside triphosphates. This consumption of energy appears to occur only in the first 5 min after infection and the degree of utilization is dependent on the multiplicity of infection.

2. A detailed electron-microscopic analysis of cells, before and after infection with phage R17, has revealed that cell-associated F-pili

shorten considerably (~30%) in the first 2 min after infection.

3. Phage attachment studies have shown that the apparent shortening of F-pili seen by electron microscopy is due to the release of F-pili fragments, rather than the retraction of F-pili into the cell.

Although the combined results of this chapter do not favour the concept of F-pili retraction during phage R17 infection, it is now of interest to interpret these data in terms of the "F-pili conduction" model proposed by Brinton (1965). Evidence has been provided that the following series of events may occur during R17 phage infection of piliated host cells. Phage attachment to the side of the F-pilus triggers a reaction which results in (a) the release of the A protein and RNA from the phage particle, (b) fragmentation of the A protein and (c) a weakening or rearrangement of the F-pilus at the point of phage attachment. This weakening of the F-pilus presumably increases the likelihood of an actual breakage of the F-pilus at this point and it is probably this type of breakage which gives rise to the "shedding" of F-pili observed in Figure 7.4. This weakening of the F-pilus could facilitate the entry of phage RNA and A protein into the core of the F-pilus and their subsequent transport into the cell.

Although the present studies thus provide new insights into the role of F-pili in mediating the transfer of phage RNA into the host cell, the detailed molecular mechanisms by which F-pili function in this capacity must await further investigation.

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APPENDIX

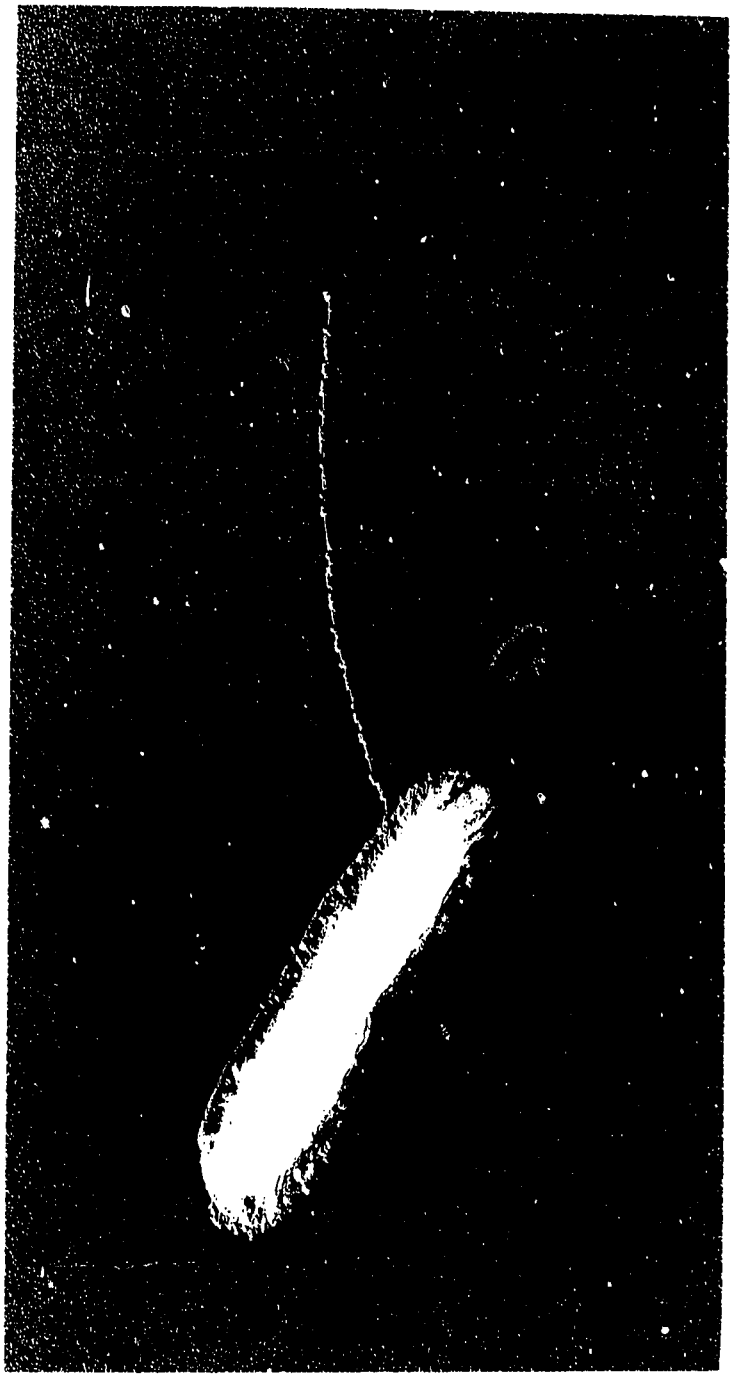

Plate I. Electron micrograph of E. coli HB11 previously infected with R17 phage. The photograph represents a typical electron micrograph employed for measurement of F-pilus lengths. Magnification = 27,000x.

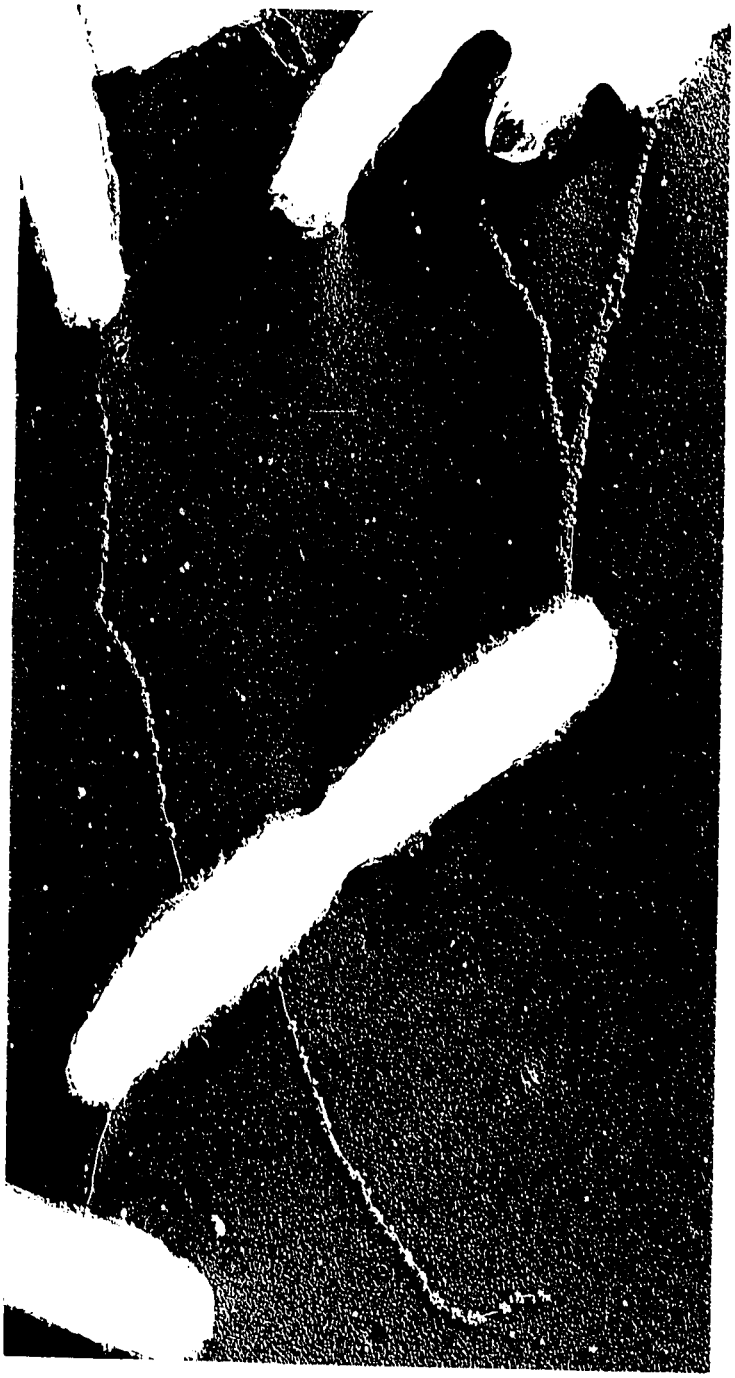

Plate II. Electron micrograph of E. coli HB11 cells previously infected with phage R17. Note the aggregated F-pili in the upper right hand side of the photograph. Magnification = 27,000x.