Advances in
VIRUS RESEARCH

VOLUME 15

Advances in

VIRUS RESEARCH

Edited by

KENNETH M. SMITH

Cambridge, England

MAX A. LAUFFER

Department of Biophysics
and Microbiology
University of Pittsburgh
Pittsburgh, Pennsylvania

FREDERIK B. BANG

Department of Pathobiology
Johns Hopkins University
Baltimore, Maryland

VOLUME 15

1969
ACADEMIC PRESS
NEW YORK AND LONDON

CONTRIBUTORS TO VOLUME 15

Numbers in parentheses indicate the pages on which the authors' contributions begin.

JUNE D. ALMEIDA, *Department of Virology, Royal Postgraduate Medical School, London, England (307)*

P. L. BERGQUIST, *Department of Cell Biology, University of Auckland, Auckland, New Zealand (159)*

D. J. W. BURNS,* *Department of Cell Biology, University of Auckland, Auckland, New Zealand (159)*

ROGER HULL, *John Innes Institute, Colney Lane, Norwich, England (365)*

R. K. RALPH, *Department of Cell Biology, University of Auckland, Auckland, New Zealand (61)*

SARAH E. STEWART,† *Viral Biology Branch, National Cancer Institute, Bethesda, Maryland (291)*

METTE STRAND, *Biochemistry Department, University of California, Berkeley, California (1)*

T. I. TIKCHONENKO, *Institute of Virology, USSR Academy of Medical Sciences, and Department of Virology, Moscow State University, Moscow, USSR (201)*

RAYMOND C. VALENTINE, *Biochemistry Department, University of California, Berkeley, California (1)*

H. J. WALTERS, *Department of Plant Pathology, University of Arkansas, Fayetteville, Arkansas (339)*

RICHARD WARD, *Biochemistry Department, University of California, Berkeley, California (1)*

A. P. WATERSON, *Department of Virology, Royal Postgraduate Medical School, London, England (307)*

* Present address: Huntington Laboratories, Massachusetts General Hospital, Boston, Massachusetts.

CONTENTS

Studies on the Herpes-Type Virus Recovered from the Burkitt's Tumor and Other Human Lymphomas

SARAH E. STEWART

The Morphology of Virus-Antibody Interaction

JUNE D. ALMEIDA AND A. P. WATERSON

Beetle Transmission of Plant Viruses

H. J. WALTERS

Alfalfa Mosaic Virus

ROGER HULL

THE REPLICATION CYCLE OF RNA BACTERIOPHAGES

Raymond C. Valentine, Richard Ward, and Mette Strand

Biochemistry Department, University of California, Berkeley, California

I. Introduction

Since the discovery of RNA phages by Loeb and Zinder (1961) about a decade ago a wealth of information has accumulated on the infective cycle of these viruses. An outline of their life cycle is now beginning to

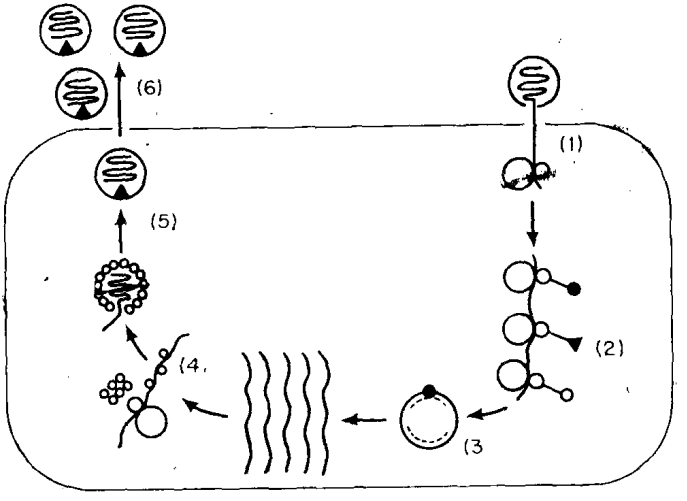

FIG. 1. Summary of the life cycle of an RNA phage. Numbers refer to different steps of the cycle: (1) RNA injection; (2) protein synthesis; (3) RNA replication; (4) regulation of protein synthesis; (5) particle assembly, (6) cell lysis. (Oo) ribosome; (○) capsid protein; (●) polymerase; (▲) A protein.

emerge at the molecular level (see Fig. 1). Actually only a skeleton of the life cycle exists at present but this is enough to see the general shape of the pathway. It is interesting that the life cycle as it evolves acquires more and more of the characteristic of a metabolic or biosynthetic pathway—an infective cycle requiring an initial primer which stimulates the production of many more copies of the template molecule. In many respects the solution of this phage life cycle as presented here was achieved by the use of general scientific methods very much like those developed for studies on metabolic cycles. The same "divide and conquer" approach has been used to dissect the phage life cycle. Specialists are now busily working out the many different reactions of the life cycle as presented in the outline. Already a number of important discoveries have

been recorded by these workers. The "life in a test tube" experiment by Spiegelman and co-workers (1965a), which was accomplished during a study of Qβ virus RNA replication, certainly stands out. These workers found that the virus-induced RNA polymerase had the almost uncanny ability to seek out its own *homologous* and *intact* template while producing new copies of infectious RNA in the test tube. This now classic study is recognized as the original ' life in a test tube" experiment. This area is most active at the moment and the complete mechanism of RNA replication may be forthcoming in the near future (August, 1969).

Studies on viral protein biosynthesis may also be carried out in the test tube and again intensive work has been done in this area (Nathans *et al.*, 1962). Much information on the mechanism of viral protein synthesis has come from these studies such as punctuation of the viral message (Zinder *et al.*, 1966, Capecchi, 1966a; Adams and Capecchi, 1966) and the nature of the viral ribosome cycle (Guthrie and Nomura, 1968). These are just two examples of the many experiments that have been done with RNA phage (Zinder, 1965). These viruses continue to be widely used in biological studies, for example, in work where a readily available, natural message is required. The phages are likely to continue to find application as tools by molecular biologists for some time to come. Aside from the use of these phages as general tools, something else is happening in the RNA phage field. There is more talk of the life cycle itself—the whole of the cycle as an integrated unit. The interest in the life cycle is more than the desire to piece together another phage puzzle. It is becoming clear that the RNA phage as an organism is simpler in nature than any yet studied. Naturally, a simple life cycle is expected. This could mean that the life cycle of the RNA phage may be the first to be fully understood at the *molecular level.* The full value of such knowledge perhaps can not be seen just yet, but it is not unreasonable to expect that a complete solution of the miniphage cycle would help "clear the air" for studies of other cycles presently at various stages of completion. At least the mystery of this one viral cycle is dying away. It is not really a large exaggeration to say that the whole life cycle of an RNA phage can be carried out away from the host cell, i.e., in cell-free extracts. This allows the techniques of biochemistry to be focused directly on the problem and explains why work is flourishing in this area.

With so much work still going on it might seem unwise to attempt a review of the life cycle at this time. Still, the hundreds of papers published in this field are widely scattered in the literature and are often of a very specialized nature. It was the general plan of this review to bring together some of the knowledge of these specialized areas and fit it to a general life cycle. It was hoped that such a treatment would best illustrate

the dynamic and integrated nature of the many reactions making up the infective process.

II. Anatomy of the Infective Particles

A. General Reactivities of the Particles

The free virus particle must take part in a variety of reactions outside the cell although for the most part the chemical reactivities of this particle are still poorly understood. An example of one of the novel reactions of the particle is the release of its packaged RNA; this will be dealt with in a later section. In general the particles seem structurally well suited for many of their tasks such as protection against harmful nucleases or proteases. A variety of other harmful compounds such as soaps, heavy metals, and the like are probably often encountered by the free living particles. These inhibitory reagents, along with physical conditions such as heat and cold, may exert strong selective pressures on the phage perhaps keeping them in a dynamic state of change. As discussed below, the viruses have a well-developed system for genetic change which gives them flexibility and allows them to readily adapt to their living conditions. In many cases very marked changes have occurred and *speciation* is now recognized among phage isolates from different parts of the world (Miyake *et al.*, 1967). In extreme cases the base ratios of the RNA molecules from different isolates vary considerably (Overby *et al.*, 1966; Bishop and Bradley, 1965). The base sequence work done to date with two of the general groups (f2, R17, MS2 *vs.* Qβ) reflects this change (see Table I). The work done so far indicates that RNA phages may be unique tools to study certain steps of molecular evolution.

B. Morphology

The gross structure of the RNA phages has not yet received a great deal of attention although it is of major importance. The first electron micrographs taken of the male-specific phage f2 were somewhat surprising and showed tiny spherical particles smaller than anything yet seen in the phage world. Later Vasquez *et al.* (1966) studied the detailed morphology of the related bacteriophage R17 by electron microscopy of negatively stained virions (Fig. 2). They observed: (*a*) the hexagonal shape, (*b*) the presence of a maximum of 10 units at the periphery, and especially (*c*) the central fivefold points of symmetry with neighboring 5 and 6 coordinated capsid units indicating icosahedral symmetry with 32 morphological units. Thus, the morphology and size of phage R17 seem to be similar to that of other small RNA viruses. Detailed structural analysis by use of both X-ray diffraction and electron microscopy has

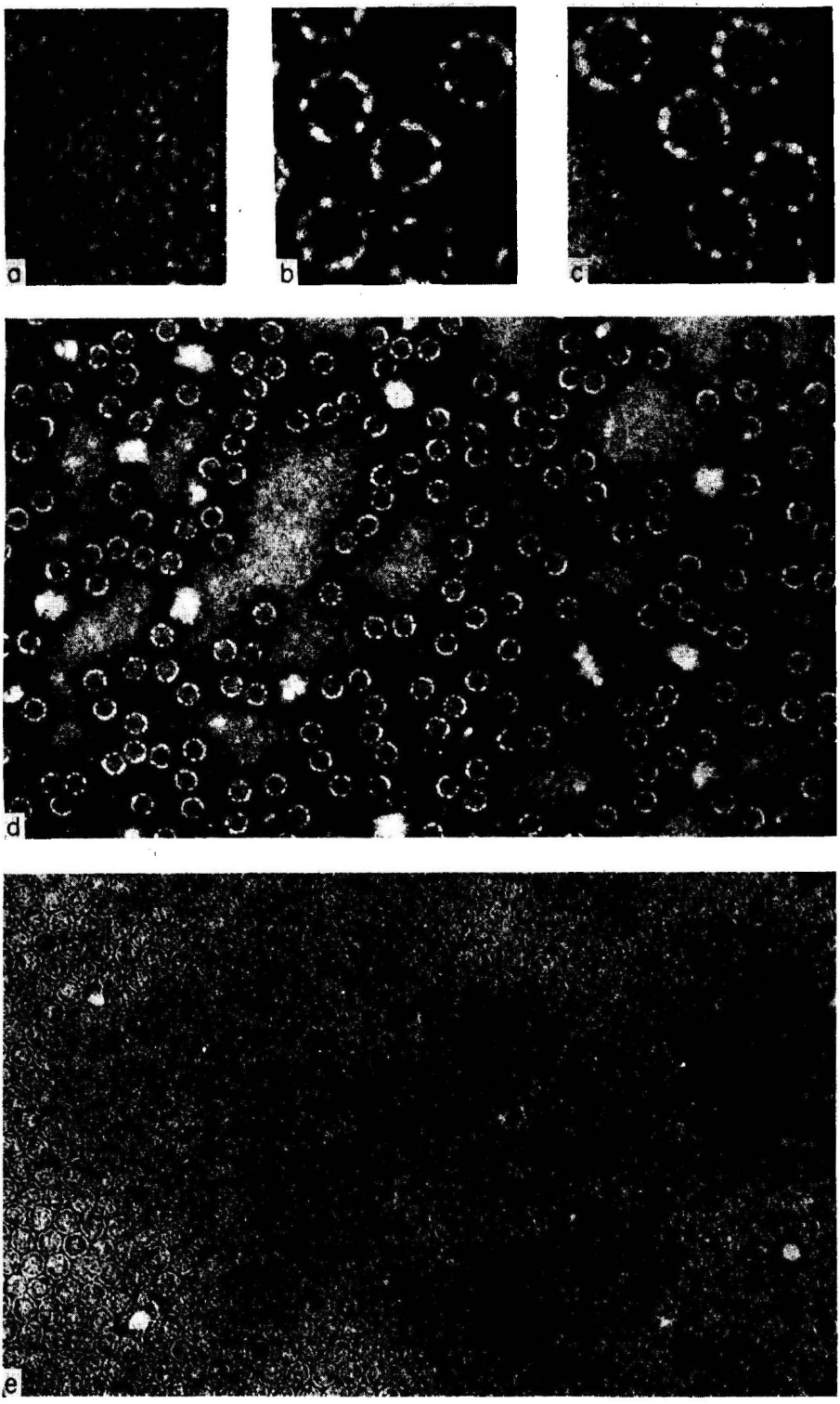

FIG. 2. RNA phage particles. (a) magnification × 600,000; (b) and (c) × 640,000; (d) × 150,000; (e) × 200,000. (Courtesy of C. Vasquez, N. Granboulan and R. M.

been carried out on some members of the spherical RNA virus group such as turnip yellow mosaic virus (TYMV) (Finch and Klug, 1966) although similar studies have not been done with an RNA phage. From their morphological studies Vasquez *et al.* (1966) suggested that the capsid of R17 and presumably other members of this phage group are composed of 32 morphological units, 12 located on the fivefold and 20 on the threefold axis of symmetry. It was not possible to resolve the fine structure of these 32 morphological capsid units; but a reasonable model was that there were 12 pentamer and 20 hexamer units.

Another important question for which there is only scant information is the nature of the packing of the RNA strand and the spatial arrangement of the RNA within the shell of the phage particle. Each phage particle contains a very long RNA strand (about 10,000 Å) which is compactly folded in the small central cavity of the particle. The nature of this packing is not known; still, it is interesting to speculate on this packing arrangement from a functional standpoint. It seems likely that the packing configuration may be quite specific and that the RNA may be coiled or arranged in a special way within the particle. In other words, the location of the 5′- or 3′-ends of the RNA strand may always be located at the same site within the particle. This idea comes mainly from experiments on RNA injection which indicate that the injection reaction may be a linear process with a particular end of the RNA strand presumably leaving the particle at the onset of infection. Also, recent experiments by Lodish (1968) may be relevant to this point. He observed that the 5′-end of the RNA genome of certain amber (A protein) mutants is preferentially destroyed by nuclease digestion indicating that the 5′-terminus may be closer to the surface of the particle and thus more susceptible to nuclease attack. These particles still contained their 3′-terminus. It is possible that the 5′-end of the RNA, being closer to the surface of the particle, may enter the cell first. The mechanism of RNA release will be discussed in more detail in a later section.

C. The RNA Molecule

The infectious RNA genome extracted from purified particles is a linear molecule approximately 10,000 Å in contour length as visualized in the electron microscope (Granboulan and Franklin, 1966). It is thought to contain roughly 3000 nucleotides. The RNA genome with a net coding capacity for about 1000 amino acids is believed to code for only three viral proteins (Viñuela *et al.*, 1967; Haywood and Harris, 1966; Nathans *et al.*, 1966), making RNA phages "three-cistron viruses." The linear order of phage genes on the molecule has not been completely established but recent evidence indicates that the order starting from the 5′-end is

capsid, maturation protein, polymerase (Spahr and Gesteland, 1968; Lodish, 1968). The order of the genes has important biological consequences as will be discussed below. One of the surprises that has come from recent chemical studies on the molecule is that the stretches of nucleotides at the ends of the molecule appear to be without physiological function since they do not contain codons specifying known amino acid sequence. The terminal sequences worked out to date are summarized in Table I. It is evident that the viral RNA chain does not start with an initiation codon for polypeptide synthesis. This means that the transla-

TABLE I

TERMINAL SEQUENCES OF SEVERAL RNA PHAGE GENOMES

Phage	5′-End	3′-End	Reference
f2	pppG	(G)UUACCACCCA$_{OH}$	Dahlberg (1968), Weith and Gilham (1967)
R17	pppG	(G)UUACCACCCA$_{OH}$	Dahlberg (1968), Roblin (1968)
MS2	pppGGGU or pppGGU	(G)UUACCACCCA$_{OH}$	De Wachter *et al.* (1968), Glitz (1968)
Qβ	pppG	(G)CCCUCCUCUCUCCA$_{OH}$	Dahlberg (1968), Watanabe and August (1968)

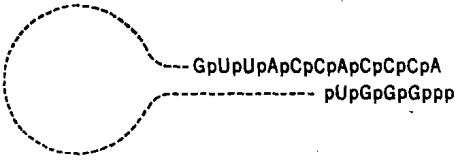

FIG. 3. Hypothetical circular RNA genome (De Wachter *et al.*, 1968).

tion of the polynucleotide message starts at some distance from the 5′-terminus and stops before the 3′-terminus is reached. Indeed no chain-terminating codon was found in the 3′-terminal sequence either. The length of the terminal RNA segments not involved in protein synthesis is at present unknown.

Note in Table I that the bases 1–4 from the 5′-terminus of MS2 are complementary with the bases 2–5 from the 3′-terminus. This makes it possible for the RNA molecule to be folded to form a cyclic molecule in which the ends form a double-stranded region as illustrated in Fig. 3. This terminal base complementarity may be important for viral RNA replication. De Wachter *et al.* (1968) have pointed out that the complementary or minus strand probably has the same terminal sequences as

the plus strand (except perhaps for the A residue). If one or both of the extremities form a recognition site for a viral RNA polymerase this site would be the same for the viral RNA and its complement so that both molecules may be recognized by the same polymerase.

Another point to note (Table I) is that Qβ RNA has a terminal sequence distinctly different from the other phages implying some interesting relations between the terminal sequences and the function of these RNA molecules (Weith *et al.*, 1968). For example, these sequence differences may provide the molecular basis for the observed template specificities displayed by Qβ and MS2 polymerases (Haruna and Spiegelman, 1965a). The other portions of the RNA molecule which do not serve a coding function may serve other roles such as providing specific binding sites for viral repressor proteins or ribosomes.

It seems very likely that the secondary structure of the RNA may play an important role in its biological function so it is necessary to consider the molecule at this level. One of the impressive features of the phage RNA is its unusually compact nature. For example, Gesteland and Boedtker (1964) observed that R17 phage RNA had a sedimentation coefficient of 26 S in the solvents in which tobacco mosaic virus (TMV) RNA, having twice the molecular weight, also sedimented at 26 S. It seems clear that R17 forms hydrodynamically more compact configurations than other RNA's, though there appears to be no readily available explanation for this compact structure. It is clear that the RNA has to assume a very compact configuration inside the phage head and this might be the result of an unusual secondary structure. The compact configuration of the viral RNA's may also serve to protect the molecule from nucleolytic attack helping to make these RNA molecules "stable" messages. Certain studies indicate that specific regions of the molecule may be preferentially attacked by nuclease indicating that these vulnerable regions are more exposed. Limited cleavage with RNase has led to the separation of a 5′-segment from a 3′-fragment (Bassel and Spiegelman, 1967).

D. Capsid

The capsid protein of phage f2 consists of a single polypeptide chain of 129 amino acids shown in Fig. 4 (Weber and Konigsberg, 1967). Inspection of the amino acid sequence shows that there are long stretches of nonpolar amino acids. Aspartic acid, for instance, is the only polar residue in the first 30 amino acids. Another stretch of 15 nonpolar amino acids occurs between 67 and 82, where glutamine is the only polar amino acid. At the carboxyl end, Lys-Asp are the only charged amino acids in the terminal 22 residues.

Sequence data on coat proteins from several RNA phages are now available (Weber and Konigsberg, 1967; Wittman-Liebold, 1966; Lin *et al.*, 1967), and it is of interest to compare these. There is only one known difference between the sequences of MS2 and R17 as compared to f2. Leucine (residue 88) in f2 is replaced by methionine in R17 and MS2; fr has the same number of residues as f2 but contains about 19 amino acid replacements. As pointed out by Weber and Konigsberg (1967), of these substitutions 15 are amino acids which can be related to those in f2 by a single base change. As might be expected the polar or nonpolar nature of the residues involved in these substitutions was preserved. These variations were clustered near the ends of the molecule.

Ala-Ser-Asn-Phe-Thr-Gln-Phe-Val-Leu-Val-Asn-Asp-Gly-Gly-Thr-Gly-Asn-Val-Thr-
5 10 15

Val-Ala-Pro-Ser-Asn-Phe-Ala-Asn-Gly-Val-Ala-Glu-Trp-Ile-Ser-Ser-Asn-Ser-Arg-
25 30 35

Ser-Gln-Ala-Tyr-Lys-Val-Thr-CMCys-Ser-Val-Arg-Gln-Ser-Ser-Ala-Gln-Asn-Arg-
40 45 50 55

Lys-Tyr-Thr-Ile-Lys-Val-Glu-Val-Pro-Lys-Val-Ala-Thr-Gln-Thr-Val-Gly-Gly-Val-
60 65 70 75

Glu-Leu-Pro-Val-Ala-Ala-Trp-Arg-Ser-Tyr-Leu-Asn-Leu-Glu-Leu-Thr-Ile-Pro-Ile
80 85 90

Phe-Ala-Thr-Asn-Ser-Asp-CMCys-Glu-Leu-Ile-Val-Lys-Ala-Met-Gln-Gly-Leu-Leu-
95 100 105 110

Lys-Asp-Gly-Asn-Pro-Ile-Pro-Ser-Ala-Ile-Ala-Ala-Asn-Ser-Gly-Ile-Tyr
115 120 125 129

FIG. 4. Amino acid sequence of f2 coat protein (Weber and Konigsberg, 1967).

Thus, the sequences of fr and f2 between residues 20 and 53 were identical. It will be interesting to know whether this same invarient segment is preserved for the unrelated phage $Q\beta$; this region might be critical for viral function. A variety of natural phage mutants is available for studies of this kind (Miyake *et al.*, 1967).

The structure-function relationship is not known for the capsid protein, although it seems evident that certain portions of the molecule must fold in special ways and perform special tasks such as forming morphological units and binding to RNA. The "active sites" on the protein molecule which are responsible for these functions are unknown although it is clear that the capsid subunit is versatile serving at least in dual function as both repressor and capsid protein. The capsid protein as shell must protect the RNA from attack and may itself be relatively resistant to break-

down by natural proteases in this role. Perhaps it is worth noting the differences in susceptibility of the NH_2- and COOH-ends of the protein to chemical or enzymic attack. Neither leucine aminopeptidase nor the usual end group reagents reacted with the NH_2-terminal residue without prior denaturation of the protein, indicating that the NH_2-terminus was buried in the substructure of the protein (Weber and Konigsberg, 1967). On the other hand, the COOH-terminal group was easily removed by standard reactions indicating its accessibility to attach. Similar folding of the subunits within the shell structure may provide a protective shield. Aside from the long stretches of hydrophobic residues which may

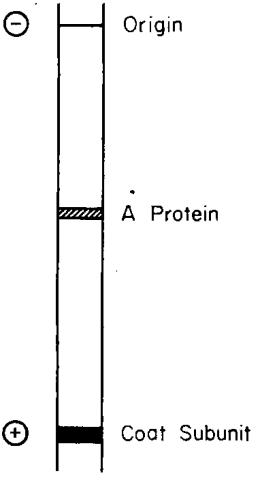

FIG. 5. Separation of f2 A protein and capsid on acrylamide gel. (Procedure of Steitz, 1968.)

be important in folding or subunit-subunit interactions, another property of the protein that stands out is the relatively low number of basic residues. If these basic residues do play a key role, for example, in charge neutralization on the RNA, then again special folding of the protein subunits may expose these residues to the polynucleotide. A positively charged inner shell may be envisioned predominantly to provide charge neutralization to the highly charged RNA strand which is very compactly folded.

E. A Protein

In 1967 Roberts and Steitz successfully reconstituted infective particles of phage R17 and in so doing proved that maturation protein (A protein) is a structural component of the phage and essential for infectivity. The A protein has now been isolated (Fig. 5) and characterized

and its amino acid composition has been analyzed (Steitz, 1968). It seems clear from this work that each R17 phage possesses only one molecule of the histidine-containing A protein which has a molecular weight of about 39,000.

To show that the A protein is a structural component of R17 grown *in vivo*, Steitz (1968) exploited the fact that the amino acid histidine is absent from the phage coat protein. Phage was grown in the presence of labeled histidine and then isolated, purified, and assayed for radioactivity. The highly purified phage contained histidine. Fractionation of the proteins isolated from the labeled phage showed that the histidine was present in phage-specific maturation protein. The protein was apparently very insoluble and difficult to work with because it tended to bind to any surface. Amino acid analysis of the purified protein indicated that there were five histidine residues per 38,000 molecular weight. As expected from the *in vitro* reconstitution experiments maturation protein was absent from the defective particles produced by phage which had an amber mutation in the maturation protein cistron (Steitz, 1968).

The maturation or A protein plays an important structural and functional role as will be described below. If, as proposed, a single molecule of this protein is imbedded in the shell of the phage at a specific point, then in one sense the overall symmetry of the shell is broken at this point. Conceivably the maturation protein might mark the binding site for particle-cell interaction—a spatial arrangement which may orient the RNA strand for proper injection. This is the current notion. The release of the RNA from the shell will be taken up next—a reaction which requires the complex interplay of all three viral constituents and various cellular components.

III. RNA Injection Pathway

A. Summary of the Pathway

The first stage of the infective process of these phages is the injection of the viral RNA into the cell (Valentine *et al.*, 1969). In simplest terms this RNA injection process involves the adsorption of an infective particle and release of the packaged RNA into the cell. During this process the empty shell or ghost remains outside the cell, as is the case with many phages. The overall injection reaction is conveniently divided into three parts: (1) simple binding or adsorption, (2) RNase-sensitive step, and (3) RNA transport. A hypothetical scheme summarizing these steps is presented in Fig. 6. As shown in the scheme the molecular events required for injection of the viral genome are complex and involve interactions between the phage RNA, the two phage proteins, the F pilus, and proba-

bly other cellular components. Essentially, the RNA originally bound in its protective shell becomes attached to the pilus and its linkage to its own coat is weakened. The coat falls away leaving the RNA to complete its infection of the cell. This can be a difficult time for the virus since for a short time the viral genome is exposed to the external environment and RNases while it is being transported into the cell. It is interesting that simple binding of a phage particle to its isolated cellular receptor site, the F pilus, does not induce major structural alterations of the particle which set up the RNase-sensitive step. Instead, the particle attached to a free or nonfunctional F pilus is readily desorbed and can find a more favorable site for injection. In nature this ability of the phage to distinguish suitable binding sites may have important advantages for survival. For example, particles may initially bind to intact F pili of physiologically inactive cells which cannot support phage development or are not able to draw the phage RNA rapidly enough into the cell. Since

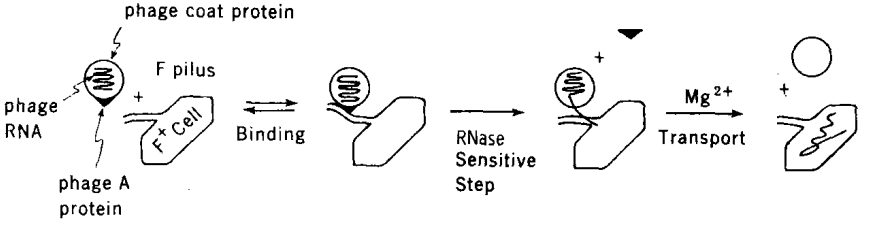

FIG. 6. The RNA injection pathway.

the adsorbed particles do not trigger release of their RNA into such "old" cells the particle can desorb and find a more suitable host. The advantage of such discriminatory powers for the virus are obvious. The mechanism that allows the virus to make this selection of hosts is hypothesized to be a special arrangement of the capsid which recognizes a certain functional configuration at the host-binding site. It seems that the phage maintains control of its injection steps up to the time of the RNase-sensitive step. Beyond this point the cell plays the dominant role and may be involved in "active transport" of the RNA strand into the cell. It may be pertinent that free RNA molecules are infective for protoplasts (Strauss and Sinsheimer, 1967) in a process not unlike transformation with DNA. In the infectious protoplast assay all of the viral proteins as well as the receptors on the cell wall have been stripped away and still the RNA is able to penetrate the cells. Perhaps RNA transport in the infectious protoplast system is part of the normal mode of phage RNA infection being equivalent to the last step of the reaction shown in Fig. 6. This point and other features of RNA injection will be described next.