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Purification and Properties of Bacteriophage f2 Replicase

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PURIFICATION AND PROPERTIES OF BACTERIOPHAGE f2 REPLICASE

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by

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SUMMARY

The present dissertation concerns the purification and properties of an RNA-dependent ribonucleotide-polymerizing enzyme (RNA replicase) produced during infection of E. coli with the RNA bacteriophage f2.

Studies on the RNA replicase of the distantly-related bacteriophage Qβ have established the feasibility of using a simplified assay for replicase activity based on the ability of the enzyme to polymerize GTP in the presence of polycytidylic acid template. A similar poly C-dependent poly G polymerase activity is detectable in E. coli infected with bacteriophage f2. The f2 poly G polymerase has been purified by ion exchange chromatography on DEAE cellulose, affinity chromatography on RNA cellulose and density gradient centrifugation. Highly purified preparations of phage-induced poly G polymerase consist predominantly of four proteins having approximate molecular weights of 75,000, 63,000, 46,000 and 33,000. The 63,000 m.w. protein has been identified as the phage-coded replicase protein based on co-electrophoresis of this polypeptide with replicase protein obtained from phage-infected cells.

Partially purified preparations of f2 poly G polymerase exhibit replicase activity. Such preparations catalyze nucleotide polymerization in the presence of single-stranded f2 phage RNA or f2 complementary strand RNA, but are inactive with other viral and bacterial RNAs. The product of the in vitro enzymatic reaction has been analysed by RNA annealing techniques and found to consist entirely of polynucleotides homologous to f2 RNAs. In the presence of single-stranded phage RNA template, the enzyme synthesizes both complementary strand RNA and some product copies of the input template strand. However, the enzyme does not synthesize RNA in excess of the input template amount and is able to release only a small proportion of the product RNA as single-stranded viral RNA. Most of the product RNA remains in partially double-stranded complexes with template RNA.

Highly purified f2 poly G polymerase shows no replicase activity. It has been found that a factor fraction necessary for replicase activity separates from the poly G polymerase during glycerol gradient centrifugation. This fraction, which shows no nucleotide-polymerizing activity and is presumably of bacterial origin, restores replicase activity with both f2 single-stranded viral RNA and f2 complementary strand RNA to the 4-polypeptide poly G polymerase.

The f2 replicase and poly G polymerase activities are quite similar with respect to such parameters as stability, temperature optimum, divalent cation requirements, phosphate insensitivity and template saturation kinetics. Template competition experiments further suggest that the synthetic polymer and phage RNA compete for binding to the enzyme. The effect of the ionic strength of the incubation medium on the two activities is quite different, however. Replicase activity is stimulated at ionic strengths up to about 0.1, while the poly G polymerase is markedly inhibited, even at quite low salt concentrations.

The results of substrate saturation studies suggest that the affinity of replicase is considerably higher for ATP, CTP and UTP than for the chain-initiator nucleotide GTP. Furthermore, the complex saturation kinetics observed for GTP in both replicase and poly G polymerase reactions indicate the simultaneous interaction of more than one molecule of GTP with the enzyme. On the basis of the present studies with the f2 enzyme and the considerable literature on other nucleotide-polymerizing enzymes, it is proposed that the replicase has separate active sites for RNA chain initiation and polymerization. It is postulated that the synthetic polymer-dependent reaction occurs primarily or exclusively at the chain initiation site, whose normal function is to recognize the 3' terminus of phage RNA templates and carry out the polymerization of the 5' terminal guanylate residues of phage RNAs. It is further suggested that replication of phage RNAs occurs primarily at the polymerization site, whose activity is governed by specific secondary interactions of the enzyme with natural template RNAs.

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CHAPTER I

INTRODUCTION

Since their discovery by Loeb and Zinder (1961) more than 10 years ago, the RNA-containing bacteriophages have been studied extensively. At present, five groups of phages have been identified, primarily on the basis of phage particle properties (Miyake et al., 1971). Group I, which includes the phages f2, R17, MS2, fr, and M12, has been analysed in great detail, both structurally and functionally. The aspect of Group I RNA phage physiology which has proved most difficult and unrewarding experimentally is the structure and enzymology of its replicase, the enzyme responsible for phage RNA replication. The analogous enzyme of Qβ, a group III phage, is experimentally much more accessible. As a result, most of the information available today on RNA replicases comes from studies on the Qβ replicase, despite the paucity of general information concerning the physiology of Group III phages and its relationship to that of Group I phages. Relatively little is known about the remaining groups of RNA phages (Stavis and August, 1970).

The present study is devoted to filling in the gap in our knowledge of Group I phages. Capitalizing on recent developments in enzyme purification techniques and the considerable knowledge derived from studies on the Qβ replicase, we have devised a purification scheme for the unstable replicase of the f2 bacteriophage. We have studied a number of its properties and certain aspects of its relationship to the Group III replicase of the Qβ phage.

The amount of information available on the structure and physiology of Group I RNA phages is prohibitive of a comprehensive review. Zinder (1965) and Stavis and August (1970) have summarized much of this information and it will not be recapitulated here. Our effort in this introduction is to provide a minimal historical framework for the present study and to introduce certain features of Group I phage physiology which we have exploited in our studies on the f2 replicase.

A. Phage Replication

The RNA phages of Group I consist of a single-stranded RNA molecule (m.w. 1.1×10^6) encapsulated in a polyhedral protein shell composed of 180 molecules of major coat protein (m.w. 14,200) and one or two molecules of A protein (m.w. 35-40,000; Stavis and August, 1970). Phage reproduction occurs in male cells. The reproductive cycle begins when the phage particle attaches to a bacterial pilus and the phage RNA penetrates into the bacterial cell. The phage RNA serves both as the genetic material of the phage and as messenger RNA for the synthesis of the three phage proteins. One of the phage proteins synthesized early in the infectious cycle is required for the enzymatic replication of phage RNA. This is the phage replicase protein. Phage RNA replication involves the synthesis of a complementary (or minus strand) copy of the incoming single-stranded (or plus strand) RNA, followed by the asymmetric synthesis of many new plus strands. A limited number of newly-synthesized plus strands undergo complementary minus strand synthesis and contribute to the further generation of plus strands (Weissmann and Ochoa, 1967). Two other phage proteins are made during infection. These are the major and minor (A protein) proteins of the viral particle. There is also some evidence for the existence of a fourth phage-specific protein, which may be necessary for lysis of infected cells (P. Model, personal communication). Viral particles are assembled from the newly-synthesized plus strand RNA, coat protein and A protein. As many as 10,000 phage particles are constructed and assembled within a single cell in the short span of 40-50 minutes after infection. At this time the cell lyses and the phage particles are released (Zinder, 1965; Stavis and August, 1970).

This thumbnail sketch of phage reproduction is applicable to all Group I phages and probably to Group III phages as well (Horiuchi and Matsuhashi, 1970). The Group I phages appear to be extremely similar; they are probably removed from each other by a relatively small number of mutational events. Thus, for example, the coat protein sequences of f2 and R17 and of f2 and MS2 differ by only a single amino acid out of 129 (Stavis and August, 1970). The nucleotide sequences of the RNAs

are also quite similar, as evidenced by extensive cross-hybridization (Weissmann and Ochoa, 1967). Nucleotide sequencing is currently in progress, and significant regions of common or similar nucleotide sequence are already apparent among the Group I RNAs (Cory et al., 1970; Stavis and August, 1970).

The Group III phage Qβ was originally thought to be unrelated to the Group I phages. There is no serological cross-reaction between these groups of phages and their RNAs and phage particles have significantly different properties (Overby et al., 1966a and b). The RNAs of Group I phages do not hybridize with Qβ phage RNA (Weissmann and Ochoa, 1967). The amino acid sequences of Group I coat proteins are quite different from that of the Qβ coat protein (Konigsberg et al., 1970). Close examination of the sequences, however, suggests that these phages may have a common ancestor (Konigsberg et al., 1970). Similar conclusions have been derived from studies on the nucleotide sequences of the RNAs (Adams and Cory, 1970; Goodman et al., 1970). Thus, the Group I phages probably diverged from the Group III phages fairly early in the evolution of these simple organisms. Conclusions based on studies of one phage group must be applied to other groups with some caution. Intra-group studies, on the other hand, can be discussed interchangeably with relative assurance.

B. Phage Protein Synthesis and Its Control

Despite their apparent simplicity, RNA phages exhibit quite sophisticated control mechanisms governing phage macromolecular synthesis. These will now be discussed in some detail. The existence of control mechanisms can be deduced from a consideration of the relative quantities of phage-specific macromolecules synthesized during infection. Analysis of phage-specific proteins made during infection of cells in which host protein synthesis is inhibited by actinomycin (Viñuela et al., 1967; Nathans et al., 1969) or rifampicin (Fromageot and Zinder, 1968) shows that the phage coat protein is made in vast molar excess over the other phage proteins. Similarly, when phage-specific RNA species are examined

by hybridization (Weissmann et al., 1964a) it is evident that about 10 times as many phage plus strands as phage minus strands are made during the infectious cycle. Considerable evidence exists that this quantitative regulation is under phage control and that the control mechanisms governing phage protein synthesis and phage RNA synthesis are interrelated.

The first phage control mechanism to be identified was that governing the synthesis of f2 replicase protein. As mentioned earlier, the replicase protein is the protein required for the enzymatic synthesis of progeny phage RNA. That a phage protein is required for RNA replication in f2-infected cells was originally shown by Cooper and Zinder (1963). They found that phage RNA synthesis could be inhibited by adding chloramphenicol to inhibit protein synthesis at the time of infection. Some evidence for the existence of a control mechanism was evident even in these early studies. Thus the chloramphenicol inhibited progeny RNA synthesis only when added early in the infectious cycle. Normal RNA replication obtained when chloramphenicol was added later than 15 minutes after infection, suggesting that the synthesis of this protein is confined to the early part of the infectious cycle. Additional evidence for such a control mechanism came from observations of replicase activity in crude cell homogenates. Again, it was found that replicase activity could be detected only when phage proteins were synthesized. In addition, there was no increase in the amount of activity detectable beyond about 20 minutes after infection (Lodish et al., 1964).

Studies on conditional-lethal mutants* (amber† and temperature-sensitive mutants§), carried out primarily by Lodish et al. (1964) and

*Conditional-lethal mutations allow the virus to grow under one set of conditions (e.g. in certain bacterial strains, referred to as permissive hosts, or at certain temperatures) but not under other conditions (e.g., in other bacterial strains, referred to as non-permissive hosts, or at other temperatures).

†Amber mutations are changes in the nucleotide sequence which result in the premature termination of a nascent polypeptide chain. When an amber mutant is grown in a non-permissive host, synthesis of the protein stops at the site of the mutation and the unfinished protein is released.

by Horiuchi et al. (1966), established the function of this viral protein. These authors found that mutations in one of the three viral cistrons resulted in the inability of the phage to replicate its RNA under non-permissive conditions. This showed directly that a viral protein is involved in phage RNA replication. The nature of the control mechanism governing the synthesis of this protein was elucidated by studying phage with amber mutations in the coat protein cistron (Lodish et al., 1964; Lodish and Zinder, 1966b). Phage mutants which do not produce the normal amount of coat protein under non-permissive conditions were found to hyperproduce phage replicase activity. Phage replicase synthesis, which normally terminates at about 20 minutes after infection in the wild-type infected cell, continues throughout the infectious cycle in the absence of a normal level of coat protein. This is particularly apparent in cells infected with the sus 11 mutant of f2, which has an amber mutation at site 70 of the coat protein (Webster et al., 1967). In such cells, replicase activity at 60 minutes after infection is 10-20 times higher than the maximal wild-type level. Lodish et al. (1964) further observed that enzyme hyperproduction in such cells was contingent upon the continued synthesis of progeny viral RNA. This suggested that under normal conditions, the replicase is synthesized from the incoming viral RNA and perhaps from early progeny RNA. As viral coat protein accumulates, it serves as a repressor and prevents further synthesis of replicase. In the absence of repressor, as in sus 11-infected cells, replicase synthesis continues unabated.

More recent experiments suggest that the coat protein also regulates the synthesis of viral A protein and, curiously enough, of the coat protein itself. Thus both Viñuela et al. (1968) and Nathans

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footnote continued

When an amber mutant is grown in a permissive host, either the original or some other amino acid (depending on the particular strain) is inserted at the mutated site and synthesis of the protein continues.

§Temperature-sensitive mutations render the virus unable to reproduce at one temperature (e.g., 43°C), while still able to reproduce at another temperature (e.g., 34°C). For a full discussion of these topics, see Hayes, Chapter 18.

et al. (1969) have shown that the A protein is also overproduced late in the infectious cycle in mutants of MS2 lacking functional coat protein. Nathans et al. (1969) and Fromageot and Robertson (personal communication) found some evidence that the kinetics of synthesis differed somewhat for the A protein and the replicase protein, suggesting that the repressor effects of coat protein with respect to the two proteins are not coordinate. The synthesis of A protein levels off at about 30 minutes after infection, while that of replicase protein stops at about 20 minutes after infection.

Recent experiments with MU9, an amber coat mutant of MS2, further suggest that the coat protein regulates its own synthesis (Sugiyama et al., 1969). In a wild-type infected cell, the amount of coat protein synthesized is very large. More than 10 times as much coat protein as any other phage protein is synthesized in the course of infection. The experiments of Sugiyama et al. (1969) suggest that this disproportion is not simply a passive consequence of coat repression of the synthesis of other viral proteins, but that it is actively promoted by the presence of an intact coat having a correct amino acid sequence. In non-permissive bacterial cells, the MU9 mutant can only make a fragment of the coat protein. In permissive cells, the complete coat protein is made, but has different alterations in the amino acid sequence at site 70 depending on the particular permissive strain used. If only the coat fragment is produced, or if the complete protein with an unsatisfactory amino acid at site 70 is produced, the coat is non-functional as a repressor and disproportionately large amounts of coat (or coat fragment) are not produced. Only when the complete protein is made and has the original amino acid at site 70 (or a satisfactory replacement), is the normal high level of coat protein synthesized. Thus the coat protein not only represses the synthesis of A protein, but also promotes a disproportionately large synthesis of the coat protein itself.

The repressor function of coat protein, deduced from the in vivo replication of mutant phage, has been confirmed by in vitro studies. When phage RNA is used as a messenger RNA in a cell-free protein

synthesizing system, all of the phage-specific proteins are synthesized. When a coat protein-RNA complex is used as messenger, the synthesis of non-coat proteins is reduced, while synthesis of coat protein is unaffected (Stavis and August, 1970). It is likely that the *in vitro* studies represent an oversimplification of the *in vivo* situation. Indeed, it has been suggested that repression may operate at a transcriptional, rather than or in addition to the translational level (Robertson *et al.*, 1968). The *in vitro* studies, however, suggest that the regulatory function of coat protein is based on a direct interaction of protein with the phage nucleic acid.

C. Phage RNA Synthesis and Its Control

The replication of phage RNA is also a carefully regulated process and the control mechanisms operating in RNA synthesis are inseparable from those described for phage protein synthesis. A rather oversimplified summary of phage RNA replication is presented in Fig. 1. The first step in RNA replication appears to be the synthesis of a complementary minus strand to the input phage plus strand. The minus strand is then transcribed to yield progeny plus strands. Many more molecules of plus strand RNA than of minus strand RNA are generated during the infectious cycle. Only a few of the newly-synthesized plus strands serve as templates for minus strand synthesis. The net result is that minus strands are used repeatedly for the asymmetric synthesis of plus strands (Weissmann and Ochoa, 1967).

The participation of a complementary or minus strand in the production of progeny single-stranded RNA was deduced from the presence and properties of double-stranded RNAs in phage-infected cells. Early studies on Group I phages showed the existence of both double-stranded and partially double-stranded phage-specific RNAs in infected cells (Erikson and Franklin, 1966; Erikson, 1968). Several lines of evidence suggested that these structures are involved in phage RNA replication (Erikson and Franklin, 1966; Weissmann and Ochoa, 1967). When bacterial cells are infected with phage whose RNA is radioactive, the labeled RNA

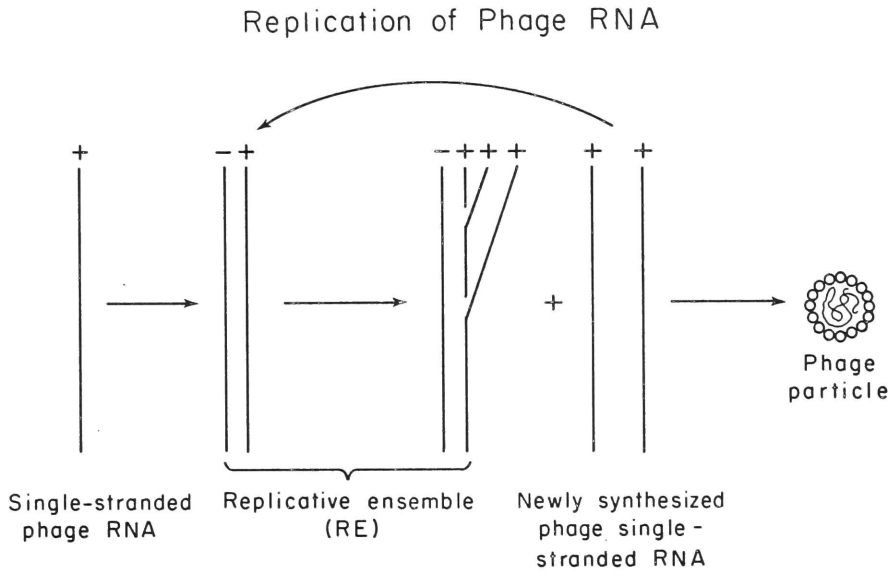
Replication of Phage RNA
+
−+
−+++
+
+
+
Phage particle
Single-stranded phage RNA
Replicative ensemble (RE)
Newly synthesized phage single-stranded RNA

Figure 1

is rapidly converted from ribonuclease-sensitive single-stranded form to RNase-resistant double-stranded form. These double strands, containing labeled parental RNA, have the same physical properties as the double-stranded RNAs extracted from infected cells. Somewhat later in infection, there is a decrease in the labeled parental RNA present in RNase-resistant form. This decrease is thought to correspond to the displacement of parental strands from partially double-stranded replicating structures by newly-synthesized plus strands. Experiments in which nascent RNA is labeled either *in vivo* or *in vitro* (Weissmann and Ochoa, 1967) suggest that label invariably appears first in association with the double-stranded RNAs and only later as free, single-stranded RNA.

The asymmetric nature of phage RNA replication is quite apparent from the relative amounts of phage single- and double-stranded RNAs. Weissmann *et al.* (1964a) showed that only about 8% of the viral RNA made after 45 minutes of infection with MS2 phage was double-stranded. Based on sensitive annealing techniques, it was later shown by Weissmann *et al.* (1968) that up to 30% of the RNA synthesized after infection with MS2 was viral RNA. Of the total viral RNA, however, only about 10% is viral minus strand RNA in a normal infection.

It is generally agreed that phage RNA replication proceeds by complementary strand synthesis, that some kind of association between plus and minus strands is maintained and that phage RNA replication is decidedly asymmetric. Beyond these facts, there is little agreement. Thus, for example, Feix *et al.* (1967) suggest that the intracellular intermediates in RNA replication are not double-stranded at all. Only during phenol extraction, they suggest, do these structures collapse into double-stranded form. Another unresolved issue is whether RNA replication is conservative or semi-conservative. Again, evidence exists for both mechanisms (Stavis and August, 1970) and perhaps the actual mechanism involves both conservative and semi-conservative elements. Another controversy concerns the number of nascent plus strands which can be associated with a single replicating structure.

Granboulan and Franklin (1968) and Robertson and Zinder (1969) have presented evidence which suggests that most replicating structures have a single nascent chain, whose length is, on the average, about 25% of the intact viral RNA. Vandenberghe et al. (1969) have reported that the largest replicating structures isolated from MS2-infected cells can have 10 or more nascent chains attached.

The overall asymmetry of RNA replication strongly suggests the existence of a mechanism controlling the transcription of the two viral strands. Early in vivo experiments on bacteria infected with mutants of f2 and MS2 showed that phage-specific proteins were involved in the regulation of phage RNA synthesis. Thus, for example, Lodish et al. (1964) found that the hyperproduction of phage replicase protein which occurs in the absence of coat protein drastically changes the ratio of plus strand RNA to minus strand RNA. As discussed above, cells infected with the sus 11 mutant produce no complete coat protein and continue to synthesize replicase protein throughout infection. Continued synthesis of replicase results in the accumulation of excessively large amounts of double-stranded RNA, suggesting that extra minus strand RNA is being made under these conditions (Lodish et al., 1964). This observation was later confirmed for a coat mutant of MS2 by Weissmann et al. (1968), who showed that the fraction of phage-specific RNA in minus strands was as high as 40% in such cells, as compared with 10% in cells infected with wild-type phage. Conversely, cells infected with a mutant having a temperature-sensitive replicase protein ceased to produce minus strand RNA (as detected by double-stranded RNA) when shifted to a non-permissive temperature (Lodish and Zinder, 1966a). Since the level of plus strand synthesis appeared to be fairly normal in cells infected with either of these mutant phages, Lodish postulated the existence of two separate enzymes for the replication of phage RNA. One of these was thought to be a phage-specific enzyme, synthesized from phage RNA and necessary only for phage minus strand synthesis, while the other was thought to be a bacterial enzyme responsible for the synthesis of plus strand RNA. By this hypothesis, the overall asymmetry of RNA replication would be determined by the

limited supply of phage-specific enzyme, whose production was, in turn, controlled by the repressor function of the coat protein.

D. In Vitro Properties of Phage RNA Replicases

The purification of Group I replicases was undertaken in a number of laboratories. An initial report by Haruna et al. (1963) suggested that an enzyme capable of specifically utilizing MS2 plus strand RNA template could be isolated from MS2-infected cells. This report was substantiated somewhat later by Fiers et al. (1967). These authors further showed that template-dependent MS2 replicase activity was associated with a large complex having a sedimentation coefficient of about 40 S and containing both protein and RNA. They also reported that while the replicase activity was insensitive to actinomycin, it was extensively inhibited by low concentrations of phosphate ion.

Weissmann and his coworkers (Weissmann and Ochoa, 1967) were unable to obtain a template-dependent replicase preparation from MS2-infected cells. They did, however, substantially purify an enzyme-template complex from such infected cells. They showed that in the presence of labeled ribonucleoside triphosphates, the replicase-template complex synthesizes phage-specific RNAs, as judged by their ability to anneal to MS2 RNAs extracted from infected cells. However, all attempts by these investigators to separate the enzyme from its endogenous RNA template promptly abolished activity (Weissmann et al., 1963).

A phage-specific enzyme with quite different properties from either MS2 preparation was isolated from f2-infected cells by August and his coworkers (August et al., 1965; Shapiro and August, 1965). Their preparation, which was obtained from cells infected with the sus 11 mutant of f2, showed template dependence. However, extremely large amounts of RNA were required as template by the enzyme and it was able to use a variety of bacterial and viral RNAs as template. Analysis of the polynucleotide product made in vitro suggested that the enzyme catalysed the synthesis of complementary copies of the various template RNAs.

Thus the replicases of Group I phage have yielded little to analysis. These enzymes are extremely unstable and template-dependent preparations are difficult to obtain (August *et al.*, 1963; Weissmann *et al.*, 1963; Weissmann and Ochoa, 1967). The properties of the various preparations that have been described are quite different from each other and it is therefore difficult to correlate the results of the *in vitro* studies with those obtained *in vivo*.

Group III replicases are a great deal more stable than Group I replicases and template-dependent preparations can be obtained with relative ease (Stavis and August, 1970; Miyake *et al.*, 1971). The most extensive studies have been carried out on the Qβ replicase. In 1965, Spiegelman *et al.* (1965) reported that partially purified Qβ replicase could not only use Qβ plus strand RNA *in vitro* as a template, but was in fact able to make plus strand RNA in excess of the input, template amount. The newly synthesized RNA was biologically competent: when used to infect spheroplasts, it was capable of supporting the synthesis of progeny phage. This was the first report of *in vitro* replication of a biologically competent nucleic acid. In subsequent studies (Weissmann and Ochoa, 1967; Spiegelman, 1970), it was clearly shown that the *in vitro* Qβ replicase reaction faithfully mirrors the *in vivo* replication of phage RNA. The input template plus strands are first transcribed to yield complementary minus strands, followed by the synthesis of excess plus strands (Feix *et al.*, 1967). The asymmetry of the *in vivo* replication is conserved *in vitro*: regardless of whether plus or minus strands serve as the input template, the product made after prolonged synthesis invariably contains a 10-fold excess of plus over minus strands.

Considerable progress has also been made in analysing the structure of the replicase. The Qβ replicase preparations used in the early studies were not extensively purified. It soon became apparent that the replicase dissociated rather easily into two or more components, all of which were necessary for the replication of phage plus strand RNA. One of these components could be obtained only from infected cells and was capable of using phage minus strands, polyribocytidylic acid or

cytidylate-containing copolymers, but not phage plus strand RNA template. The other component (or components) had no nucleotide-polymerizing activity *per se*, but restored the capacity of the phage component to use phage plus strand RNA template (Stavis and August, 1970). A schematic representation of this organization is shown in Fig. 2. The component which is found only in infected cells is designated as the 'core' replicase or poly G polymerase. The other component(s), which can be purified from uninfected cells, are designated as 'factors'.

The 'core' replicase or poly G polymerase of the Qβ enzyme has recently been obtained in pure form (Kamen, 1970; Kondo *et al.*, 1970). Kamen purified the core replicase by the simple expedient of using the poly C-dependent poly G polymerase activity as a routine assay. Kondo *et al.* (1970) achieved the same result by a somewhat different route. The results of both of these studies show that the Qβ poly G polymerase consists of four proteins, three of which are bacterial and one of which is the phage-coded replicase protein. The approximate molecular weights of the proteins are 75,000, 68,000, 46,000 and 33,000 and they are designated subunits I, II, III, and IV, respectively, by Kamen (Kondo *et al.* adopted a somewhat different subunit designation). At low ionic strengths, the poly G polymerase can be separated into aggregates of subunits I + II and III + IV. This separation is accompanied by a loss of poly G polymerase activity, as well as of activity with Qβ minus strand template (Kamen, 1970). While complete subunit separation and reassembly have not yet been accomplished, some reconstitution of activity is observed when preparations rich in I + II are combined with preparations rich in III + IV. This suggests that at least one of each pair (and probably both) is necessary for activity.

The function of the various subunits, as well as the nature of their association, is not clear. The observed sedimentation constant of the poly G polymerase is about 7 S (Kamen, 1970; Kondo *et al.*, 1970), an anomalously low value for a globular protein with an aggregate molecular weight in excess of 200,000 (assuming that the subunits are present in equimolar quantities). This behavior may simply be attributable

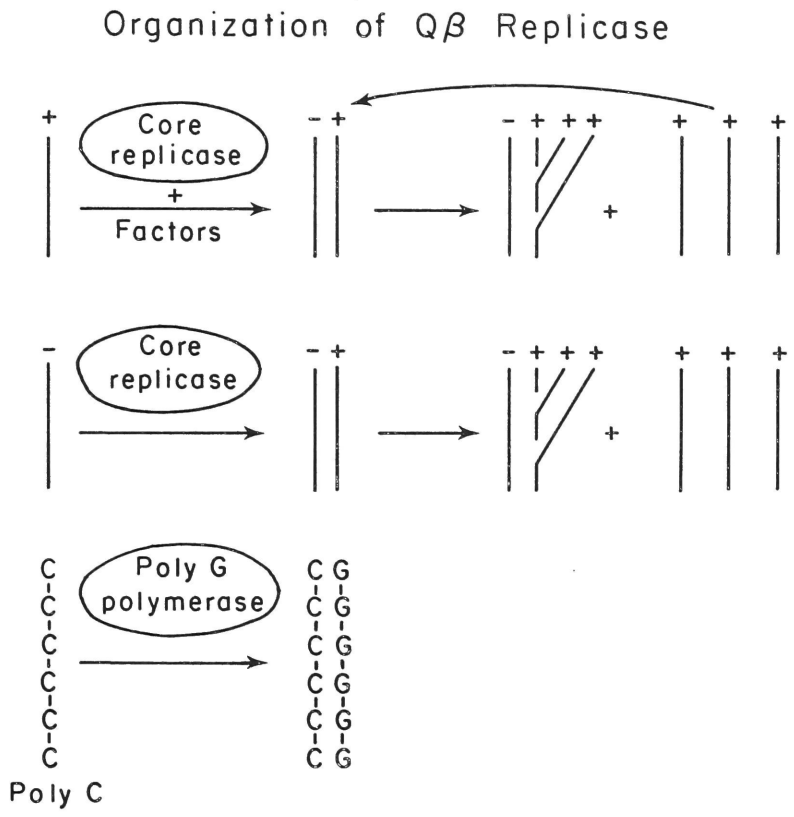
Organization of Qβ Replicase
Core replicase
+
Factors
Core replicase
Poly G polymerase
Poly C
Core replicase = poly G polymerase

Figure 2

to a high degree of asymmetry. However, Kondo et al. (1970) observed that the recovery of different subunits was quite variable and suggest that the subunits may exist in reversible equilibrium. They further observed that when the replicase was incubated with Qβ RNA, followed by isolation of the enzyme-template complex, only the two largest subunits could be recovered from the complex.

It is generally agreed that the 'core' replicase or poly G polymerase requires some additional factors for activity with Qβ plus strand RNA. The nature and number of these factors remains in dispute. Workers in Spiegelman's laboratory have identified a single factor requirement based on density gradient centrifugation studies and designate this as the 'light component' of the enzyme, by contrast to the more rapidly sedimenting poly G polymerase, or 'heavy component' (Eikhom and Spiegelman, 1967; Eikhom et al., 1968). August's group, on the other hand, have identified two factor requirements based on chromatographic separation (Franze de Fernandez et al., 1968; Shapiro et al., 1968). They have designated these as Factors I and II. Both appear to be proteins (August et al., 1969). At least one of these proteins (II) can be replaced by a variety of basic proteins, including ribosomal proteins and histones (Kuo and August, 1971).

Thus the Qβ replicase appears to be quite complex, consisting of a four-polypeptide 'core' replicase and one or two additional protein factors. The mechanism of the reaction, its template specificity and the role of the various bacterial factors are not well understood. The most remarkable features of the replicase reaction are its template specificity and its overall asymmetry. The Qβ replicase discriminates between template RNAs with great fidelity. The only RNAs replicated by the enzyme are Qβ plus and minus strands and some naturally occurring variants of the Qβ RNA (Spiegelman, 1970). Synthetic polymers containing cytidylic acid serve as template for complementary strand synthesis, but do not appear to be replicated. Banerjee et al. (1969) have suggested that a small, naturally occurring 6 S RNA may also be replicated.

No satisfactory hypothesis has yet been advanced to explain the asymmetry of the reaction with Qβ plus strand template. Feix et al. (1968) proposed some years ago that the enzyme had different binding sites for plus and minus strands and that the affinity for minus strands was higher than the affinity for plus strands. However, the subsequent discovery of a requirement for bacterial factors casts this interpretation in doubt. August et al. (1968) reported that differential utilization of plus and minus strand templates could be observed only in a 'factor'-deficient system. When 'factors' are present in saturating amounts, the replicase uses plus and minus strand templates about equally well. The observation that the final ratio of plus to minus strands is about 10:1 both in vivo and in vitro remains to be explained.

Another poorly understood aspect of the replicase is the role played by the bacterial 'factors' in stimulating 'core' replicase activity with plus strand template. 'Core' replicase alone will bind to Qβ plus strand RNA (August, 1969; August et al., 1969) and the presence of factors does not appear to influence simple binding. Nonetheless, no nucleotide polymerization in response to plus strand template occurs in the absence of the factors.

It has been reported that partially purified Qβ replicase preparations support the template-independent replication of some small variant RNAs. Banerjee et al. (1969) found that their enzyme preparations replicate a 6 S RNA molecule which is present in the enzyme preparation itself. They suggest that this small RNA is similar to a 6 S RNA frequently observed in infected cells. The origin and mode of replication of this molecule are somewhat obscure. It does not appear to be derived from Qβ RNA and has properties characteristic both of single- and of double-stranded RNA. Optimal template activity of this small RNA is obtained only after it is heat denatured (Prives, 1971). The significance of this reaction is unclear. Spiegelman's group has reported that a rapidly replicating RNA can be derived from Qβ RNA by application of various selective pressures (Spiegelman, 1970). These molecules, or at least some of them, appear to be derived

from the parent Qβ RNA as judged by the presence of sequences homologous or identical to those in Qβ RNAs. Recently this group has also observed the template-independent replication of small RNAs by partially purified Qβ replicase preparations (Kramer, personal communication). These RNAs, unlike the original variants, do not appear to be related to Qβ RNA. The size of the replicated variant varies from enzyme preparation to enzyme preparation. Again, the significance of these RNAs and their origin remain obscure.

E. Isolation of the f2 Replicase

In attempting once again to isolate and study a Group I replicase, we have exploited much of the knowledge gained from studies of the Qβ replicase. Our basic assumption was that since Group I and Group III replicases carry out very similar reactions *in vivo*, the enzymes probably share a fundamentally similar organization. Specifically, we assumed that the f2 replicase could be subdivided into a 'core' replicase and various bacterial components. We felt that perhaps some of the difficulties encountered by previous investigators were attributable to the stringency of their assays, in which the ability to use homologous plus strand RNA template had invariably been used as the criterion of replicase activity. Studies on the Qβ enzyme had demonstrated that enzyme activity with plus strands requires a more complex entity than replicase activity with minus strands or with synthetic polymers. Kamen (1970) had amply demonstrated the usefulness of a simple synthetic polymer assay in purifying the Qβ 'core' replicase. Thus our first project was simply to establish whether the f2 replicase could be monitored in an analogous way. If we could establish the existence of a phage-induced synthetic polymer activity, we felt that we stood a good chance of obtaining at least some part of the original f2 replicase complex. The first section of experimental results describes the detection, purification and structure of the poly G polymerase produced during infection by the f2 bacteriophage (Chapter IV).

Chapter V concerns the properties of the f2 enzyme after partial purification. The enzyme is shown to be highly template dependent and able to polymerize nucleotides in the presence of f2 plus and minus strand RNAs, but not in response to other bacterial and viral RNAS. By annealing techniques, the in vitro replicase product has been found to consist entirely of f2 RNAs. It is concluded from the strand composition of the product that the f2 replicase is able to carry out all the component reactions of RNA replication, despite its inability to make an amount of product RNA in excess of the input template amount. The replicase and poly G polymerase activities of the f2 enzyme preparation are compared with respect to a number of parameters. Some comparative studies on the Qβ and f2 enzymes are also described. The results of these studies are evaluated in relation to the properties of other ribonucleotide polymerizing enzymes.

The relationship between the f2 replicase and poly G polymerase activities is considered in Chapter VI. It is demonstrated that the poly G polymerase is necessary, but not sufficient, for replicase activity. It has been found that some as yet unidentified factors, necessary for replicase activity with either plus or minus strand template, dissociate from the poly G polymerase during purification. Chapter VI concludes with a discussion of the results obtained in the course of this study.

CHAPTER II

MATERIALS AND METHODS

A. Chemical Materials

1. Radioactive materials. Tritiated ribonucleoside diphosphates and ribonucleoside triphosphates were obtained from Schwarz/Mann. [^{14}C]-labeled amino acids (lysine, leucine, phenylalanine, arginine) were purchased from New England Nuclear, as were [^{14}C]uracil and [^{3}H]uracil. [^{3}H]polyribocytidylic acid was obtained from Miles Laboratories.

2. Chromatographic media. CF 11 cellulose powder, PC 11 phosphocellulose and DE 52 DEAE cellulose were Whatman products. Dextran T500 was purchased from Pharmacia.

3. General chemicals and reagents. Ribonucleoside diphosphates and ribonucleoside triphosphates were purchased from Miles Laboratories and P-L Biochemicals. Polyribocytidylic acid was obtained from P-L Biochemicals and polyribouridylic acid, from Miles Laboratories. Amino acids, thiamine HCl, dithiothreitol and β-mercaptoethanol were from CalBiochem. Enzyme grade $(NH_4)_2SO_4$, RNase-free sucrose, and Coomassie Brilliant Blue were obtained from Schwarz/Mann. Acrylamide and N,N'-methylenebisacrylamide were Canalco products. N,N,N',N',-tetramethyl-ethylenediamine (TEMED) and diethyl pyrocarbonate were obtained from Eastman Organic Chemicals. Photoflo was purchased from Eastman Kodak. Polyethylene glycol (Carbowax 6000) came from Union Carbide, and Brij 58 from Atlas Chemical Industries. NCS solubilizer was from Amersham-Searle. Common laboratory reagents (e.g., glycerol, EDTA) were generally Mallinckrodt products.

4. Growth media. Enriched tryptone medium contained 32 g of tryptone, 20 g of yeast extract and 5 g of NaCl per liter of medium.

Minimal medium, used for preparation of radioactive phage, phage RNA and phage proteins, was the MTPA medium described by Viñuela *et al.*

(1967). It contained 0.1 M Tris-HCl (pH 7.5), 8.5 mM NaCl, 0.1 M KCl, 0.02 M NH_4Cl, 0.34 mM KH_2PO_4, 0.16 mM Na_2SO_4, 2.5 mM $CaCl_2$, 2.5 mM $MgCl_2$, 0.01 M glucose, twenty L-amino acids (0.1 mM each) and 10 μg/ml thiamine HCl.

5. Buffers. Several standard buffers were used extensively. These are defined below and will be referred to hereafter by the indicated abbreviations.

Buffers used in purification and analysis of RNAs:

TSE: 0.05 M Tris-HCl (pH 6.85), 0.1 M NaCl, 0.001 M EDTA.

35% ethanol-TSE: 0.65 volume TSE + 0.35 volume absolute ethanol.

15% ethanol-TSE: 0.85 volume TSE + 0.15 volume absolute ethanol.

10xTSE: 0.5 M Tris-HCl (pH 6.85), 1.0 M NaCl, 0.01 M EDTA.

4xTSE: 0.2 M Tris-HCl (pH 6.85), 0.4 M NaCl, 0.004 M EDTA.

1.5xTSE: 0.075 M Tris-HCl (pH 6.85), 0.15 M NaCl, 0.0015 M EDTA.

Buffers used in enzyme purification and storage:

SB: Standard buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA and 0.1 mM dithiothreitol.

SB4: SB containing 4% v/v of glycerol.

SB20: SB containing 20% w/v of glycerol.

SB50: SB containing 50% v/v of glycerol.

SB4Mg, SB20Mg, and SB50Mg: the above standard buffers containing 5 mM magnesium acetate.

B. Biological Materials

1. Enzymes. DPFF DNase I was purchased from Worthington Biochemical Corp. Pyruvate kinase (rabbit muscle) and aldolase were obtained from CalBiochem. Catalase was purchased from Mann Research Laboratories; bovine hemoglobin, from Pentex, Inc.; pancreatic ribonuclease, from Sigma Chemical Co.; lysozyme, from Reheis Chemical Co.; and bovine serum albumin, from General Biochemicals. RNA polymerase (E. coli) was the

kind gift of Dr. T.-S. Chan.

2. Bacterial strains. The E. coli strains used as host bacteria for f2 replicase isolation were K38 (su^-) or Q13 ($su^-RNase^-PNPase^-$). E. coli Q13 was used for Qβ replicase preparations. E. coli K37 (su^+) was used for growth of phage f2 and Qβ amber mutants.

3. Phage. The f2 amber mutant sus 11 was used as infecting phage for f2 replicase preparations and for isolation of in vivo [^{14}C]labeled replicase protein. This mutant has amber mutations in both the coat and A protein cistrons of the phage genome (Zinder and Cooper, 1964; Horiuchi et al., 1966). Wild-type f2 and Qβ phage were used for obtaining various phage RNAs. The Qβ am 12 mutant, having an amber mutation in the coat protein cistron, was used as infecting phage for the isolation of Qβ replicase (Horiuchi and Matsuhashi, 1970).

4. Miscellaneous. REOvirus-infected mouse L2 cells were the gift of Dr. S. Silverstein. Single-stranded Qβ RNA was provided by Drs. K. Horiuchi and W. Mangel. Single-stranded f1 phage DNA was the gift of Dr. K. Horiuchi. E. coli ribosomes were provided by Dr. P. Model. Rifampicin was kindly supplied by Dr. J. Gelzer of Ciba Pharmaceutical Co.

C. Preparative Methods

1. Preparation of chromatographic media.

DEAE cellulose. Whatman DE 52 DEAE cellulose was prepared for use by precycling through acid and base. Fine particles were first removed by suspending the DEAE cellulose in distilled water, allowing it to settle to the desired extent and decanting the unsettled fine particles. The slurry was then suspended in 0.5 M NaOH and stirred for 30 min. NaOH was removed by filtration through a funnel having a sintered glass disc bottom, followed by extensive washing with distilled water. The cellulose was stirred for 30 min with 0.5 M HCl, which was then removed by filtration and washing. The washed cellulose was suspended in 2 vol of distilled water, adjusted to pH 4 with concentrated HCl and degassed under vacuum for 30 min. The washed, degassed slurry

was suspended in 50 mM Tris-HCl (pH 7.5) and stored in the cold.

RNA cellulose. Purified single-stranded f2 RNA in 35% ethanol-TSE buffer was mixed with 75-150 mg of ethanol-washed CF 11 cellulose/mg RNA. The slurry was allowed to stand for 1 hr at room temperature, diluted 50-fold with cold absolute ethanol, filtered under suction through Whatman #3 paper and allowed to dry. The powder was suspended in 20 ml absolute ethanol per 500 mg cellulose, made 10 mM in magnesium acetate, and UV-irradiated for 30 min as described by Litman (1968). The ethanol was removed by filtration and the powder was dried at room temperature. Unbound RNA was removed by washing with cold TSE buffer. The amount of RNA bound to the cellulose was determined by the orcinol reaction (Schneider, 1957). The orcinol reaction was carried out on washed and unwashed RNA-cellulose, as well as on unreacted cellulose, since the cellulose itself gave a significant color reaction. The procedure described above bound 75-80% of the input RNA to the cellulose, giving preparations containing 4-12 mg of bound f2 RNA per gram of cellulose.

2. Colorimetric determination of protein.

Protein concentrations were determined as described by Lowry et al. (1951), using bovine serum albumin as a standard.

3. Phage.

Wild-type phage. Enriched broth cultures of E. coli K38 were grown to a density of 1 x 10^9/ml, supplemented with 2.5 mM $CaCl_2$, and infected with f2 phage at a multiplicity of 1-5. Incubation at 37° C was continued to lysis (3-4 hr); when necessary, 5 mM EDTA and 25-100 µg/ml lysozyme were added to complete lysis. Phage were then purified from 3-10 liter lysates by ammonium sulfate precipitation and CsCl equilibrium centrifugation, as described by Webster et al. (1967).

Stocks of wild-type f2 and Qβ phage to be used in the preparation of phage replicating RNAs were prepared in the same way, but not purified after lysis. Lysates were simply clarified by centrifugation, titrated and stored at 4° C.

Phage amber mutants. Stocks of the f2 mutant sus 11 and the Qβ mutant am 12 were made from plate scrapes. Single plaques of either mutant plated on K37 were picked into 1 ml of a solution containing 50 mM Tris-HCl (pH 7.5), 2 mM EDTA and 10 μg/ml lysozyme. The stocks giving the highest titer and lowest reversion rate were plated at 5×10^5 pfu per plate on K37 and incubated 8-10 hours at 37°C. The top agar containing the bacteria and phage was scraped from 40-60 plates into 100-200 ml of 50 mM Tris-HCl (pH 7.5), 5 mM EDTA and 10 μg/ml lysozyme. The suspension was allowed to stand for several hours at room temperature and centrifuged for 15 min at 9000 rpm. The resulting supernatant was used to infect cells for enzyme extraction.

Radioactive phage. To prepare tritiated or [^{14}C]uracil-labeled wild-type phage, K38 bacteria were grown in minimal medium (MTPA) to a density of 2×10^8/ml and infected at a multiplicity of 5-10. [^{3}H]uracil (20 Ci/mmole, 2 μCi/ml) or [^{14}C]uracil (52 mCi/mmole, 1-2 μCi/ml) was added at 5 min after infection and the cultures were allowed to go to lysis. The labeled phage was then harvested and purified through CsCl equilibrium centrifugation as described by Webster et al. (1967).

4. Ribonucleic acids.

Single-stranded phage RNA. To extract single-stranded RNA from purified f2, the phage was suspended in TSE buffer at a concentration of 3 mg/ml and gently shaken for 3 min at 0-4° C with an equal volume of phenol saturated with the same buffer. The phases were separated by brief low-speed centrifugation and the phenol phase was re-extracted with 1/2 of the original volume of cold buffer. The aqueous phases were then combined and re-extracted with 1/2 vol of buffer-saturated phenol. The combined aqueous phases were made 0.1 M in potassium acetate and the RNA was precipitated by addition of 2 volumes of cold, absolute ethanol and storage for several hours or overnight at -13° C. The precipitate was collected by centrifugation at 10,000 rpm for 20 min, dissolved in a small volume of either distilled water or TSE buffer and dialysed in a thin-film dialyser to remove traces of phenol.

The integrity of the RNA was checked by centrifuging approximately 0.05 mg of RNA through a 2 ml 5-20% sucrose gradient, using 27 S f1 DNA as a sedimentation marker.

Partially double-stranded phage replicating RNAs. The partially double-stranded RNAs produced during infection of bacteria by either f2 or Qβ phage were purified from infected K38 for f2 and infected Q13 for Qβ. Bacteria were grown in enriched broth medium to a density of 1.0-1.5 x 10^9/ml and infected at a multiplicity of 10. The cells were harvested at 35-40 min after infection by pouring the cultures rapidly over frozen, crushed growth medium diluted 1:1 with saline solution (physiological). The chilled cultures were then centrifuged for 10 min at 6000 rpm. All subsequent operations were carried out at 4° C or on ice to minimize ribonuclease activity. The cell pellets from 1-2 liters of culture were resuspended in 50-100 ml of 0.1 M Tris-HCl (pH 7.4) containing 20% sucrose (TS) and centrifuged for 10 min at 6000 rpm. The pellet was taken up in 67.5 ml TS and to it was added 7.5 ml of a solution containing 5 mg/ml lysozyme and 10 mM EDTA in TS. Lysozyme treatment was terminated after 8 min at 0° C by addition of 8 ml 0.1 M $MgSO_4$ in TS and the resulting spheroplast suspension was centrifuged for 10 min at 4500 rpm. The spheroplast pellet was suspended in 30 ml of 0.005 M Tris-HCl (pH 7.4), 0.01 M $MgSO_4$, and 0.06 M NaCl (TMN). To this suspension, 3.75 ml 10 μg/ml DNase (RNase-free) was added and incubation continued for 3 min at 0° C, whereupon 3.8 ml of 6% Brij 58 in TMN was added. The suspension was kept at 0° C for an additional 5-6 min with gentle stirring and then diluted with 30 ml TMN. RNA was then extracted with 1 vol of TMN-saturated phenol as described above, followed by ethanol precipitation and dialysis to remove residual phenol. This method of cell lysis, which represents a slight modification of the method described by Phillips et al. (1969) for the preparation of polysomes, was used because it was found to yield partially double-stranded RNAs of higher sedimentation rate and lower RNase-resistance than other cell lysis methods involving freezing and thawing or detergent treatment of the cells.

The partially double-stranded phage RNAs were then separated from single-stranded cellular and phage RNAs by chromatography on CF 11 cellulose as described by Robertson and Zinder (1969). The total RNA from about 1.5 liters of infected cells, dissolved in 35% ethanol-TSE, was applied to a 5 x 60 cm CF 11 cellulose column equilibrated with the same buffer. The column was washed with several volumes of 35% ethanol-TSE to remove residual DNA, then with several volumes of 15% ethanol-TSE. The 15% ethanol-TSE buffer elutes most of the single-stranded RNAs present in the preparation. Double-stranded and partially double-stranded RNAs are then eluted with TSE buffer alone. Residual single-stranded RNA was removed from the preparation by passage through a second, smaller (2 x 20 cm) column of CF 11 cellulose as described above. The TSE buffer eluate of the second column was adjusted to 0.1 M potassium acetate and precipitated with cold absolute ethanol as described above. This procedure yields RNA having a sedimentation coefficient of 15-18 S and a ribonuclease resistance of 65-75%. This preparation has been designated as the 'replicative ensemble' or RE (Engelhardt et al., 1968) and will hereafter be referred to as such. RE contains both phage plus and phage minus strand RNAs. The minus strand content of f2 RE is about 1/3, as determined by saturation annealing or isotope dilution methods, to be described below. Because of its fragility, RE was stored as a precipitate in 66% ethanol at -13° C until used.

RE was obtained from Qβ-infected cells by the same method. This preparation is referred to as Qβ RE.

REOvirus RNA. Double-stranded REOvirus RNA was purified from infected mouse L2 cells as described by Schonberg et al. (1971), followed by CF 11 cellulose chromatography as described above.

Heat denaturation of REOvirus RNA and RE. Partially or completely double-stranded RNAs were converted to single-stranded form by heat denaturation as described by Weissmann et al. (1967). The RNA was dissolved in 0.5 mM EDTA (pH 7.5) and adjusted to a concentration of 200-400 μg/ml. The RNA was then dialysed against 0.5 mM EDTA (pH 7.5) to

remove residual salt. The RNA was denatured at 100° C for 1-3 min. Renaturation was prevented by rapid chilling for 5 sec in a dry ice-ethanol bath. This procedure increased the RNase-sensitivity of RE from about 30% to more than 95%. After denaturation, about 50% of the RNA sediments as intact single-stranded phage RNA (27 S). The sedimentation characteristics of f2 RE before and after heat denaturation are illustrated in Fig. 3. The 27 S peak of the denatured RE contains both plus and minus strand RNAs, as determined by annealing. The material sedimenting behind the peak represents undenatured RE, nascent plus and minus strands, and RNA broken during denaturation.

E. coli RNA. E. coli total cellular RNA was extracted from cells grown to saturation. The cells were harvested by centrifugation, suspended in 0.2 M phosphate buffer (pH 7.0) and lysed by addition of sodium dodecyl sulfate (SDS) to 2%. RNase activity was inhibited by addition of diethyl pyrocarbonate to saturation. The lysate was phenol-extracted as described above and precipitated with ethanol. Cellular DNA was digested with RNase-free DNase (10 μg/ml) at room temperature and the phenol extraction and ethanol precipitation steps were repeated. E. coli ribosomal RNA was purified by phenol extraction from E. coli ribosomes, which were the gift of Dr. P. Model.

In vitro replicase product. The acid-insoluble polynucleotide produce ([^{3}H]labeled) synthesized in vitro by the f2 replicase was prepared for further analysis by phenol extraction and CF 11 cellulose chromatography. The replicase reaction was stopped at the desired time by addition of 1-2 vol of cold TSE buffer. The replicase product was then separated from proteins by phenol extraction as described above. The pooled aqueous phases were dialysed against several changes of TSE, using a thin-film dialyser. The solution was adjusted to 35% ethanol and applied to an 0.5-1.0 ml column of CF 11 cellulose equilibrated with 35% ethanol-TSE. The column was washed with 10-15 ml of 35% ethanol-TSE and all adsorbed RNA was then eluted with TSE buffer alone. This procedure rapidly and efficiently removes residual, acid-soluble, labeled nucleoside triphosphates. Acid-insoluble replicase product free

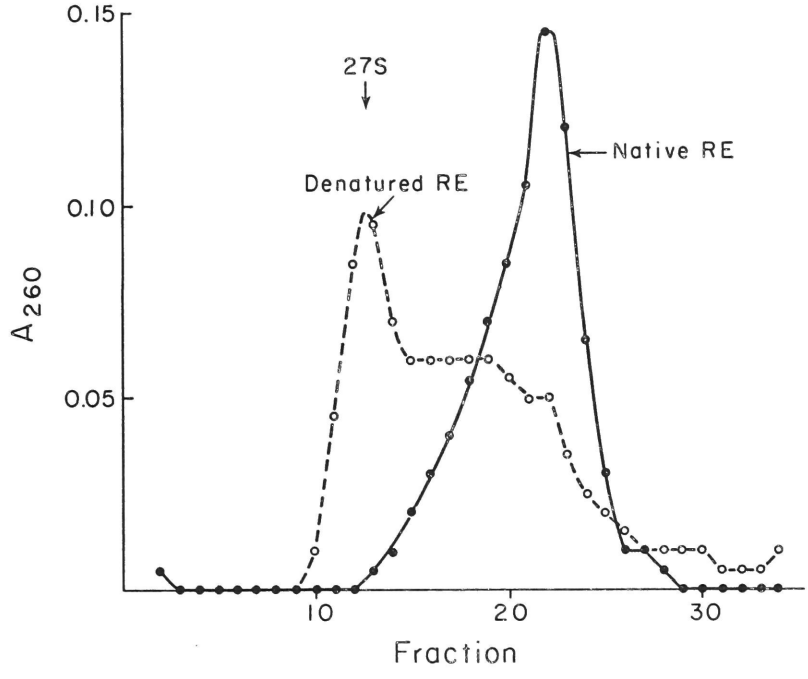

Figure 3. Sedimentation behavior of f2 RE

Partially double-stranded f2 replicating RNAs (RE) were purified from f2 infected cells by cellulose-ethanol chromatography as described in the text. RE was denatured by heating in 0.5 mM EDTA (pH 7.5) to 100° C for 2 min and chilling in a dry ice-ethanol bath for 5 sec. Samples containing approximately 50 µg of native or denatured RE in 0.1 ml H_2O were layered on 2 ml 5-20% sucrose gradients in quartz tubes and centrifuged as described in the text. Absorbance at 260 nm was read directly along the length of the tube. Single-stranded f1 DNA (27 S) was used as a sedimentation marker.

of soluble label and proteins was recovered in 70-90% yield. The salt concentration of the TSE eluate was reduced by dialysis against 0.5 mM EDTA (pH 7.5). When necessary, the volume was reduced by ethanol precipitation or flash evaporation.

5. [^{14}C]labeled phage replicase protein.

E. coli K38 was grown to a density of 2 x 10^8/ml in MTPA medium and infected with sus 11 at a multiplicity of 5. A parallel uninfected culture was used as a control. Rifampicin (100 μg/ml) was added to both aliquots of cells at 20 min after infection to inhibit bacterial RNA and protein synthesis. Proteins were labeled by adding 0.2 μCi/ml each of [^{14}C]labeled lysine, arginine, leucine and phenylalanine at 30 min after infection and continuing incubation until 60 min after infection. Both cultures were lysed at 60 min after infection and labeled proteins were extracted as described by Viñuela et al. (1967). The labeled proteins were then subjected to polyacrylamide gel electrophoresis as described below.

D. Analytical Methods

1. SDS-polyacrylamide gel electrophoresis. Proteins were analysed by gel electrophoresis according to the method of Weber and Osborn (1969). Proteins to be analysed were dissolved in or dialysed against a solution consisting of 0.01 M sodium phosphate (pH 7.2), 0.1% SDS, 0.1% β-mercaptoethanol and 20% glycerol. An aliquot of the protein solution (0.1-0.2 ml) was layered on an 8 cm gel (10% acrylamide, 0.3% bis-acrylamide) polymerized in a 0.6 x 9 cm glass tube treated with a 1:200 dilution of Photoflo. Bromphenol blue (.001%) was used as a dye marker. Electrophoresis was done as described by Weber and Osborn (1969); the run was stopped when the dye marker had traveled approximately 7.5 cm. The gels were removed from the glass tubes and stained overnight in 10 ml of a solution containing 50% methanol, 7.5% acetic acid and 0.25% Coomassie Brilliant Blue. The gels were destained by diffusion in 50% methanol-7.5% acetic acid solution. The destained gels were transferred to 5% methanol-7.5% acetic acid solution and either photo-

graphed directly or traced at 600 nm in a Beckman Spectrophotometer equipped with a linear transport.

Gels containing radioactive proteins were frozen on dry ice immediately after electrophoresis. The frozen gels were then sliced (using a gel slicer) into 1 mm discs. Each disc was placed in a liquid scintillation vial and swollen for 1 hr in 50 µl of distilled water at room temperature. NCS (0.2 ml) was then added to each vial and the vials allowed to stand for 2 hr, after which an additional 0.3 ml NCS was added to each vial. The vials were capped and incubated overnight at 37°. Toluene-based liquid scintillation fluid (15 ml) was added to each vial and the vials were counted in a Nuclear Chicago liquid scintillation counter.

When radioactive and non-radioactive proteins were analysed on the same gel, the gels were first stained and traced, then frozen and sliced. Acetic acid was removed from the slices by drying them thoroughly under a heat lamp on Whatman #1 filter paper. The slices were then swollen in 75-100 µl distilled water and prepared for counting as described above.

2. Density gradient centrifugation. Routine checks on the integrity of template RNAs were carried out in 2 ml sucrose gradients. Approximately 0.1 OD unit of the RNA in H_2O was applied to a 2 ml 5-20% sucrose gradient in 1 M NaCl and 0.1 M phosphate (pH 7.0). The gradients were centrifuged at room temperature for 3.5 hr at 35,000 rpm in the SW 50 rotor of the Beckman L2-65B ultracentrifuge. The absorbance at 260 nm was then read directly along the length of the quartz tube in a Beckman spectrophotometer. F1 single-stranded phage DNA (27 S) was used as a sedimentation marker. The direction of sedimentation is from right to left for all gradients (i.e., fraction 1 ≡ bottom of tube).

In vitro replicase product was analysed by sedimentation through 7-25% glycerol gradients in TSE. Purified replicase product in 0.1-0.2 ml H_2O or TSE was layered on 4 ml gradients and centrifuged for 4 hr at 4° C in the SW 56 rotor of the Beckman L2-65B ultracentrifuge at 50,000

rpm. Fractions of about 0.15 ml (7 drops) were collected from the bottom of the tube. [^{14}C]labeled RNA preparations containing 27 S f2 phage RNA, 23 and 16 S E. coli ribosomal RNA and some 4 S E. coli tRNA were used as sedimentation marker in a separate tube.

3. Annealing. RNA-RNA hybridization (annealing) was carried out by a modification of the method described by Weissmann et al. (1964a). Small aliquots of RE (0.1-1.0 μg) in 0.12 ml 0.5 mM EDTA (pH 7.5) were denatured by heating for 2-3 min at 100° C in 10 x 75 mm siliconized glass tubes. The tubes were supplemented with competing RNA (0.1-10 μg), transferred to an 85° water bath and adjusted to 4xTSE by the addition of 80 μl of 10xTSE. The tubes were kept at 85° for 2 hr and then cooled slowly to room temperature over a 2 hr period. The salt concentration was then adjusted to 1.5xTSE in a final volume of 1 ml. Ribonuclease (1-2 μg) was added to each tube and the tubes were incubated for 10 min at 37°. The digestion was stopped with 1 ml 10% trichloroacetic acid (TCA). Precipitated RNA was collected on Millipore filters. The filters were washed 4 times with 5 ml aliquots of 5% TCA, dried and counted. Control tubes were carried throughout the procedure, but not exposed to RNase digestion.

Fig. 4 shows the application of this method to a standard sample of [^{14}C]RE. To prepare [^{14}C]labeled RE, cells were infected and labeled as described in section C3 for the preparation of labeled phage, except that the infected cells were harvested at 40 min after infection by pouring the cultures over frozen, crushed growth medium. RE was then purified as described in Methods C4. When checked by sedimentation, the [^{14}C]RE (native and denatured) had the sedimentation properties shown in Fig. 3. Native [^{14}C]RE was about 70% RNase-resistant and had a specific activity of 4 x 10^3 cpm/μg. Each annealing tube contained 0.2 μg [^{14}C]RE and the indicated amount of unlabeled RNA (f2 plus strand, E. coli, or Qβ plus strand). Control tubes containing only RE were subjected to all manipulations except RNase digestion and showed no loss of acid-precipitable radioactivity. The use of RE, which contains an excess of plus over minus strands, assures virtually complete

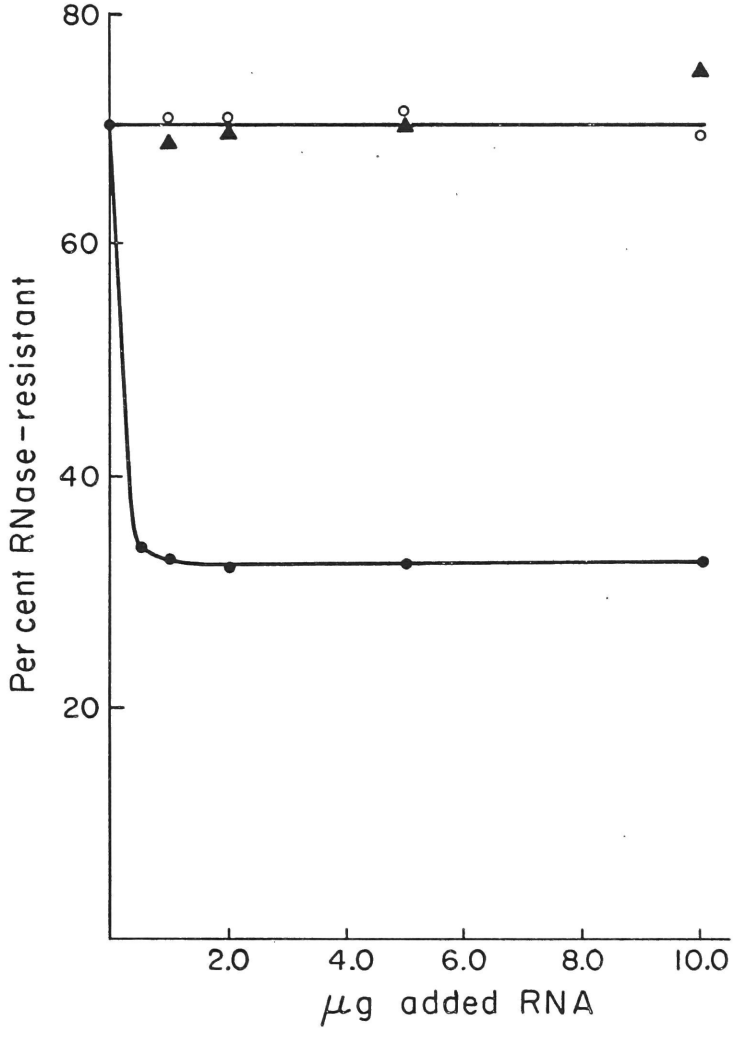

Figure 4. Reannealing of [^{14}C]f2 RE in the presence of unlabeled competing RNAs

[^{14}C]f2 RE (0.2 μg/tube, sp. act. 4 x 10^3 cpm/μg) was denatured and reannealed in the presence of increasing amounts of unlabeled competing RNAs as described in the text. Results are expressed as the fraction of the input radioactivity remaining RNase-resistant after reannealing and ribonuclease digestion. Unlabeled competing RNA: f2 plus strand RNA, ●——●; E. coli cellular RNA, ▲——▲; and Qβ plus strand RNA, o——o.

reannealing of minus strands (Billeter et al., 1966a). This is shown by the quantitative recovery of the initial 70% RNase-resistance after heating and reannealing of the RE in the absence of added RNA (Fig. 4). The specificity of the reaction is illustrated by reannealing in the presence of unlabeled RNAs. Neither E. coli nor Qβ plus strand RNA have any effect on the recovery of RNase-resistant radioactivity, even when added in 50-fold excess. Unlabeled f2 plus strand RNA, on the other hand, dilutes the population of labeled f2 plus strands and thereby displaces them from RNase-resistant form. Since only half of the radioactivity in each RNase-resistant duplex is in plus strands, only half of the radioactivity is displaced from double-stranded form. The displacement of half of the radioactivity by added unlabeled plus strand RNA further shows that the annealing is quantitative in the absence of added, unlabeled RNA. Robertson and Zinder (1969) have reported that almost all of the RNase-sensitive material in native RE is plus strand RNA. It can therefore be concluded that the overall composition of the [^{14}C]RE is 1/3 minus strand RNA and 2/3 plus strand RNA.

In view of the quantitative reannealing of minus strands in the presence of excess plus strand RNA, a simple isotope dilution test was devised to determine the composition of unlabeled RE. A constant amount of unlabeled RE is denatured and reannealed with a negligibly small amount of highly labeled f2 plus strand RNA, in the absence or in the presence of increasing amounts of unlabeled plus strand RNA. The composition of the unlabeled RE can then be evaluated from the resulting dilution curve.

Using the basic equilibrium statements for the annealing reaction developed by Weissmann et al. (1964a), the present annealing reaction can be represented as follows:

$$([m] - [d])([a] + [p^*] - [d]) = k[d] \qquad (1)$$

where m = unlabeled minus strands, a = added, unlabeled plus strands, p* = plus strands labeled by the introduction of a negligibly small quantity of highly labeled plus strand RNA, d = double-stranded RNA

and k is the equilibrium constant. Restating equation 1 we obtain:

$$[d] = \frac{[m]([a] + [p^*])}{k + [a] + [p^*] + [m] - [d]} \qquad (2)$$

It can be seen from the lower curve in Fig. 4 that the value of [d] at [a] = 0 is equal to the value of [m] at [a] >> [p*]. This shows that the reaction goes essentially to completion even at the modest excess of plus strand RNA present in RE. Thus under the present conditions, k must be very small and can be neglected. In addition, since [m] ≅ [d], [m] - [d] ≅ 0. Equation 2 therefore becomes:

$$[d] = \frac{[m]([a] + [p^*])}{[a] + [p^*]} \qquad (3)$$

The experimental parameter measured in the present experiments is the fraction (f) of the radioactivity originally in plus strands (p*) which is RNase-resistant or double-stranded (d) after reannealing or:

$$f = \frac{[d]}{[a] + [p^*]} = \frac{[m]}{[a] + [p^*]} \qquad (4)$$

$$\text{then:} \quad \frac{1}{f} = \frac{[p^*]}{[m]} + \frac{1}{[m]}[a] \qquad (5)$$

A plot of 1/f vs. [a] is linear and has a slope of 1/[m], a Y-intercept of [p*]/[m] and an X-intercept of -[p*]. Given a constant volume, the amounts of plus and minus strand RNA in a given sample of unlabeled RE can be directly measured with reference only to the amount of added plus strand RNA. Plots of f vs. [a] and 1/f vs. [a] for a hypothetical sample of RE containing 0.1 μg minus strand RNA and 0.2 μg plus strand RNA are shown in Fig. 5a and 5b. Knowing [p] and [m] for a given preparation of RE, one can calculate f for any [a].

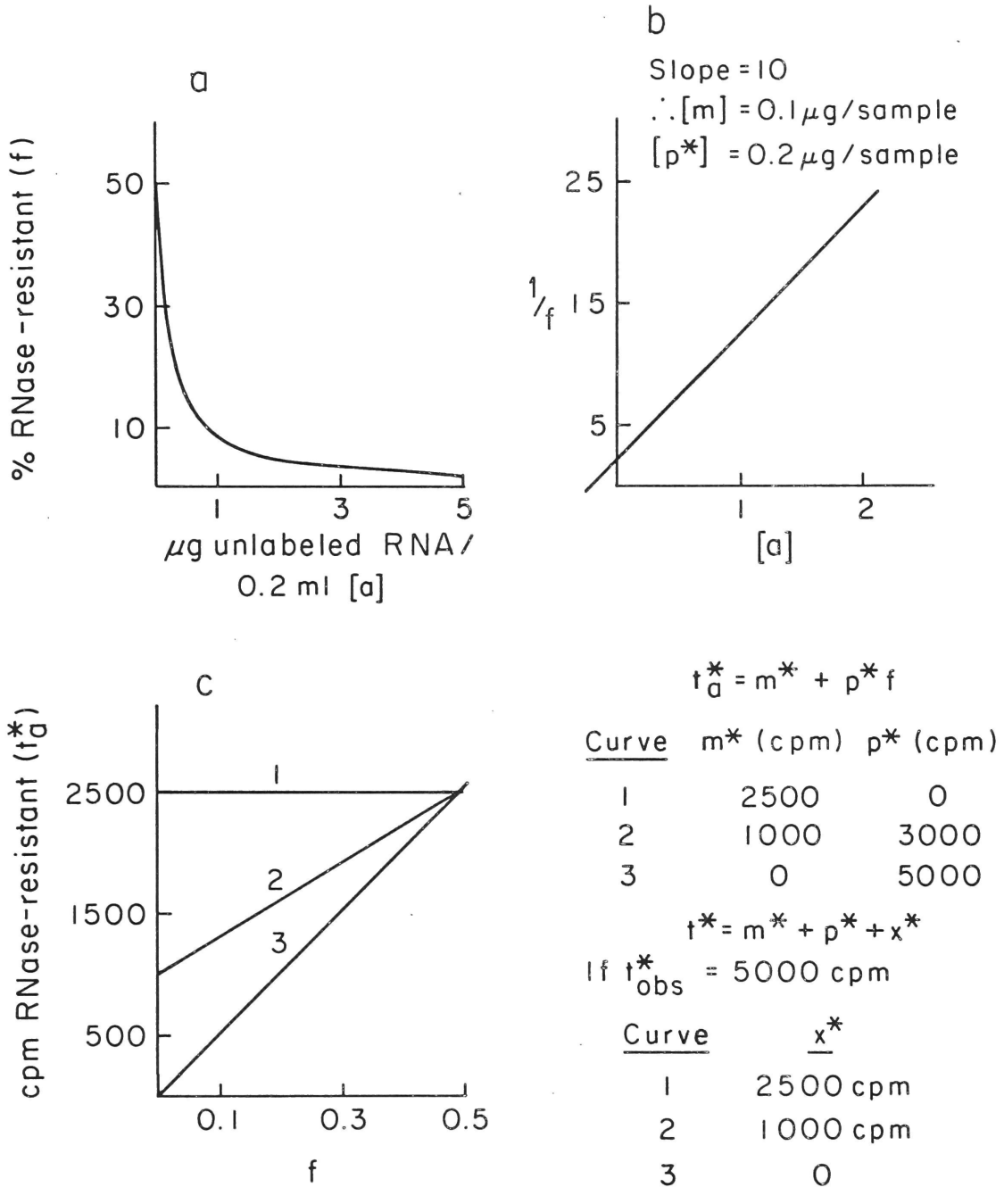

Figure 5. Sample curves and calculations used in analysing isotope dilution data

Parts a and b: determination of the composition of a hypothetical sample of RE containing 0.1 μg minus strand and 0.2 μg plus strand RNA. Part c: determination of composition of unknown radioactive polynucleotide.

Once the composition of a given sample of unlabeled RE is known, then the composition of an unknown polynucleotide (e.g., the labeled polynucleotide product made in vitro by f2 replicase) can be deduced from its ability to anneal to the RE in the absence and presence of unlabeled f2 plus strand RNA. The total radioactivity (t*) in a sample of unknown composition can be represented as the sum of polynucleotides which are homologous to f2 RNAs (those that are capable of annealing to f2 plus or minus strands) and non-homologous polynucleotides (those which will not anneal to f2 RNAs) as follows:

$$t^* = m^* + p^* + x^* \qquad (6)$$

where t* is the total radioactivity (cpm), and m*, p* and x* are the amounts of radioactivity in f2 minus strands, f2 plus strands and non-homologous polynucleotides, respectively. If a small amount of highly labeled unknown polynucleotide is annealed with excess RE in the presence of increasing amounts of unlabeled f2 plus strand RNA, then the actual amount of annealed radioactivity (t^*_a) will be:

$$t^*_a = m^* + p^* f \qquad (7)$$

since all of the minus strand RNA and the fraction f of the plus strand RNA will anneal. A plot of t^*_a vs f (calculated for any value of [a] as described above) gives a straight line, having p* as the slope and m* as the Y-intercept. Knowing m* and p*, one can then evaluate x* from equation 6. Thus the amount of plus strand RNA, minus strand RNA and non-homologous polynucleotide product can be established from a single dilution curve. Fig. 5c shows the curves obtained for various hypothetical values of m* and p*. Also shown in Fig. 5 is the evaluation of x* for a given value of t* and the calculated values of m* and p*.

Since p* is evaluated with respect to a standard sample of labeled plus strand RNA, this method is insensitive to non-ideal behavior of plus strands in annealing. Errors arise in the estimation of m* if the annealing of minus strands is not quantitative or if some of the hybrids are digested by RNase. However, these errors serve only to

exaggerate the value of x*. Empirically, the sum of m* and p* (t^*_{calc}) has almost always equalled or exceeded the measured value of t* (t^*_{obs}), justifying the assumption of quantitative reannealing and recovery of minus strands in RNase-resistant form.

CHAPTER III

ENZYME ASSAYS AND PURIFICATION OF PHAGE REPLICASES

A. Assays

The basic composition of reaction mixtures used in the assays to be described was:

50 mM Tris-HCl (pH 7.5)
1 mM EDTA
10 mM magnesium acetate
10% w/v glycerol
1 mM phosphoenolpyruvate
5 μg/ml pyruvate kinase
0.5 mM dithiothreitol
0.2 mM GTP, ATP, CTP, UTP
[^{3}H]GTP, specific activity 28 cpm/pmol
2.5 μg/ml rifampicin

1. Poly G polymerase assay. The assay for rifampicin-resistant poly G polymerase activity was routinely conducted in 0.1-0.2 ml of the above reaction mixture, either containing GTP alone or in the presence of all 4 triphosphates and 1-4 μg poly C template. A small aliquot of enzyme (1-20 μg) was added to the reaction mixture and incubation was at 30° C for 10-20 min. Variations on the basic procedure are described as they occur.

2. Replicase assay I (low salt). F2 enzyme (20-100 μg) and template RNA (1-10 μg) were added directly to 0.1-0.5 ml of the reaction mixture. Incubation was carried out at 30° C for 20 min, unless otherwise indicated.

3. Replicase assay II (high salt). To detect replicase activity with plus strand RNA at low template concentrations, the replicase assay I was modified by including a preincubation of the enzyme in a high salt buffer, followed by incubation at an ionic strength of 0.1. F2 enzyme (20-100 μg) and template RNA (1-10 μg) were preincubated for 5 min at 30° C in 25-125 μl of SB20Mg containing 10 μmoles NaCl/25 μl buffer

(0.4 M). The reaction mixture, with all concentrations adjusted to give the correct final concentrations, was added at the end of the pre-incubation period and incubation was continued at 30° C for an additional 20 min. Variations in this basic procedure are given as they arise.

4. Replicase activity in crude homogenates. Replicase activity in crude cell extracts is measured in the absence of added template. No template-dependent enhancement of nucleotide polymerization is observed in f2-infected cell extracts. Crude activity is measured in the presence of both DNase (50 μg/ml) and rifampicin (2.5 μg/ml). An aliquot (5-10 μl) of cell extract is added to 0.2 ml of the indicated reaction mixture and incubation carried out for 10 min at 30° C.

5. Polynucleotide phosphorylase assay. The composition of the basic reaction mixture was modified by substituting ribonucleoside diphosphates for the ribonucleoside triphosphate substrates, using [^{3}H]UDP as the labeled substrate, omitting rifampicin, phosphoenol-pyruvate and pyruvate kinase, and decreasing the magnesium concentration to 3 mM. The incubation was generally carried out for 20 min at 30° C. No template or primer RNA was added.

6. DNA-dependent RNA polymerase assay. The composition of the basic reaction mixture was modified by omitting rifampicin and adding 0.2 M KCl and 200 μg/ml calf thymus DNA. Incubation was at 36° C for 30 min.

7. Ribonuclease assay. The composition of the basic reaction mixture was modified by the omission of the [^{3}H]GTP and the addition of 0.1 μg/ml [^{3}H]poly C. Incubation was carried out for 10-20 min at 30° C. The amount of RNase activity was crudely estimated by comparing the amount of pancreatic RNase required to effect a comparable digestion.

The assays described above were terminated by the addition of 3-5 ml of 5% TCA containing 1.0 M NaCl and 0.01 M sodium pyrophosphate. If the precipitate were to be collected on glass-fiber filters, 1-2 drops of bacterial extract containing both protein and RNA was added to each tube to serve as carrier. No carrier was added if the precipitate

were to be collected on a Millipore filter. Precipitates were collected by filtration through either glass fiber or Millipore filters on a Millipore filtration apparatus. The filters were then washed with 15 ml of the TCA-NaCl-pyrophosphate solution, dried and placed in glass vials containing 5 ml of toluene-based scintillation fluid. The vials were then counted in a Nuclear Chicago liquid scintillation counter.

Enzyme activity is generally reported in terms of pmol or nmol soluble NTP or NDP converted to acid-insoluble NMP equivalents by the enzyme aliquot assayed. RNase activity is reported as mμg [^{3}H]poly C digested by the enzyme aliquot assayed.

B. Isolation and Purification of f2 Phage-Induced Enzyme

1. Preparation of crude extract. Bacterial cultures (K38 or Q13) were grown in enriched medium to a density of 1.0-1.5 x 10^9/ml with vigorous shaking at 37° C. At 5 min before infection, agitation of the cultures was reduced (this procedure was found to increase the fraction of cells infected) and the cultures were supplemented with $CaCl_2$ to 2.5 mM. The cultures were infected with sus 11 phage at a multiplicity of 5-10. Gentle shaking was continued for 10 min after infection, at which time vigorous shaking was resumed to ensure adequate aeration. The infected cultures were collected 1-1.5 hr after infection by pouring them over 1/2 volume of frozen, crushed growth medium. The cells were harvested by centrifugation at 6000 rpm for 10 min and the cell pellets were frozen immediately in a dry ice-ethanol bath. The cell pellets were then removed from the centrifugation bottles, combined and weighed. In general, 1 liter of culture yielded 6-9 grams of frozen cell pellet. The purification scheme described below is based on 35-40 g of cell pellet; when using less starting material, all procedures were scaled to the amount of starting cell pellet. All subsequent procedures are carried out at 4° C or on ice.

The pooled frozen cell pellets were combined with 2.5 g washed glass beads and 1 ml SB20Mg per gram cell pellet in the 250 ml container of the Virtis "45" homogenizer. The vessel was placed in an ice bath and the cells ground at medium speed for 15-20 min, interspersing 3 min grinding intervals with 1 min intervals for chilling. The extract was centrifuged briefly at low speed, and the rather viscous supernatant was removed. The sediment containing the glass beads was suspended in 0.5 vol of SB20Mg and filtered through a funnel lined with glass wool under slight suction. The beads were washed with another 0.5-1.0 vol of buffer. The filtrate was combined with the original supernatant and centrifuged at 8000 rpm for 10-15 min to remove unbroken cells and cell debris.

2. Phase partitioning. Liquid-polymer phase partitioning was carried out by a slight modification of the methods described by Eoyang and August (1968) and by Kamen (1970) for the Qβ replicase. The objective of this procedure is to free the enzyme preparation from nucleic acids. The two-phase system formed by polyethylene glycol and high molecular weight dextran, at high salt concentrations, efficiently sequesters nucleic acids and template-bound enzyme in the lower phase, which is rich in dextran, leaving the upper, polyethylene glycol-rich phase enriched for free proteins (Alberts, 1967). Since f2 enzyme is tightly bound to template RNA in the crude extract (as judged by its insensitivity to added template and its high sedimentation coefficient), a fairly long exposure to high salt conditions prior to the addition of polymer solutions was found to be critical for the recovery of enzymatic activity in the polyethylene glycol phase.

The clarified extract was made 4 M in NaCl by the addition of solid NaCl. The solution was stirred slowly by means of a magnetic stirrer for 1.0-1.5 hr in an ice bath, whereupon 1/3 weight of a solution containing 25.6% polyethylene glycol, 6.4% Dextran T500, and 4 M NaCl in SB20Mg was added. Slow stirring was continued for 30 min. The phases were separated by centrifugation at 8000 rpm for 10 min.

The resulting clear upper phase was dialysed against 8 l of SB20 for 6-7 hr until the salt concentration was 0.05 M or less, as determined by checking the conductivity of the extract against standard salt solutions.

3. DEAE cellulose chromatography. The dialysate was applied to a 4 x 30 cm column of DEAE cellulose equilibrated with SB20 containing 0.05 M NaCl. The column was washed with several column volumes of loading buffer and developed with a salt gradient in SB20. The salt gradient was generally 0.08-0.25 M or 0.05-0.20 M NaCl, the latter being found to give slightly better resolution. The volume of the gradient was 700 ml. Absorbance at 280 nm and conductivity were measured along the gradient. Enzyme assays were carried out using 10-25 µl of each 10-15 ml fraction. The fractions containing rifampicin-resistant poly G polymerase activity were either pooled and precipitated with ammonium sulfate, stored in SB50Mg or dialysed for further purification by RNA cellulose chromatography.

4. RNA cellulose chromatography. The RNA cellulose column was prepared by suspending 0.35-1.0 g of RNA cellulose in SB50 containing 0.05 M NaCl and pouring it into a 0.5 or a 0.9 cm (ID) column equipped with a needle valve. The column was washed for several hours or overnight with the same buffer at a flow rate of 2-4 ml/hr to remove residual unbound RNA. DEAE-purified enzyme was prepared for application to RNA-cellulose by dialysing it against SB20 for 1-2 hr to reduce the salt concentration and then against SB50 for 2-3 hr. The enzyme aliquot (35-40 mg total protein) was then applied to the RNA cellulose column at a flow rate of 1-2 ml/hr. The flow rate was restored and the column was washed with 0.05 M NaCl-SB50 until the A_{280} of the eluate was 0.05 or less. The column was then washed with 0.1 M NaCl-SB50 (1-2 column volumes). Poly G polymerase activity was eluted with 0.2 M NaCl-SB50. The column fractions (1-2 ml) were assayed for poly G polymerase and RNase activity. Peak poly G polymerase fractions were either stored in liquid nitrogen, or prepared for glycerol gradient centrifugation.

5. Glycerol gradient centrifugation. Enzyme preparations were analysed by sedimentation through 7-25% v/v glycerol gradients in SBMg containing 0.5 or 1.0 M NaCl. Commercial preparations of aldolase, catalase and hemoglobin were centrifuged in separate tubes as sedimentation markers. Approximately 3 mg of each marker protein in SB4Mg was layered on a 14 ml gradient and centrifuged simultaneously with the f2 enzyme preparation. The f2 enzyme was prepared for layering by a 2 hr dialysis against SB4Mg. Gradients were centrifuged for 24 hr at 2° C in the SW40 rotor of the Beckman L2-65B ultracentrifuge at 40,000 rpm. The gradients were collected in 0.5 ml fractions from the bottom of the tube. Aldolase and hemoglobin were determined spectrophotometrically by measuring absorbance at 280 and 400 nm, respectively. Catalase activity was detected by a modification of the procedure described by Beers and Sizer (1952). Aliquots (100 µl) of each fraction were mixed with 2 ml 0.1% hydrogen peroxide in 0.05 M phosphate buffer (pH 7.0) and the absorbance at 240 nm was read at 30 sec after mixing. F2 enzyme gradients were assayed for poly G polymerase, replicase and RNase activity as described above. The absorbance at 280 nm was also determined.

6. Storage of enzyme. Partially purified enzyme preparations obtained from DEAE cellulose were concentrated by ammonium sulfate precipitation. The enzyme could be precipitated by the direct addition of solid ammonium sulfate. However, greater recovery of activity was obtained when the enzyme was precipitated by dialysis against SB20Mg saturated with ammonium sulfate. The precipitate was collected by centrifugation at 20,000 rpm for 20 min and dissolved in SB20Mg. Residual ammonium sulfate was removed by dialysis against SB20Mg for several hours. The enzyme was then dialysed against SB50Mg for 1-2 hr, divided into small aliquots (100-300 µl), frozen in a dry ice-ethanol bath, and stored in liquid nitrogen. No detectable loss of activity was observed during storage in liquid nitrogen. Repeated freezing and thawing of a single aliquot was detrimental to activity. When an aliquot was to be used, it was thawed quickly in a 30° C water bath

and stored at -13° C. The presence of salt (0.5 M NaCl) in the SB50Mg storage buffer prolonged the survival of the enzyme at -13° C. The enzyme half-life under these conditions varied from 2 weeks to 1 month.

Highly purified poly G polymerase was stored in liquid nitrogen in SB50Mg as described above. The highly purified poly G polymerase was quite unstable at -13° C, having a half-life of about 12-24 hr, depending on the salt concentration of the storage buffer.

C. Isolation of Qβ Replicase

Qβ replicase was purified from Qβ am 12-infected E. coli Q13 as described above for the f2 replicase, except that phosphocellulose column chromatography was used in place of RNA cellulose column chromatography. Phosphocellulose chromatography was carried out as described by Kamen (1970). Qβ replicase was stored in SB20Mg containing 0.2 M $(NH_4)_2SO_4$ at 0° C (Eoyang and August, 1968) or in SB50Mg containing 0.2 M $(NH_4)_2SO_4$ at -13° C.

CHAPTER IV

PURIFICATION OF RIFAMPICIN-RESISTANT POLY G POLYMERASE FROM f2-INFECTED *E. COLI*

Infection of bacterial cells by the f2 bacteriophage is accompanied by the appearance of a new, ribonucleotide-polymerizing activity. Studies on both wild-type phage- and mutant phage-infected cells have demonstrated that this activity represents the phage RNA-replicating enzyme or enzyme complex (August *et al.*, 1963; Lodish *et al.*, 1964). Evidence that the phage replicase complex involves both viral and bacterial components was reviewed in Chapter I. In the present work, we have used the sus 11 mutant of f2 as the infecting phage. Some properties of this phage, which has amber mutations in both the major and minor coat protein cistron, have already been discussed (Chapter I). The property which made it particularly useful in the present studies is the overproduction of phage replicase activity observed in sus 11-infected cells. Both August *et al.* (1963) and Lodish *et al.* (1964) have shown that crude extracts of sus 11-infected cells promote 10-20 times as much ribonucleotide polymerization as do crude extracts of wild-type f2-infected cells. We have therefore used this mutant to maximize the yield of phage-specific enzyme.

The phage-induced ribonucleotide-polymerizing activity in crude extracts of MS2-infected cells is completely insensitive to the presence of added template RNA (Weissmann *et al.*, 1963). This appears to be due to the fact that the phage enzyme is tightly bound to the phage template RNAs of infected cells. When the phage-induced enzymatic activity is sedimented through a sucrose gradient, ribonucleotide polymerizing activity is found to be associated with 40 S particles (Weissmann *et al.*, 1963). Such complexes incorporate radioactive ribonucleotides into phage RNA (Chapter I). Thus the complex appears to contain both enzyme and phage template RNA. Preliminary studies on the f2 sus 11-induced activity gave the same results. The activity in

crude cell extracts proved to be completely insensitive to added template (August et al., 1963; N. Fedoroff, unpublished observations) and sedimented as a large complex with a sedimentation coefficient of about 50 S (Fig. 6). Thus, in order to establish whether the f2 replicase, like the Qβ replicase, has an associated poly C-dependent poly G polymerase activity, we had first to free the enzyme from endogenous template RNA.

A. Rifampicin-Resistant Poly G Polymerase Activity in sus 11-Infected Cell Extracts

When measuring nucleotide polymerization in crude cell extracts and during the early stages of purification, it is necessary to design the assays in such a way that only the activity of interest, f2 replicase in this case, is detectable. The major bacterial ribonucleotide polymerizing enzymes are the DNA-dependent RNA polymerase, polynucleotide phosphorylase, and poly A polymerase. The DNA-dependent RNA polymerase will polymerize ribonucleotides in the presence of DNA, RNA or a variety of synthetic deoxyribo- and ribopolymers (Chamberlin and Berg, 1962; Fox et al., 1964; Niyogi and Stevens, 1965; Anthony et al., 1969). The activity of this enzyme in crude cell extracts is dependent on the presence of endogenous bacterial DNA and can be suppressed by adding deoxyribonuclease to the extract (August et al., 1963). The bacterial RNA polymerase is also sensitive to inhibition by the drug rifampicin, which prevents initiation of RNA synthesis in the presence of either DNA or synthetic polymers (Sippel and Hartmann, 1968; Kamen, 1970). Neither f2 phage RNA replication in intact bacterial cells, nor the replicase activity detectable in crude cell extracts, is sensitive to rifampicin inhibition (Fromageot and Zinder, 1968; Robertson, personal communication). Polynucleotide phosphorylase polymerizes nucleoside diphosphates rather than nucleoside triphosphates, with concomitant release of inorganic phosphate. The polynucleotide phosphorylase reaction is reversible and depends on the ratio of nucleoside diphosphates to inorganic phosphate in the incubation medium (Littauer

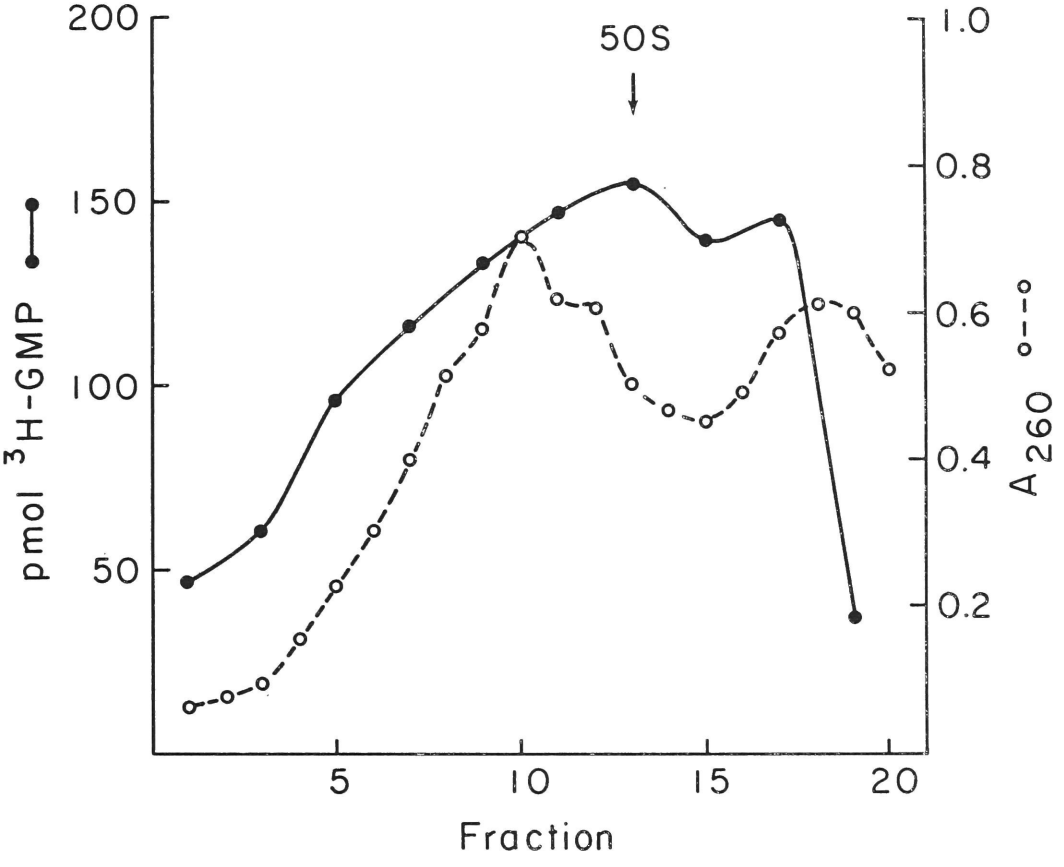

Figure 6. Sedimentation behavior of crude f2 replicase activity

Sus 11-infected E. coli were ground with glass beads as described in Chapter III. Cell homogenates were clarified by centrifugation, dialysed against SB4Mg containing 0.2 M NaCl for 2 hr and layered on 10-40% glycerol gradients in SBMg containing 0.2 M NaCl. The 14-ml gradients were centrifuged for 4 hr at 40,000 rpm at 2° C; 0.7 ml fractions were collected and assayed for crude replicase activity as described in Chapter III. Replicase activity (●——●); absorbance at 260 nm (o---o). [^{14}C]labeled f2 phage were used as a sedimentation marker.

and Kornberg, 1957). Hence polynucleotide phosphorylase activity is not detectable if ribonucleoside diphosphate contamination of NTPs is reduced by adding a nucleoside triphosphate-regenerating system (phosphoenolpyruvate and pyruvate kinase) or if sufficient phosphate is present to reverse the reaction. Poly A polymerase (August et al., 1962) is rendered undetectable by the simple expedient of using a labeled substrate other than ATP. The first line in Table I compares the template-independent nucleotide polymerizing activities of uninfected and sus 11-infected cell extracts under these conditions. In the presence of DNase, rifampicin and a triphosphate-regenerating system, extracts of infected cells incorporate 40 times as much [^{3}H]GTP into acid-insoluble material as do extracts of uninfected cells.

Liquid-polymer phase partitioning at a high salt concentration was used to dissociate the phage enzyme-template complex and separate proteins from nucleic acids, as described in Chapter III. The lower part of Table I shows the nucleotide polymerizing activity recovered in the upper, protein-rich phase after phase partitioning of infected and uninfected cell extracts. Under the assay conditions used, no template-independent activity is detectable in either infected or uninfected cell extract after phase partitioning. Addition of poly C template in the absence of rifampicin stimulates incorporation of [^{3}H]GTP into acid-insoluble material by both infected and uninfected cell extracts. However, the stimulation is markedly greater for the infected cell extract than for the uninfected cell extract. When rifampicin is added to inhibit the synthetic polymer-dependent activity of the bacterial DNA-dependent RNA polymerase, the activity of the uninfected cell extract is completely suppressed, while that of the infected cell extract is depressed by only about 20%. Hence the infected cell extract contains a substantial amount of poly C-dependent poly G polymerase activity which is resistant to inhibition by rifampicin.

Table I

Poly G polymerase activity in infected and uninfected cell extracts

	Additions			[^{3}H]GMP incorporation*	
	DNase	Rifampicin	Poly C	Infected	Uninfected
Crude extract	+	+	-	501	13.5
Extract after phase partitioning	+	-	-	1.7	7.0
	+	-	+	389	84.0
	+	+	+	311	6.7

Aliquots (5 µl) of crude cell homogenates and extracts obtained by liquid-polymer phase partitioning as described in Chapter III were assayed in standard 0.2 ml reaction mixtures for the crude cell homogenates and 0.4 ml reaction mixtures for the partitioned extracts (to overcome the inhibitory effects of high salt and polyethylene glycol concentrations) for 10 min at 30° C. Acid-insoluble material was collected on glass fiber filters.

*Picomoles of GTP converted to acid-insoluble GMP equivalents by 5 µl of extract.

B. Chromatographic Purification of the Rifampicin-Resistant Poly G Polymerase

Fig. 7 shows an elution profile of the rifampicin-resistant poly G polymerase from a DEAE cellulose column. The poly G polymerase elutes as a single sharp peak at a fairly low salt concentration (0.1-0.13 M NaCl). This affords good separation from the bacterial DNA-dependent RNA polymerase, which remains tightly bound at this salt concentration (Burgess, 1969). The polynucleotide phosphorylase activity elutes at a slightly higher salt concentration than the f2 poly G polymerase. The polynucleotide phosphorylase peak is rather broad under these conditions and it overlaps the poly G polymerase peak. Contamination by polynucleotide phosphorylase proved to be a problem in studies with partially purified enzyme. This difficulty was generally circumvented by using a bacterial strain lacking the normal polynucleotide phosphorylase activity as host bacterium. Contamination of the f2 enzyme by ribonuclease activity was also minimized by using host strains low in RNase activity.

The extent of purification achieved by DEAE cellulose chromatography is difficult to evaluate for several reasons. The specific activity of a given enzyme preparation with respect to poly C-dependent activity can be measured only after endogenous template has been removed. The presence of polyethylene glycol in the extract prior to DEAE chromatography inhibits the poly G polymerase to a certain extent and also interferes with estimates of the protein content. However, based on approximate estimates of specific activity and on total protein recovery, the purification achieved by DEAE cellulose chromatography is 10- to 20-fold. The specific activity for poly G polymerase increases from approximately 1-2 nmol GMP/mg/10 min prior to chromatography to 10-20 nmol GMP/mg/10 min after DEAE cellulose chromatography; preparations of much higher specific activity after DEAE cellulose have occasionally been obtained.

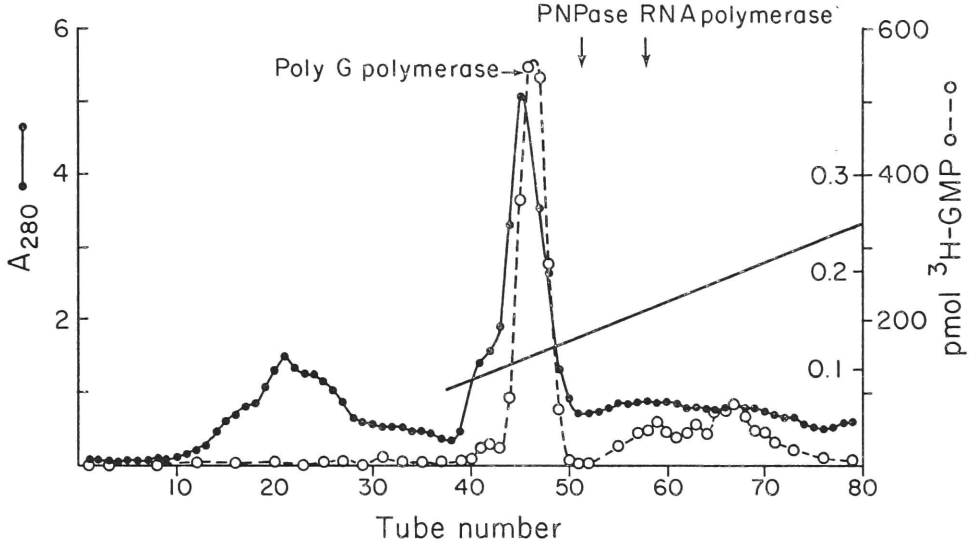

Figure 7. DEAE cellulose chromatography of rifampicin-resistant poly G polymerase activity

Extracts of f2 sus 11-infected cells were subjected to liquid-polymer phase partitioning and DEAE cellulose chromatography as described in Chapter III. The solid line represents the eluting NaCl gradient (0.08-0.25 M). A 10 µl aliquot of each 10-ml fraction was assayed for poly G polymerase activity for 10 min at 30° C in the presence of rifampicin (o----o); UV absorbance was read at 280 nm (•——•). Assays for bacterial DNA-dependent RNA polymerase and polynucleotide phosphorylase were carried out as described in Chapter III.

The properties of DEAE-purified f2 enzyme will be discussed in detail in Chapter V. Briefly, the partially purified enzyme is adequately free of extraneous nucleotide polymerizing activities and shows excellent template dependence. The stability of the partially purified enzyme is not notably different from the stability of the crude enzyme activity. Both have a half-life of approximately 12 hours at 0^{o} C. As described in Chapter III, the enzyme can be stored in liquid nitrogen without loss of activity. When stored in a high salt, 50% glycerol buffer at -13^{o} C, the enzyme has a half-life of about one month. The enzyme also proved very fragile during further purification. Conventional column techniques which have been used successfully in isolating similar enzymes were quite unsatisfactory. Thus, for example, only 2-5% of the original activity could be recovered from either phosphocellulose or hydroxylapatite columns, both of which are quite useful in purifying the Qβ replicase.

The recently developed methods of affinity chromatography appeared to be promising since these methods are gentle and exploit the natural binding properties of various proteins. DNA cellulose, for example, has been an extremely effective method for purifying enzymes and proteins which have an affinity for DNA (Alberts and Herrick, 1971). Since the replicase uses only RNA templates, a method was devised for binding phage RNA to cellulose (see Chapter II). Chromatography of the f2 poly G polymerase on RNA cellulose proved both gentle and quite efficient. As much as 80% of the input activity was recovered from RNA cellulose and the purification was at least 20-fold. The elution profile of an RNA cellulose column is shown in Fig. 8. Very little of the total protein and almost all of the poly G polymerase activity bind to the column in low salt (0.05 M NaCl). Additional contaminating protein elutes at 0.1 M NaCl, and the poly G polymerase elutes at 0.2 M NaCl. Most of the ribonuclease activity present in the partially purified enzyme elutes in the 0.05 M and 0.1 M NaCl washes. Only 3-5% of the RNase activity elutes with the poly G polymerase peak.

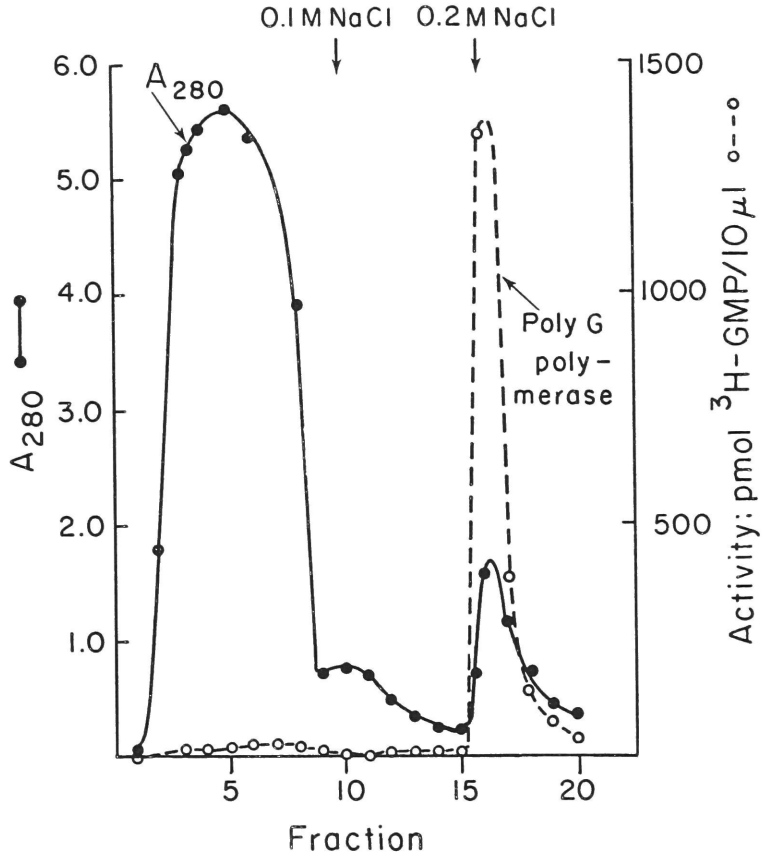

Figure 8. RNA cellulose chromatography of rifampicin-resistant poly G polymerase activity

Peak poly G polymerase fractions from DEAE cellulose were purified by RNA cellulose chromatography as described in Chapter III. A 5 µl aliquot of each 1-ml fraction was assayed for poly G polymerase activity for 10 min at 30° C (o----o); UV absorbance was read at 280 nm (●——●).

C. Proteins of the Purified Poly G Polymerase

The f2 poly G polymerase obtained after RNA cellulose chromatography is extremely unstable and sensitive to dilution. Considerable activity losses occur upon further purification, with only marginal gains in purity. The evidence presented below concerning the identity of the poly G polymerase proteins is, necessarily, indirect and quite tentative. The proteins associated with the poly G polymerase activity after various purification procedures were analysed by SDS-polyacrylamide gel electrophoresis. Proteins which consistently purified with the poly G polymerase activity were considered as probable constituents of the poly G polymerase. Any protein whose loss or diminution was not detrimental to activity was assumed to be a contaminant. Proteins whose loss was associated with loss of enzyme activity were identified as necessary components of the poly G polymerase.

Sedimentation of the RNA cellulose-purified enzyme through a high salt glycerol gradient achieves a modest additional purification. The poly G polymerase sediments as a single peak with a sedimentation coefficient of about 6.5 S, based on the sedimentation behavior of marker proteins run in parallel tubes (Fig. 9). About 60% of the total protein on the gradient is present under the poly G polymerase peak. The separation between the poly G polymerase peak and a more slowly sedimenting protein peak, which presumably represents inactive contaminating proteins present, is incomplete. Aliquots of the purified poly G polymerase were analysed by polyacrylamide gel electrophoresis before and after centrifugation. Fig. 10 shows spectrophotometric tracings of such gels. The gels were run, stained and destained simultaneously, and it is assumed that the relative staining intensity is the same for both gels. The proteins labeled I, II, III, and IV are the only proteins which do not diminish upon centrifugation. All other proteins either decrease in relative amount or disappear entirely after glycerol gradient centrifugation. These are the four proteins which persistently purify with the poly G polymerase activity and they have therefore been tentatively identified as constituents of the poly G polymerase. Before

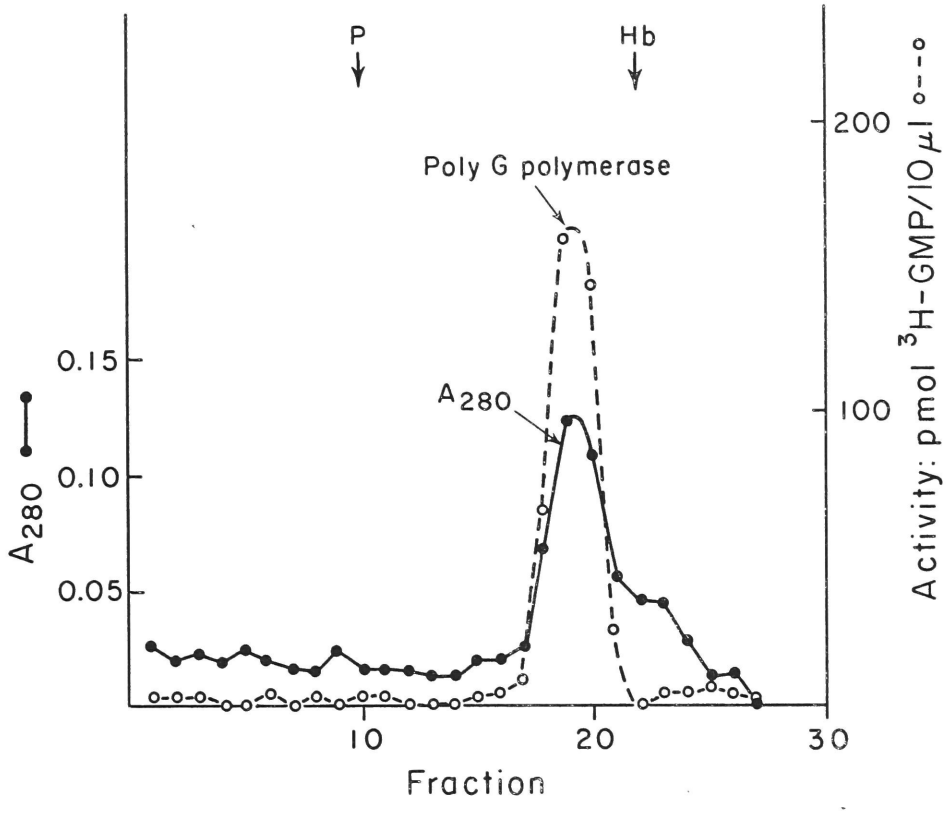

Figure 9. Glycerol gradient centrifugation of rifampicin-resistant poly G polymerase activity

Aliquots of poly G polymerase purified through RNA cellulose chromatography as described in Chapter III were dialysed for 2 hr against SB4Mg containing 0.5 M NaCl and layered on 7-25% glycerol gradients in the same buffer. Centrifugation was for 24 hr at 40,000 rpm as described in Chapter III. Hemoglobin (Hb, 4.3 S) and E. coli DNA-dependent RNA polymerase (P, 13-15 S) were used as sedimentation markers. A 10 µl aliquot of each 0.5-ml fraction was assayed for poly G polymerase activity in the presence of rifampicin for 10 min at 30° C (o----o); UV absorbance was read at 280 nm (●——●).

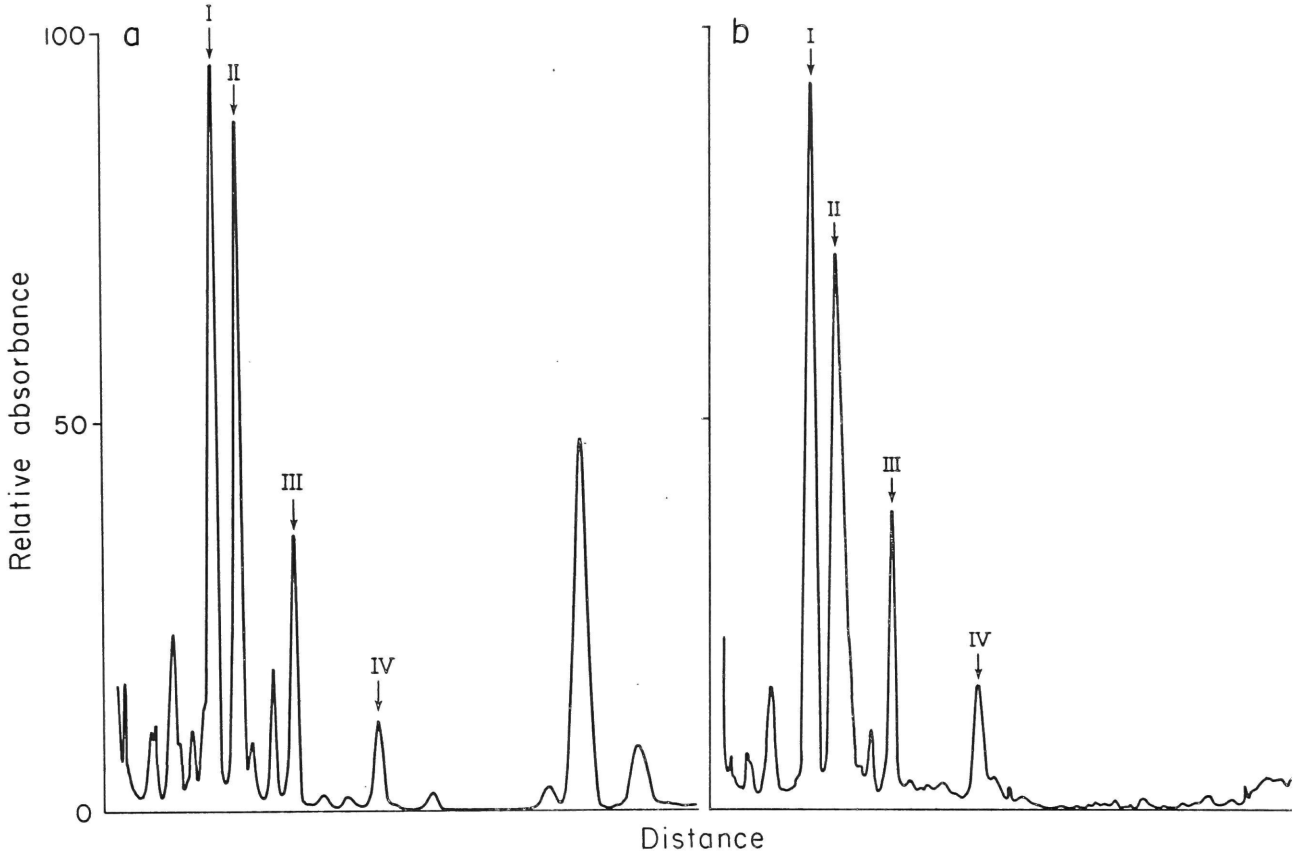

Figure 10. SDS-polyacrylamide gels of rifampicin-resistant poly G polymerase before and after glycerol gradient centrifugation

Aliquots of poly G polymerase were subjected to electrophoresis on 10% polyacrylamide gels as described in Chapter II. Gels of enzyme before and after glycerol gradient centrifugation were run in parallel, stained and destained simultaneously. Stained gels were scanned at 600 nm. Commercial preparations of bovine serum albumin, aldolase and lysozyme were used as molecular weight standards. Bands identified as poly G polymerase subunits are marked I, II, III and IV. Part a: enzyme purified by RNA cellulose chromatography; part b: enzyme purified by RNA cellulose chromatography and glycerol gradient centrifugation.

centrifugation, 60% of the total protein stain is present in proteins I-IV; more than 85% of the protein stain is attributable to these proteins after centrifugation. Since the stoichiometric relationship between protein concentration and staining intensity is not known and may vary for different proteins, this figure is only an approximate indication of the extent of purification.

It can be seen in Fig. 10 that proteins I and II are present in excess over proteins III and IV. This does not appear to be due only to differential staining of proteins differing in size. The amount of III + IV relative to the amount of I + II was found to vary considerably, both from preparation to preparation and between samples from the same preparation subjected to somewhat different final purification steps. Moreover, the specific activity of a given poly G polymerase preparation depends strongly on the amount of III and IV present. Thus, for example, when the DEAE-purified enzyme was stored for a fairly long period at -13° C (with considerable loss of activity) before RNA cellulose chromatography, poly G polymerase of fairly low specific activity (<100 nmol GMP/mg/10 min) was recovered after chromatography. Such preparations contained large amounts of proteins I and II and quite small amounts of proteins III and IV. A similar loss of proteins III and IV with a concomitant decrease in specific activity was observed when aliquots from the same DEAE preparation were subjected to slightly different elution procedures during RNA cellulose chromatography. This is illustrated in Fig. 11, which shows the poly G polymerase proteins from two different RNA columns. Aliquots from the same DEAE preparation were applied to RNA cellulose under the same conditions. The only difference in the two column runs was that column b, corresponding to gel b, was washed much more extensively with low salt buffer than column a, in an effort to remove more contaminating protein before eluting the poly G polymerase activity. If successful, the removal of more extraneous protein should increase the specific activity of the eluting enzyme. However, the extra washing actually decreased the specific activity of the poly G polymerase. Thus the specific activity of enzyme eluted

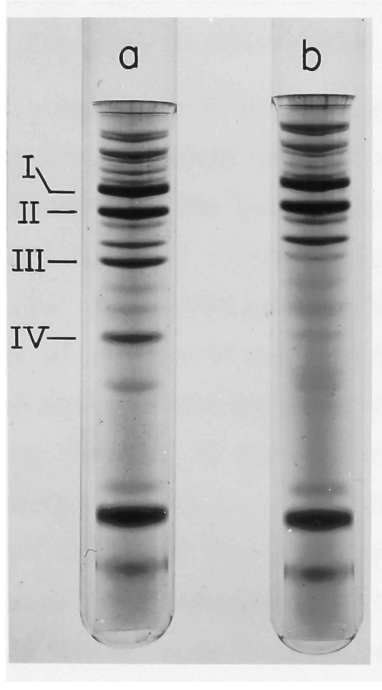

Figure 11. SDS-polyacrylamide gels of rifampicin-resistant poly G polymerase from different RNA cellulose columns

Aliquots of the same DEAE-purified poly G polymerase preparation were subjected to RNA cellulose chromatography as described in the text. Column b, corresponding to gel b, was subjected to extensive washing with 0.1 M NaCl-SB50 prior to elution of the poly G polymerase. Column a, corresponding to gel a, was washed with only 1 column volume of 0.1 M NaCl-SB50 prior to elution of the poly G polymerase. Samples of enzyme were analysed by SDS-polyacrylamide gel electrophoresis as described in Chapter II.

from column a was about 240 nmol GMP/mg/10 min, while that eluted from column b had a specific activity of only 90 nmol GMP/mg/10 min. All proteins are present in similar amounts in both preparations with the conspicuous exception of proteins III and IV, which are present in greatly reduced amounts in gel b. The correlation between the specific activity of the enzyme preparation and the relative amounts of proteins III + IV suggests that at least these two proteins (or perhaps only one of them) are required for poly G polymerase activity.

Rifampicin-resistant poly G polymerase activity, like the phage replicase activity, is present only in infected cells. Studies described earlier have demonstrated that the phage replicase protein is required for phage replicase activity and is probably a component of the replicase enzyme (Chapter I). To establish whether the phage replicase protein is a component of the purified f2 poly G polymerase, *in vivo* [^{14}C]labeled replicase protein was co-electrophoresed with poly G polymerase proteins. Since the sus 11 mutant of f2 has amber mutations in both the coat and A protein cistrons, the only complete phage protein made in sus 11-infected cells is the phage replicase protein. Such cells were therefore used as a source of this protein. Aliquots of infected and uninfected cells were treated with rifampicin to inhibit bacterial RNA and protein synthesis and then labeled with [^{14}C]amino acids, as described in Chapter II. Polyacrylamide gel electrophoresis of the labeled proteins extracted from sus 11-infected and uninfected cells is shown in Fig. 12. The single major protein species which labels in sus 11-infected cells, but not in uninfected cells after rifampicin treatment (P. Model and N. Fedoroff, unpublished observations) or after actinomycin treatment (Nathans *et al.*, 1966), is the replicase protein (Fig. 12, gel slices 13-14). The identity of this protein as the product of the replicase cistron has been established by studies on mutant and wild-type phage-infected cells (Nathans *et al.*, 1966; Viñuela *et al.*, 1968). This protein has a molecular weight of approximately 63,000 and is the largest phage protein made during infection.

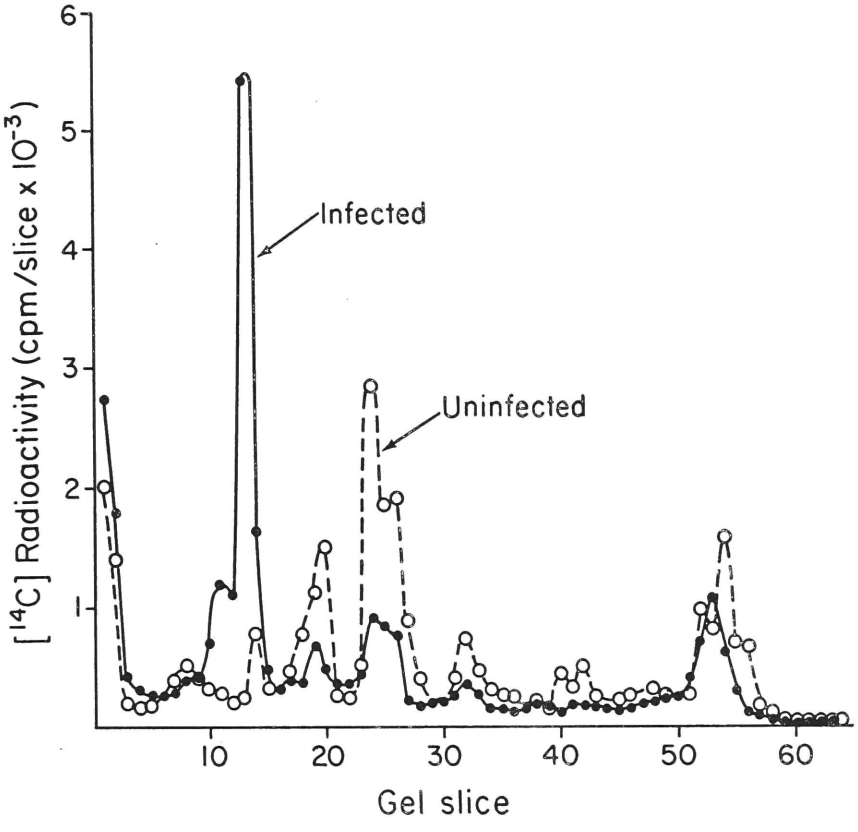

Figure 12. SDS-polyacrylamide gel electrophoresis of [^{14}C]labeled proteins from sus 11-infected and uninfected E. coli

Sus 11-infected and uninfected E. coli K38 cultures were treated with rifampicin and labeled with [^{14}C]amino acids; labeled proteins were extracted from both cultures and analysed by SDS-polyacrylamide gel electrophoresis as described in Chapter II. Labeled proteins from infected cells (●——●); labeled proteins from uninfected cells (o----o).

Co-electrophoresis of the proteins labeled *in vivo* during sus 11 infection with the proteins of the glycerol gradient-purified poly G polymerase is shown in Fig. 13. The gel was first stained and traced to locate the poly G polymerase proteins and then sliced for detection of radioactive proteins as described in Chapter II. The replicase protein labeled *in vivo* co-electrophoreses with protein II of the purified poly G polymerase (Fig. 13, peak II). Similar results were obtained when labeled proteins synthesized *in vitro* from purified f2 RNA template were co-electrophoresed with the poly G polymerase proteins (not shown). Thus protein II of the purified poly G polymerase appears to be the phage-coded replicase protein.

Although the fragility of the f2-induced poly G polymerase precludes a rigorous demonstration that all four of the identified proteins are indeed subunits of the active enzyme, a comparison of the f2 and Qβ poly G polymerases suggests that the f2 subunit identification is correct. Fig. 14 shows that the Qβ and f2 enzymes are quite similar. Proteins I, III, and IV of the two enzymes are electrophoretically indistinguishable. In the case of the Qβ enzyme, these subunits have been identified as bacterial proteins by Kamen (1970) and by Kondo *et al.* (1970). It is probable that proteins I, III, and IV are the same bacterial proteins in both enzymes. Molecular weight estimates for the poly G polymerase proteins, obtained independently by the indicated investigators, are given in Table II. The phage-coded polypeptides (protein II in each case) of the f2 and Qβ enzymes differ somewhat in electrophoretic mobility. This difference in the size of the Qβ and f2 replicase proteins has been observed with both the purified poly G polymerase protein II and with f2 and Qβ replicase proteins labeled both *in vivo* and *in vitro* (K. Horiuchi, personal communication).

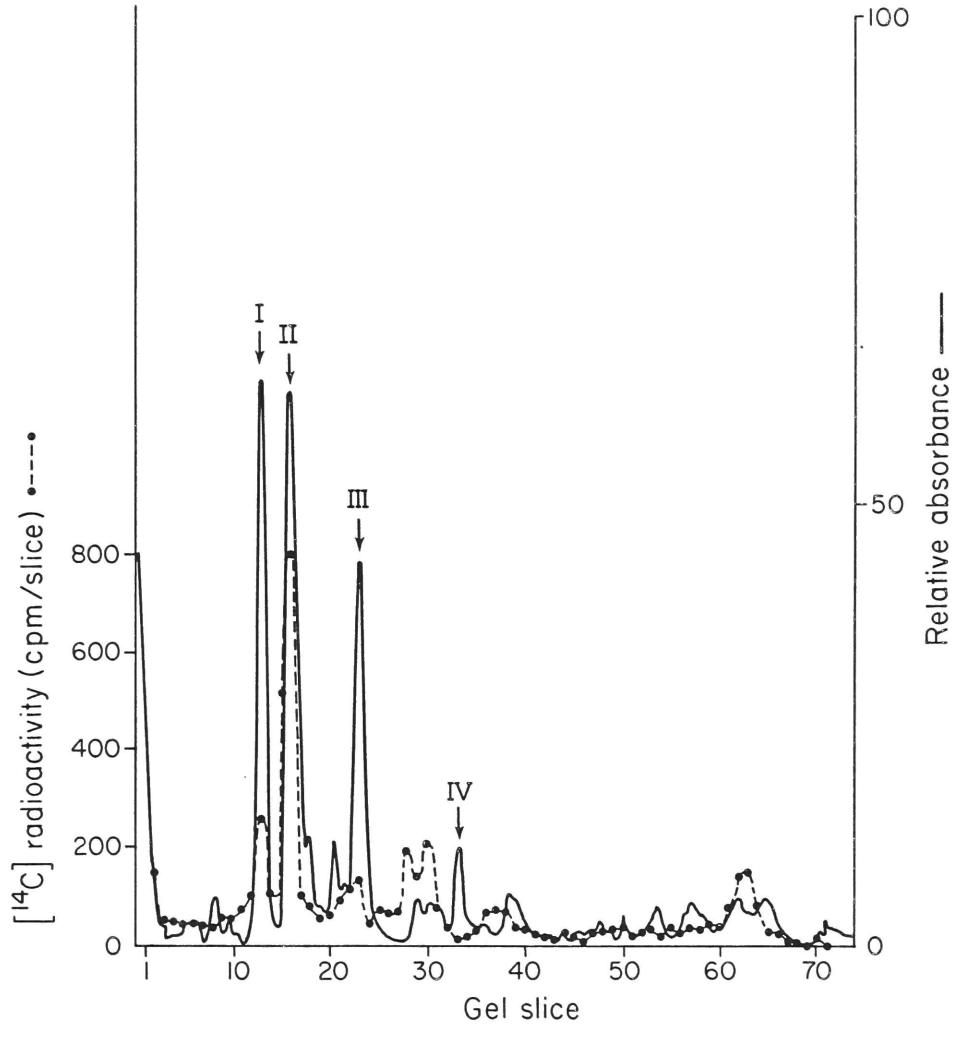

Figure 13. Co-electrophoresis of <u>in vivo</u> [^{14}C]proteins from sus 11-infected cells with purified poly G polymerase

A small aliquot of [^{14}C]labeled, infected-cell proteins (Fig. 12) was co-electrophoresed with rifampicin-resistant poly G polymerase purified by glycerol gradient centrifugation. Gels were first stained and scanned to locate the poly G polymerase proteins (I-IV, ——), then sliced and counted to detect radioactive, <u>in vivo</u> proteins (•----•).

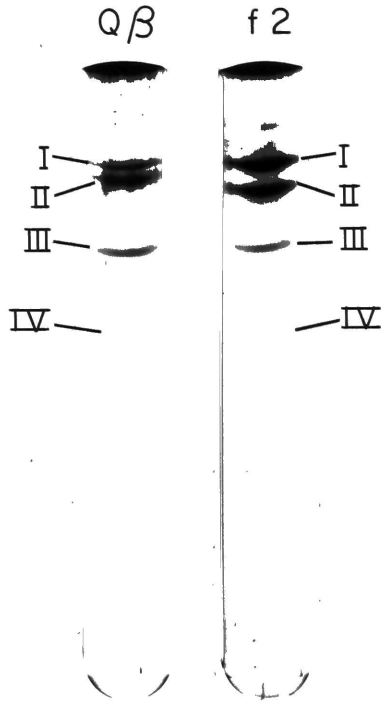

Figure 14. Purified Qβ and f2 poly G polymerases

Rifampicin-resistant poly G polymerase was purified from f2 sus 11-infected cells as described in Chapter III. Rifampicin-resistant poly G polymerase was purified from Qβ am 12-infected cells by the same procedure, except that phosphocellulose chromatography was used in place of RNA cellulose chromatography. Aliquots of the purified enzymes were subjected to SDS-polyacrylamide gel electrophoresis as described in Chapter II. The Qβ subunit designation is that given by Kamen (1970).

Table II

Estimated molecular weights for proteins I, II, III, and IV of the Qβ and f2 poly G polymerases

Enzyme	Estimated molecular weight (Daltons)				Reference
	I	II	III	IV	
Qβ	74,000	69,000	47,000	33,000	Kondo et al. (1970)
Qβ	70,000	65,000	45,000	35,000	Kamen (1970)
Qβ	73,000	66,000	44,500	32,500	Eoyang and August (1971)
f2	75,000	63,000	46,000	33,000	Fedoroff and Zinder (1971)

D. Discussion

Evidence has been presented that bacterial cells infected with the sus 11 mutant of f2 contain a rifampicin-resistant poly G polymerase activity not found in uninfected cells. The rifampicin-resistant poly G polymerase has been purified extensively. Four proteins have been tentatively identified as constituent proteins of the poly G polymerase. One of the proteins is electrophoretically identical to the phage-coded replicase protein.

There is a striking structural similarity between the poly G polymerases induced during f2 and Qβ infections. The two poly G polymerase enzymes appear to differ only in the phage-specific subunit, which is somewhat larger in the case of the Qβ enzyme than in the case of the f2 enzyme. Yet these enzymes differ markedly, both in stability and in template properties (Eoyang and August, 1968; August et al., 1968; Fedoroff and Zinder, 1971). If the three non-phage proteins of the two enzymes are in fact the same bacterial proteins, as their electrophoretic behavior suggests, then the difference in stability must be attributable to the respective phage replicase proteins. The difference in template specificity will be considered in Chapter VI.

In view of the structural similarity of the two enzymes, observations on the Qβ enzyme provide some insight into the properties of the f2 enzyme and its behavior during purification. Kamen (1970) showed that the Qβ poly G polymerase dissociates at low salt concentrations into aggregates of subunits I + II and subunits III + IV. These complexes or aggregates can be separated by glycerol gradient centrifugation. Incubation of fractions rich in components I + II with subunits III + IV results in the reconstitution of active Qβ poly G polymerase. It was further shown by Kondo et al. (1970) that subunits I + II probably represent the RNA-binding component of the enzyme. When these investigators incubated the purified Qβ enzyme with Qβ RNA, they found that only subunits I + II could be recovered from the template-enzyme complex. The behavior of the f2 enzyme during RNA cellulose chromatography can be

explained in terms of these observations. If proteins I and II of the f2 enzyme are its RNA-binding component, these proteins would be expected to bind to RNA-cellulose whether associated with the other two subunits or not. In fact, the low salt conditions necessary for binding of the f2 enzyme to RNA cellulose might favor the dissociation of subunits I + II from subunits III + IV. If proteins III and IV, on the other hand, can bind to the column only when associated with I and II, while proteins I and II bind regardless of the presence of the other two, enzyme eluted from the column will contain both active enzyme consisting of all 4 proteins and inactive subunits I and II. If, furthermore, loss of activity during storage of the enzyme is due to dissociation of proteins I + II from III + IV, RNA cellulose chromatography of such preparations should give poly G polymerase preparations rich in inactive subunits I + II. Prolonged washing of the column, by favoring the low salt dissociation, would have the same net result. Thus the differential recovery of proteins I + II and III + IV may simply be an artifact of the purification technique used.

CHAPTER V

PROPERTIES OF THE f2-INDUCED ENZYME

In this chapter, we will discuss some of the properties of the nucleotide-polymerizing enzyme produced during infection of bacteria with the f2 phage. All of the studies described here were carried out on enzyme preparations purified by liquid-polymer phase partitioning and DEAE cellulose chromatography. Our first objective was to show that the enzyme preparation, which was routinely assayed for rifampicin-resistant poly G polymerase activity, did indeed have the properties of an RNA phage replicase, both in terms of its ability to use phage RNA templates and its ability to carry out the synthetic reactions involved in phage RNA replication. To do this, we tested its response to various natural RNA templates under different conditions. We then analysed the polynucleotide products of the _in vitro_ reaction by RNA annealing methods and by glycerol gradient sedimentation. In the course of these studies, it became apparent that the replicase and poly G polymerase activities responded differently to the manipulation of certain experimental conditions. We therefore carried out a series of comparative studies on the replicase and poly G polymerase activities. Finally, we have briefly compared some properties of the f2 and Qβ replicases.

A. Purity of the Enzyme Preparation

1. _Contamination by bacterial enzymes._ The enzyme preparations used in these studies were purified from sus 11-infected _E. coli_ Q13 through the DEAE cellulose step as described in Chapter III. At this stage of purification, the f2 poly G polymerase has a specific activity of 10-100 nmol GMP/mg/10 min and shows excellent template dependence. Assays carried out on this preparation to detect the presence of other nucleotide polymerizing activities and RNase activity are shown in Table III. The preparation is substantially free of bacterial DNA-dependent RNA polymerase and polynucleotide phosphorylase. Some ribonuclease activity can be detected; however, the amount of [^{3}H]poly C

Table III

Assay of DEAE-purified f2 poly G polymerase for contaminating bacterial enzymatic activities

Enzyme activity assayed	Substrate	Activity
Poly G polymerase	[^{3}H]GTP	710 pmol GMP
DNA-dependent RNA polymerase	[^{3}H]GTP	3 pmol GMP
Polynucleotide phosphorylase	[^{3}H]UDP	<1 pmol UMP
Template-independent nucleotide polymerization	[^{3}H]GTP	<1 pmol GMP
Ribonuclease	[^{3}H]poly C	0.085 µg poly C

Assay conditions are described in Chapter III. Each assay was carried out in a final reaction volume of 0.1 ml, using 20 µg of DEAE-purified f2 enzyme. All assays were incubated for 20 min at 30° C, except the RNA polymerase reaction, which was incubated at 36° for 30 min. Acid-insoluble radioactivity was collected on Millipore filters as described in Chapter III. Activity for the nucleotide polymerizing enzymes is given as pmol [^{3}H]NTP or [^{3}H]NDP converted to acid-insoluble [^{3}H]NMP by 20 µg enzyme. RNase activity is given as µg [^{3}H]poly C digested by 20 µg enzyme.

digested under the standard poly G polymerase assay conditions is equivalent to only 1-5% of the input template RNA.

2. Template-independent nucleotide polymerization. In some of the early enzyme preparations, we noted that template-independent nucleotide polymerization could be detected upon prolonged incubation of the enzyme in the absence of added template RNA (Fedoroff and Zinder, 1971). Since similar observations have been made on partially purified Qβ replicase preparations (Banerjee et al., 1969), it was of interest to analyse the nature of this reaction and its product. The product of the template-independent f2 reaction was found to be an 8-10 S, single-stranded polynucleotide. By annealing criteria, it did not appear to be homologous to f2, Qβ or REOvirus RNAs, nor to E. coli bacterial RNA. Finally, the relative amount of template-independent activity varied from enzyme preparation to enzyme preparation and appeared to decrease during purification. The nucleotide polymerization observed in the absence of added template RNAs in the case of both Qβ and MS2 replicases has been attributed to the presence of endogenous template RNA in the enzyme preparation (Weissmann and Ochoa, 1967; Banerjee et al., 1969). However, the properties of the product of the f2 template-independent reaction appeared to be those of a random polynucleotide, suggesting that perhaps the template-independent activity of the f2 enzyme preparations was attributable to some contaminating enzyme. Some of the properties of the template-independent activity in such an f2 enzyme preparation are given in Table IV. The reaction is stimulated by the omission of the triphosphate-regenerating system from the assay mixture, completely inhibited at low concentrations of phosphate, and has a sharp magnesium optimum at about 3 mM Mg^{++}, suggesting that it may be attributable to the presence of polynucleotide phosphorylase (Kimhi and Littauer, 1968). Direct assays for polynucleotide phosphorylase, using labeled ribonucleoside diphosphate substrates, suggested that the amount of template-independent activity was indeed correlated with the amount of polynucleotide phosphorylase activity present in the partially purified enzyme. Finally, when a bacterial

Table IV

Some properties of the template-independent reaction

Composition of assay medium		Incorporation (pmol [^{3}H]GMP)
Complete		138
Omit phosphoenolpyruvate, pyruvate kinase		679
Magnesium concentration*:	1 mM	36
	3 mM	2120
	5 mM	1260
	10 mM	790
Phosphate concentration*:	0.10 mM	277
	0.25 mM	23
	0.50 mM	2
	1.0 mM	0

The complete reaction mixture is that described for the replicase assay I (Chapter III). Each assay was carried out in 0.05-0.1 ml of the assay mixture, using 68 µg of replicase purified from a PNPase$^+$ strain of E. coli (K38) through DEAE cellulose chromatography. No template RNA was added. Acid-insoluble radioactivity was determined after a 20 min incubation at 30° C.

*Phosphoenolpyruvate and pyruvate kinase omitted.

strain having a mutated polynucleotide phosphorylase gene (E. coli Q13) was selected as host bacterium in place of the standard wild-type strain (E. coli K38), neither polynucleotide phosphorylase activity nor template-independent nucleotide polymerization was detectable in the partially purified poly G polymerase preparation (see Table III). We therefore concluded that the template-independent activity in our early enzyme preparations was most probably attributable to contamination by polynucleotide phosphorylase. The enzyme preparations used in all subsequent experiments were derived from E. coli Q13 and had the properties shown in Table III.

B. Replicase Activity Associated with Partially Purified f2 Poly G Polymerase

Extensive studies on the Qβ replicase enzyme have shown that the replicase is active with both single-stranded Qβ plus and minus strand RNAs, but is inactive with the partially double-stranded replicating RNAs obtained from infected cells by phenol extraction (Weissmann et al., 1967). It has also been established that the Qβ replicase dissociates rather easily into a 'core' replicase capable of using the minus strand template (and having poly G polymerase activity) and one or more cellular proteins which must be present for replicase activity with Qβ plus strand template (Chapter I). Assuming, as a working hypothesis, that a similar situation obtained for the f2 replicase, we tested both types of template derived from f2-infected cells for their ability to stimulate nucleotide polymerization by the partially purified f2 poly G polymerase.

The preparation of template RNAs is fully described in Chapter II. Plus strand template, which is the single-stranded RNA occurring in phage particles, is easily obtained by purifying phage particles and extracting the RNA from the encapsulating protein coat. Minus strand template, which is the complementary copy of the viral RNA, is more difficult to obtain. The minus strand is an intermediate in the intracellular replication of the phage. It is produced only in small

quantities and is present in infected cells, but not in phage particles. Furthermore, minus strands are present primarily in double-stranded and partially double-stranded replicating structures (RE), rather than free minus strands (Weissmann and Ochoa, 1967). As mentioned above, these structures, isolated from Qβ-infected cells, are not templates for the Qβ replicase. However, if the strands are separated by denaturing procedures, such an RE preparation can be used directly as template without further isolation of the minus strands (Weissmann et al., 1967). Adopting this shortcut, we have used denatured f2 replicating structures (denatured RE) as a source of minus strand template (see Chapter II for the properties of this RNA) in the present experiments.

1. Assay conditions favorable for replicase activity. Originally, we used assay conditions favorable for poly G polymerase activity when assaying the enzyme preparation with natural RNA templates. Under these conditions, nucleotide polymerization is stimulated by denatured RE, but not by native RE and only slightly by f2 plus strand RNA. Nucleotide polymerization in the presence of plus strand RNA, however, becomes detectable under somewhat different assay conditions. At low concentrations of template RNA, replicase activity was substantially enhanced by 1) increasing the ionic strength of the incubation mixture and 2) including a brief preincubation of the enzyme at high ionic strength prior to the addition of ribonucleoside triphosphate substrates. The effect of increasing ionic strength, as measured by the incorporation of radioactive GTP into acid-insoluble material in the presence of denatured f2 RE and f2 plus strand RNA, is illustrated in Fig. 15. Incorporation in the presence of either template is stimulated up to an ionic strength of 0.1 and inhibited at higher ionic strengths. Similar results have been obtained when KCl, NaCl or $(NH_4)_2SO_4$ were used at comparable ionic strengths. Activity with f2 plus strand template is much more sensitive to salt stimulation than is activity with denatured RE template. At the optimal ionic strength of about 0.1, replicase activity with plus strand template is stimulated by about

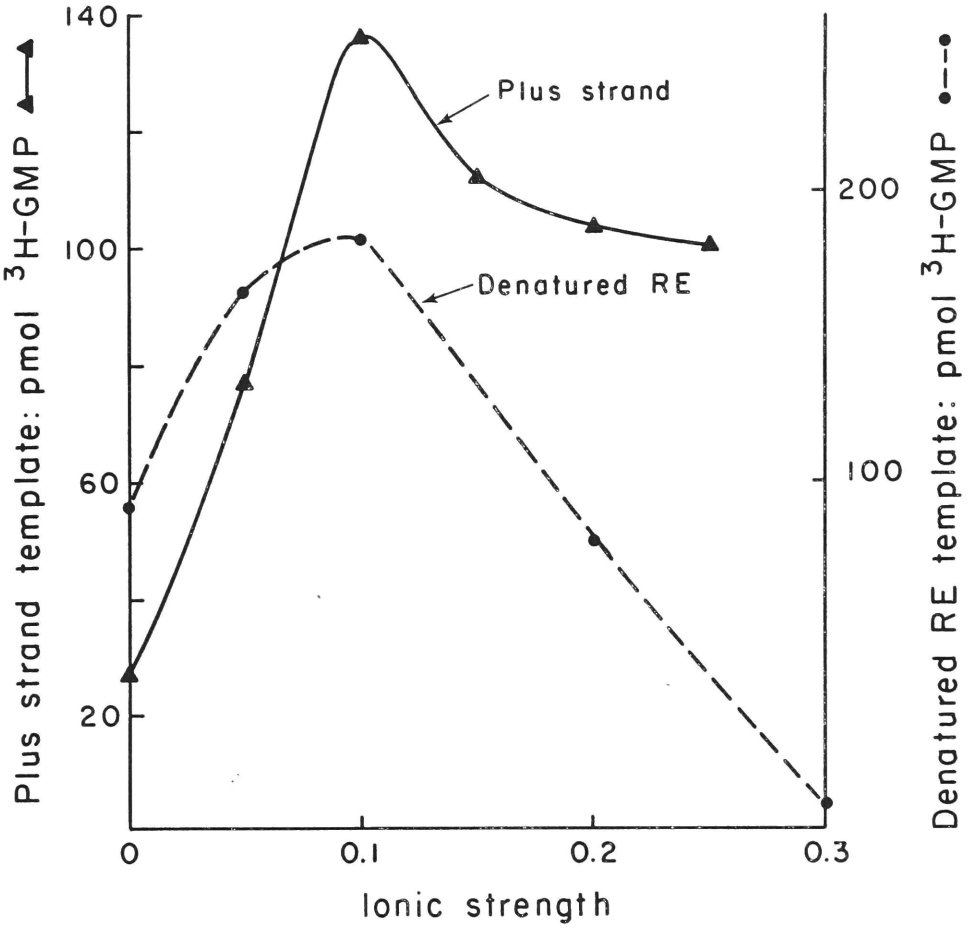

Figure 15. Effect of the ionic strength of the assay medium on replicase activity

Replicase activity was assayed as described in Chapter III (replicase assay II); 20 µg enzyme protein was preincubated for 5 min at 30° C with template RNA (2 µg denatured RE or f2 plus strand RNA) in 25 µl SB20Mg, followed by addition of 75 µl of the reaction mixture. NaCl was added to the preincubation buffer; the indicated ionic strength corresponds to the final ionic strength obtained after addition of the reaction mixture. Acid-insoluble radioactivity was determined after an additional 20 min incubation at 30° C. Template RNA: f2 plus strand RNA (▲——▲); f2 denatured RE (●----●).

fivefold, while incorporation with denatured RE template is stimulated 2-3-fold. Fig. 16 shows that preincubation of the enzyme at high ionic strength exerts a further stimulation. Again the effect is greater with plus strand template than with denatured RE template. The optimal preincubation time appears to be about 5 min. Preincubation of the enzyme in the absence of salt results in the loss of activity. In this experiment, template RNA was present during preincubation. However, similar results are obtained when enzyme is preincubated without template at high ionic strength. Hence the effect of preincubation appears to be on the enzyme complex itself, rather than on the formation of the enzyme-template complex.

Table V shows that replicase activity with plus strand RNA can also be detected under the low salt assay conditions in the presence of sufficient RNA template. The amount of minus strand RNA added in this experiment is about 0.7 μg, since only 1/3 of the RNA in the RE preparation is minus strand RNA (see Chapter II). More than 10 times as much plus strand RNA is required to achieve a comparable level of incorporation under these conditions. Thus the high salt assay conditions* appear to influence the affinity of the enzyme for plus strand RNA. About 20% as much plus strand RNA is required to obtain the same level of nucleotide polymerization under high salt as under low salt conditions†. However, even under high salt conditions, somewhat more plus strand than denatured RE template is required to achieve the same level of activity, suggesting that perhaps the enzyme has a higher affinity for minus than for plus strands, regardless of assay conditions.

2. Template specificity of the f2 replicase. A remarkable feature of RNA phage replicases is their ability to discriminate between template RNAs. With one exception (August et al., 1965), the Group I

---

*The assay conditions referred to for simplicity as 'high salt' include a 5 min preincubation and incubation at 0.1 M NaCl (Chapter III, replicase assay II).

†The assay conditions referred to as 'low salt' correspond to the replicase assay I (Chapter III); no preincubation is included in the assay and no extra salt is added to the basic reaction mixture.

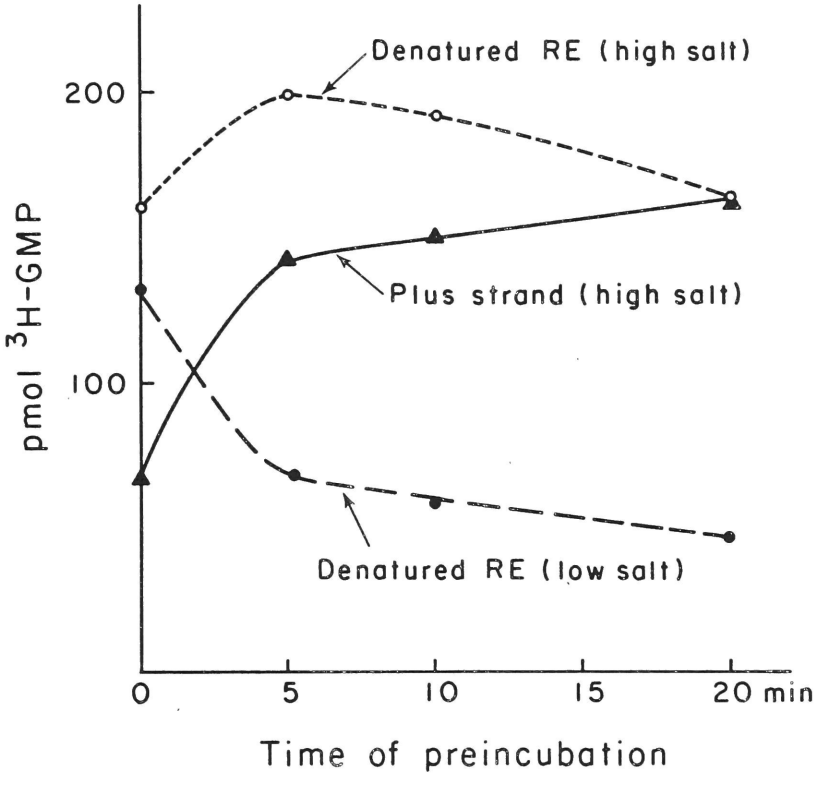

Figure 16. Effect of preincubation time on replicase activity at high and low salt concentrations

Replicase assays were carried out as described in Chapter III (replicase assay II); 20 μg enzyme protein was preincubated for the indicated times in 25 μl SB20Mg containing 10 μmoles NaCl and 2 μg template RNA. The low salt preincubation buffer contained no added NaCl. All reactions were diluted with reaction mixture to a final volume of 0.1 ml; the final salt concentration (where added) was 0.1 M. Acid-insoluble radioactivity was determined after an additional 20 min incubation at 30° C. Denatured RE template, high salt preincubation (o----o); denatured RE template, low salt preincubation (●----●); plus strand template, high salt preincubation (▲——▲).

Table V

Replicase activity with plus strand RNA template under low salt assay conditions

Template	Amount	Incorporation (pmol [^{3}H]GMP)
Denatured f2 RE*	2.0 µg	179
f2 plus strand RNA	0.4 µg	0.6
	1.0 µg	18
	4.0 µg	95
	8.0 µg	123

The replicase assays were carried out as described for replicase assay I (low salt). Each 0.1 ml reaction mixture received 20 µg of replicase protein and the indicated amount of template RNA. Acid-insoluble radioactivity was determined after a 20-min incubation at 30° C.

*The amount of RE is given as total RNA. One-third of this amount is present as minus strand RNA.

and Group III replicases have shown little or no activity with RNAs from sources other than RNA bacteriophage of the group to which they belong (Haruna et al., 1963; Haruna and Spiegelman, 1965a; Fiers et al., 1967). So sensitive is the ability of RNA replicases to discriminate even among bacteriophage RNAs that Miyake et al. (1971) have proposed the template specificity of the bacteriophage replicase as a criterion for the grouping of RNA phages, rather than the previously used criteria of phage particle and RNA properties. The ability of various bacterial and viral RNAs to stimulate ribonucleotide polymerization by the f2 enzyme is shown in Table VI. Under either set of assay conditions, the enzyme is inactive in the absence of template RNA. Neither bacterial RNA (E. coli) nor the viral RNAs of Qβ or REOvirus promote nucleotide polymerization by the f2 enzyme under either high salt or low salt assay conditions. As in the case of the Qβ replicase, the f2 enzyme shows almost no activity with native RE. Denatured RE, which contains both single-stranded plus and minus strand RNAs, is a good template under either set of conditions. At these template concentrations, good nucleotide polymerization with f2 plus strand RNA is observed only under the high salt assay conditions. Thus, the f2 replicase shows the high degree of template specificity characteristic of all known RNA replicases.

Figures 17a and 17b show the time course of the replicase reaction under high and low salt assay conditions. The time course of the reaction is similar in both cases. Very little synthesis is observed after 40-60 min of incubation under either set of conditions. Net synthesis of RNA is not observed in either case. The amount of RNA synthesized with either template is less than half of the amount present as template.

Table VI

Template specificity with and without salt, preincubation

Template	Incorporation (pmol [^{3}H]GMP) High salt 5 min preincubation	Low salt No preincubation
Denatured f2 RE	178	102
Native f2 RE	11	6
f2 plus strand RNA	136	17
No template	0	0
Qβ plus strand RNA	0	0
Denatured Qβ RE	4	3
Native REOvirus RNA	2	3
Denatured REOvirus RNA	8	3
E. coli cellular RNA	1	3

The replicase assays were carried out as described in Chapter III, replicase assay I (low salt) and replicase assay II (high salt). Each 0.1 ml reaction mixture contained 20 µg replicase protein and template RNA as follows: denatured f2 RE, native f2 RE, f2 plus strand RNA, Qβ plus strand RNA, denatured Qβ RE, 2 µg each; native and denatured REOvirus RNA, 1.6 µg; *E. coli* cellular RNA, 2 µg. Acid-insoluble radioactivity was determined after a 20-min incubation at 30° C.

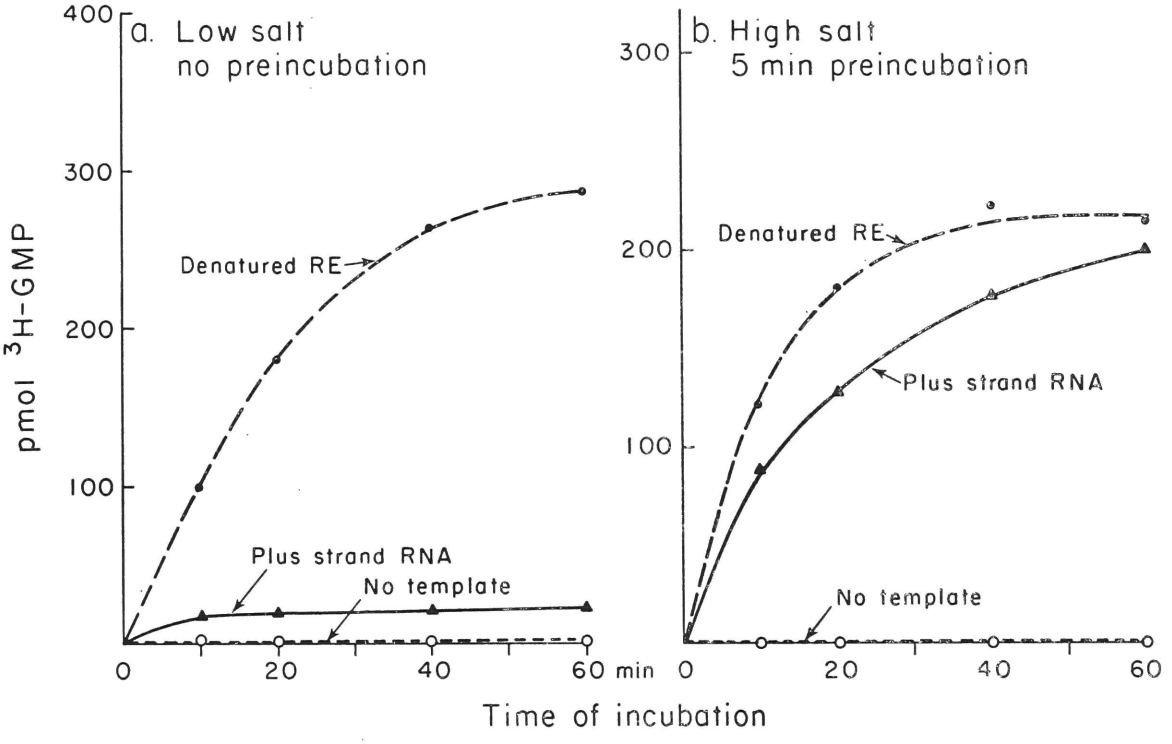

Figure 17. Time course of the replicase reaction at high and low salt concentrations

Part a: Conditions as described for replicase assay I (Chapter III). Each reaction mixture of 0.5 ml contained 100 µg enzyme protein and 10 µg of template RNA; 100 µl aliquots were taken for determination of acid-insoluble radioactivity at the indicated times. Part b: Conditions as described for replicase assay II (Chapter III). Assays included a 5 min preincubation of 100 µg enzyme protein in 125 µl SB20Mg containing 50 µmoles NaCl and 10 µg template RNA. The final volume after addition of the reaction mixture was 0.5 ml and the final NaCl concentration was 0.1 M. Aliquots (100 µl) were taken at the indicated times for determination of acid-insoluble radioactivity. Template: denatured f2 RE (●----●); f2 plus strands (▲——▲); none (o----o).

C. Analysis of the Polynucleotide Product Synthesized In Vitro by the f2 Replicase

1. Annealing experiments. The nature of the polynucleotide product synthesized in vitro by the f2 enzyme was examined in annealing experiments. RNAs having complementary base sequences interact specifically to form ribonuclease-resistant double-stranded structures (Weissmann and Ochoa, 1967). The ability of two different populations of RNA to compete for binding to the same complementary sequence is a sensitive measure of nucleotide sequence homology. The sensitivity and specificity of RNA-RNA hybridization (annealing) for bacteriophage RNAs has been explored extensively by Weissmann and his coworkers (Weissmann et al., 1964a and b; Billeter et al., 1966a and b; Weissmann et al., 1968). In the present experiments, the fidelity of in vitro transcription by the f2 replicase was judged by the ability of the replicase product to anneal to f2 RNAs. The composition of the replicase product was deduced both from the extent of annealing and from competition experiments. The specificity of the annealing reaction with f2 RNAs and the mathematical treatment of the data were discussed in detail in Chapter II.

Since nucleotide polymerization under low salt assay conditions and at low template concentrations is much greater with denatured RE template than with f2 plus strand template, it appeared likely that the minus strands of the RE were preferentially utilized as template. If the replicase reaction occurs by the well-established Watson-Crick base-pairing mechanism, then the in vitro polynucleotide product made from denatured RE template should consist primarily of f2 plus strand RNA. This prediction was tested by annealing small aliquots of replicase product made with denatured RE template to unlabeled f2 RE in the presence of increasing amounts of unlabeled f2 plus strand RNA. The annealing and isotope dilution behavior of the replicase product made after 10, 20 and 40 minutes of incubation is shown in Fig. 18. The inset to Fig. 18 shows plots of annealed radioactivity (t^*_a) as a

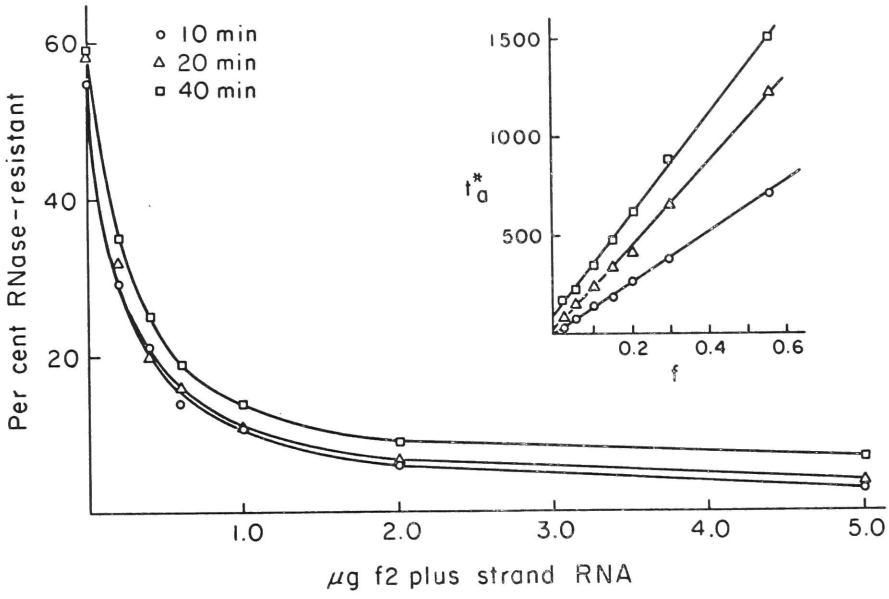

Figure 18. Isotope dilution analysis of [^{3}H]replicase product made in the presence of denatured RE template under low salt assay conditions

[^{3}H]replicase product was synthesized from denatured RE template under replicase assay I conditions (Chapter III), except that the NTP concentrations were 0.5 mM GTP and ATP, 0.2 mM CTP and 0.1 mM UTP. [^{3}H]UTP (sp. act. 280 cpm/pmol) was the labeled substrate; 0.5 ml reaction mixtures containing 10 μg denatured RE and 100 μg enzyme protein were incubated for 10, 20 or 40 min. The replicase product (containing unlabeled template RNA) was extracted and purified as described in Chapter II. A small aliquot of [^{3}H]replicase product (0.07-0.08 μg, sp. act. 1.7-3.5 x 10^4 cpm/μg) was annealed to excess unlabeled RE (0.4 μg) in the presence of increasing amounts of unlabeled f2 plus strand RNA (0-5 μg). Results are given as a fraction of the total replicase product recovered in RNase-resistant form after reannealing. Inset: the amount of radioactivity recovered in RNase-resistant form (t^*_a, cpm/sample) is plotted against the fraction (f) of plus strands expected to anneal at each concentration of unlabeled RNA (equation 7, Chapter II).

function of the amount expected to anneal if all of the radioactivity is present as plus strand RNA (f, see equation 4, Chapter II). The amounts of radioactivity present as plus strand and minus strand RNA, calculated from these curves, are given in Table VII. Also given in Table VII are the experimentally determined values for total acid-insoluble radioactivity (t^*_{obs}) present in each sample. Since the calculated sum (t^*_{calc}) of radioactivity in plus (p*) and minus (m*) strands is equal to or greater than the measured total radioactivity, all of the in vitro replicase product is able to anneal to f2 RNAs and must therefore be homologous to f2 RNA. Furthermore, most of the label is in plus strand RNA, as expected if minus strand template is used preferentially under these conditions. Even after 40 minutes of incubation in the presence of denatured RE, which contains both plus and minus strand RNAs, less than 4% of the radioactivity is in minus strand RNA.

It was shown in the previous section that replicase activity with both plus strand RNA and denatured RE templates was enhanced in the presence of salt. To determine whether the stimulation was attributable only to the enhanced template activity of plus strands, or whether salt also stimulates synthesis of plus strand product from minus strand template, replicase product made from denatured RE in the presence and absence of salt (no preincubation) was analysed as described above. The amount of plus and minus strand product made in 10 and 20 min incubations was calculated from t^*_a vs. f plots and compared with the total acid-insoluble radioactivity. As shown in Table VIII, all of the acid-insoluble product was in f2 plus and minus strand RNA. Despite the fourfold increase in the minus strand content, most of the radioactivity was present as plus strand RNA. Thus most of the increase in total incorporation is attributable to the increased synthesis of plus strand RNA. This result shows not only that salt stimulates replicase activity with minus strand template, but also that minus strand template is preferentially utilized, even in the presence of a similar amount of plus strand template.

Table VII

Composition of replicase product made from denatured RE template under low salt assay conditions

Incubation time	p*	m*	t^*_{obs}	t^*_{calc}	x*	% f2 minus strands	% f2 plus strands
10 min	1300	0	1299	1300	0	0	100
20 min	2140	25	2065	2165	0	1.2	98.8
40 min	2500	100	2550	2600	0	3.9	96.1

Data given in Fig. 18 were analysed as described in Chapter II, section D3. Total acid-precipitable radioactivity (t^*_{obs}) was measured both before and after the annealing procedure and was the same. The amount of radioactivity in f2 plus strand RNA (p*) and f2 minus strand RNA (m*) were calculated as described from the slope and intercept of t^*_a vs. f plots, shown in the inset to Fig. 18. The amount of radioactivity in plus and minus strands (t^*_{calc}) represents the sum of p* and m*. The value of x*, the amount of radioactivity present as non-homologous polynucleotides, is the difference between t^*_{obs} and t^*_{calc} (see Chapter II, section D3). All values are given in [^{3}H]cpm/sample; each sample represents the same fraction (1%) of the total replicase product synthesized in the indicated time interval.

Table VIII

Composition of replicase product made from denatured RE template under high and low salt assay conditions

Incubation time	Assay conditions	p*	m*	t^*_{obs}	t^*_{calc}	x*	% f2 minus strands	% f2 plus strands
10 min	low salt	3060	0	2955	3060	0	0.0	100
	high salt	6420	75	6470	6495	0	1.2	98.8
20 min	low salt	3800	50	3869	3850	19	1.3	98.7
	high salt	8480	475	8847	8955	0	5.5	94.5

Replicase product was synthesized as described in Fig. 18 in the absence (low salt) and presence (high salt) of 0.1 M NaCl in the reaction mixture. [^{3}H]UTP (sp. act. 300 cpm/pmol) was used as labeled substrate; reactions were terminated at 10 and 20 min. Replicase product was purified and annealed as described in Chapter II; isotope dilution data were analysed as described in Chapter II, section D3. Each annealing tube received 0.4 µg unlabeled RE and 0.07 µg labeled replicase product (sp. act. 0.43-1.3 x 10^5 cpm/µg). Reannealing was carried out in the presence of 0-10 µg of f2 plus strand RNA (unlabeled). Values of radioactivity in plus strands (p*), minus strands (m*), total radioactivity (t^*_{obs} and t^*_{calc}), and radioactivity in non-homologous polynucleotides (x*) are given in [^{3}H]cpm/sample; each sample represents the same fraction (2%) of the total replicase product synthesized in the indicated time interval under the indicated salt conditions.

The strand composition of replicase product made in the presence of plus strand template under the high salt assay conditions is shown in Table IX. As with denatured RE template, all of the replicase product is f2 RNA. Most of the replicase product made from plus strand template is complementary minus strand RNA. The fraction of the product present as plus strand RNA increases from less than 6% after 10 minutes of incubation to 27% at 40 minutes. Since plus strands can only be made from minus strand template and the added template consisted only of phage plus strand RNA, the labeled product plus strands must have been synthesized from minus strand template made *in vitro*. Thus the replicase is not only able to synthesize complementary minus strands from plus strand template, but can also reinitiate synthesis of plus strands from the newly-synthesized minus strands. This shows that despite the absence of net RNA synthesis, the f2 replicase is able to carry out all of the component synthetic reactions involved in phage RNA replication. Given plus strand template, it will first synthesize the complementary minus strand and then synthesize plus strand RNA from newly-synthesized minus strands.

2. *Glycerol gradient sedimentation of replicase product*. The data given in Table IX suggest that while chain termination and reinitiation of RNA synthesis do occur *in vitro*, these processes are not very efficient. Analysis of acid-insoluble replicase product made in the presence of either denatured RE under low salt assay conditions or in the presence of plus strand template under high salt assay conditions confirms this impression (Figures 19 and 20). RNA made from either plus or minus strand template sediments predominantly as extensively double-stranded 15-16 S structures. Since no effort was made to separate the unlabeled template RNAs from the labeled product RNAs, this result suggests that most of the product RNA remains associated with template in structures resembling the replicating RNAs isolated from infected cells (Erikson and Franklin, 1966; Robertson and Zinder, 1969). However, some RNase-sensitive 27 S RNA is present in both cases, which shows that some full length phage single-stranded RNA is completed and

Table IX

Composition of replicase product made from plus strand template under high salt assay conditions

Incubation time	p*	m*	t^*_{obs}	t^*_{calc}	x*	% f2 minus strands	% f2 plus strands
10 min	111	1835	1929	1946	0	94.3	5.7
20 min	326	2544	2867	2870	0	88.7	11.3
40 min	1123	3046	4156	4169	0	73.0	27.0

Replicase product was synthesized as described in Fig. 18, except that the assays included a 5 min preincubation and incubation at 0.1 M NaCl (Chapter III, replicase assay II). F2 plus strand RNA, purified from phage particles, was used as template. Reactions were terminated after 10, 20 and 40 min of incubation. Replicase product was purified and analysed by the isotope dilution test as described in Chapter II. Each tube received 1 µg unlabeled f2 RE and 0.14 µg [^{3}H]replicase product (sp. act. 1.5-3.0 x 10^4 cpm/µg). Reannealing was carried out in the presence of 0-10 µg of unlabeled f2 plus strand RNA. Values of radioactivity in plus strands (p*), minus strands (m*), total radioactivity (t^*_{obs} and t^*_{calc}), and radioactivity in non-homologous polynucleotides (x*), are given in [^{3}H]cpm/sample; each sample represents the same fraction (1%) of the total replicase product synthesized in the indicated time interval.

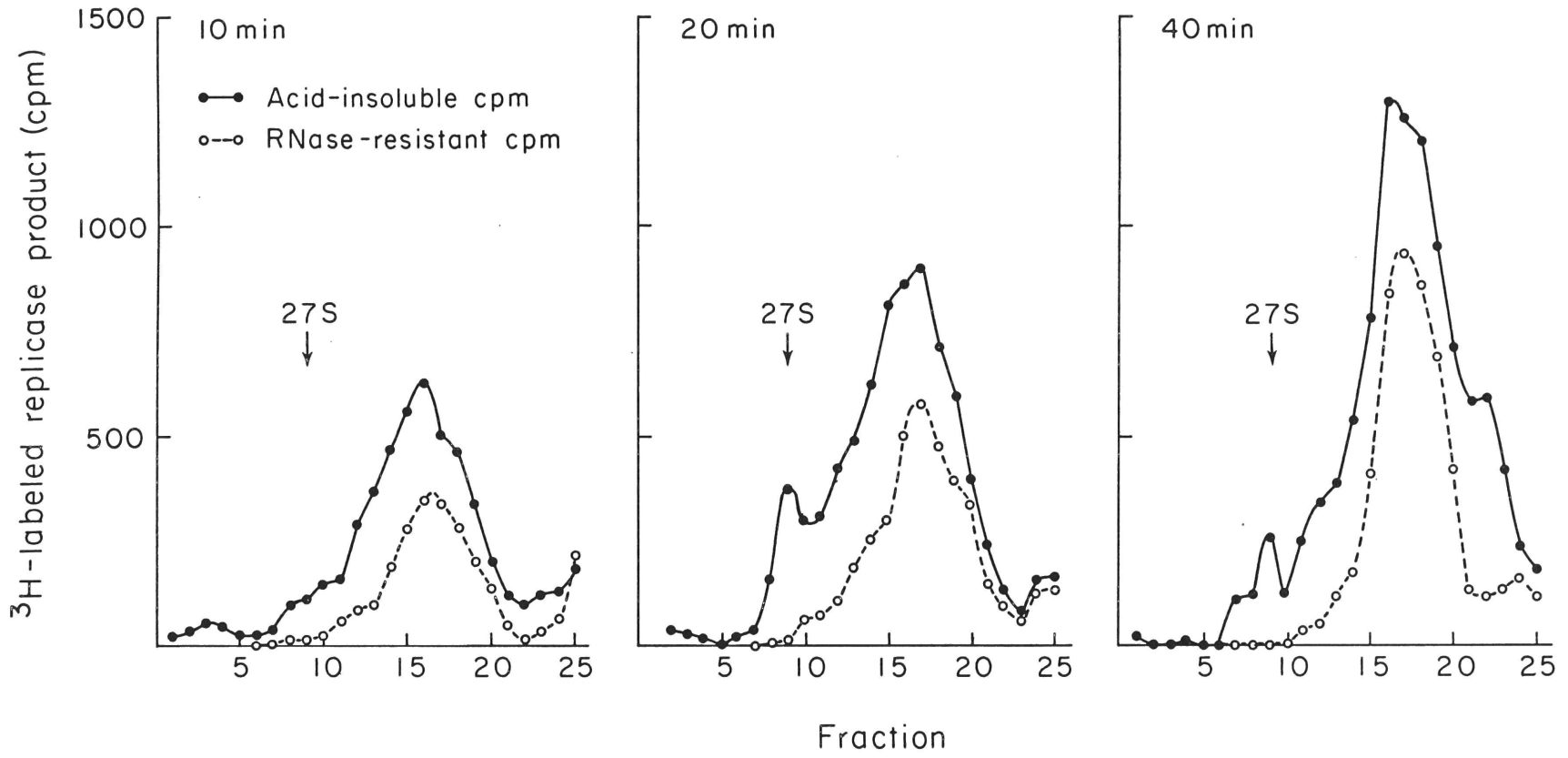

Figure 19. Glycerol gradient centrifugation of [^{3}H]replicase product made in the presence of denatured f2 RE under low salt assay conditions

Replicase product was synthesized under the conditions described in Fig. 18; the replicase product was phenol extracted, but not purified by adsorption to RNA cellulose (Chapter II). Aliquots containing 2.5 µg total RNA in 25 µl TSE buffer were layered on 7-25% glycerol gradients and centrifuged for 4 hr at 50,000 rpm (Chapter II). Equal aliquots of each fraction were taken for determination of total acid-insoluble radioactivity (●——●) and RNase-resistant radioactivity (o----o).

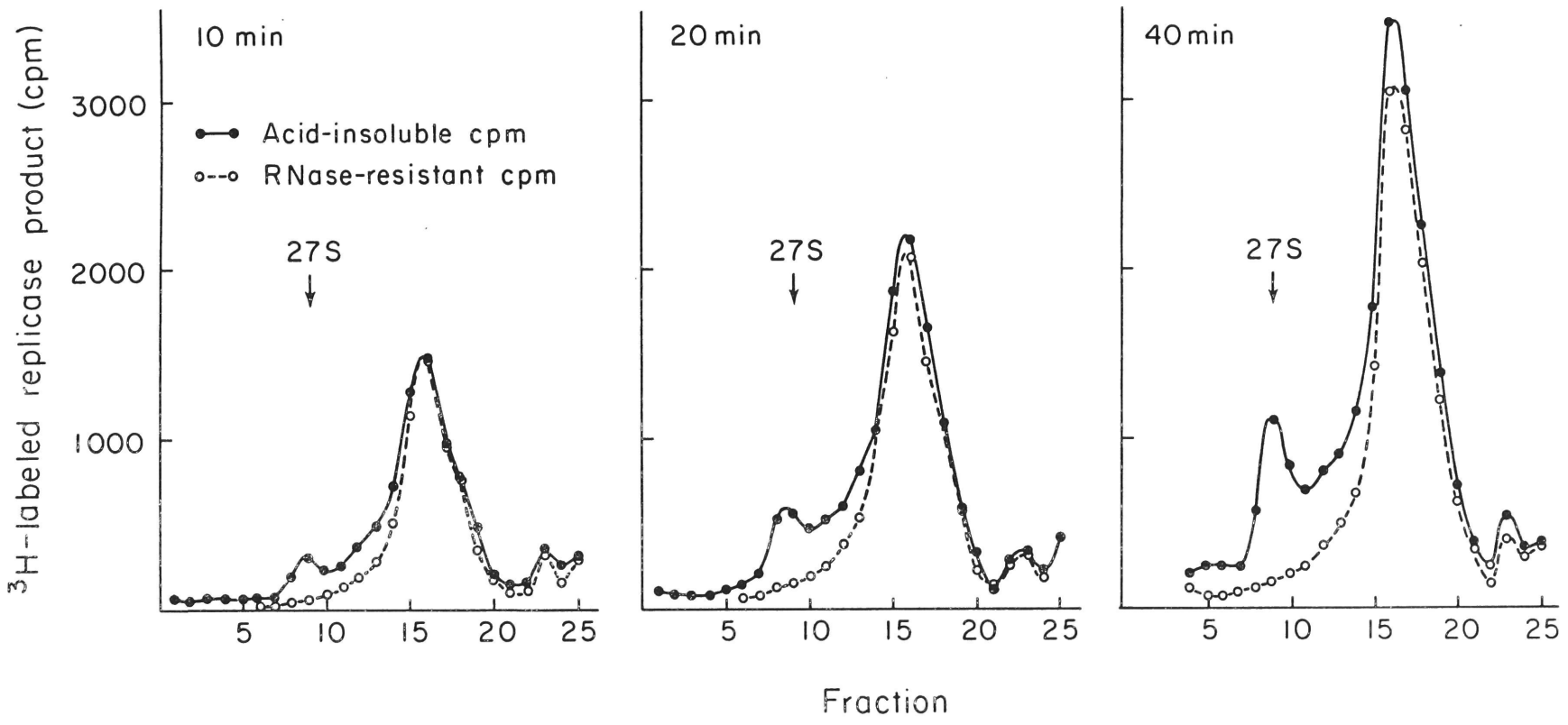

Figure 20. Glycerol gradient centrifugation of [^{3}H]replicase product made in the presence of plus strand template under high salt assay conditions

Replicase product, synthesized as described in Table IX, was phenol extracted, but not purified by adsorption to RNA cellulose (Chapter II). Centrifugation was as described in Fig. 19. Equal aliquots of each fraction were taken for determination of total acid-insoluble radioactivity (●——●) and RNase-resistant radioactivity (o----o).

released. The amount of free single-stranded RNA is greatest when plus strands are used as template. The fraction of RNA sedimenting at 27 S increases from about 6% at 10 min to about 17% at 40 min of incubation.

D. Comparative Studies on the Replicase and Poly G Polymerase Activities

In the following sections we describe a series of studies designed to characterize the f2-induced enzyme. In the course of the experiments described in the previous section, it was noted that the poly G polymerase activity in the partially purified enzyme preparation showed a markedly different response to increases in ionic strength than did the replicase activity. In order to obtain some insight into this difference and perhaps to detect other differences, conditions were selected under which both the poly G polymerase and at least one of the replicase activities could be assayed in parallel. Since substantial replicase activity with denatured RE template and poly G polymerase activity could both be assayed under low salt conditions and at low template concentrations, these are the activities which were compared.

1. Effect of ionic strength. As described above, replicase activity with both plus and minus strand templates is enhanced by the presence of salt during incubation. The upper curves in Figures 21 and 22 show that the stimulation is quite similar with both NaCl and $(NH_4)_2SO_4$. Replicase activity with denatured RE template is maximal at an NaCl concentration of about 0.1 M and an $(NH_4)_2SO_4$ concentration of about 30-40 mM. The curves are similar and the maximum falls at about the same ionic strength for both salts. Thus the stimulation appears to be related to the ionic strength of the incubation medium, rather than to the composition of the salt. The lower curves in Figures 21 and 22 show that increasing the ionic strength of the incubation mixture has a radically different effect on the poly G polymerase activity. The synthetic polymer-dependent activity shows no salt stimulation and is inhibited even at very low salt concentrations. At the optimal ionic strength for replicase activity, poly G polymerase activity shows a

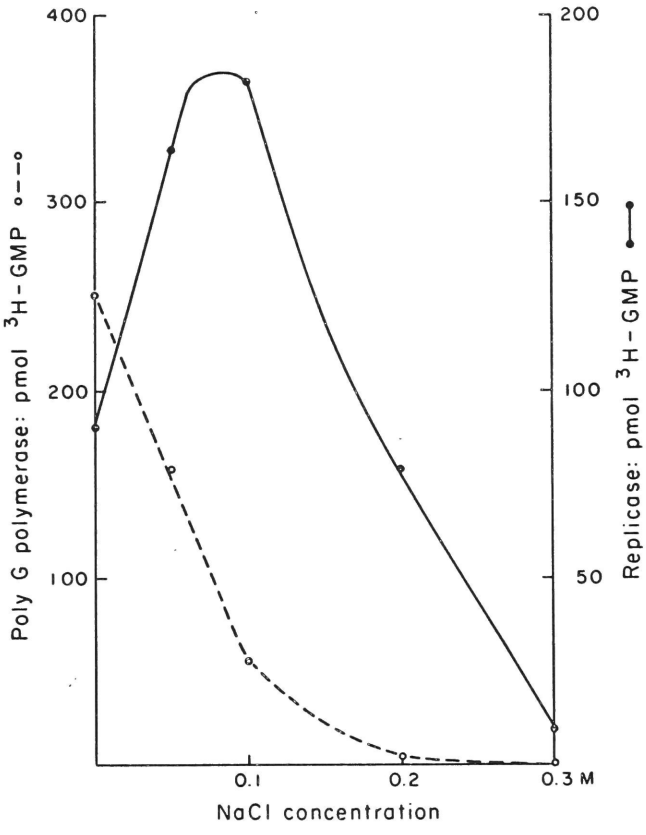

Figure 21. Effect of NaCl concentration on f2 poly G polymerase and replicase activities

Assays were done under replicase assay I (low salt) conditions (Chapter III). Each 0.1 ml replicase assay mixture contained 20 µg enzyme protein, 2 µg denatured f2 RE and NaCl at the indicated concentration. Each 0.1 ml poly G polymerase assay mixture contained 10 µg enzyme protein, 2 µg poly C template and NaCl at the indicated concentration. Acid-insoluble radioactivity was determined after a 20 min incubation at 30° C. Poly G polymerase (o----o); replicase (•——•).

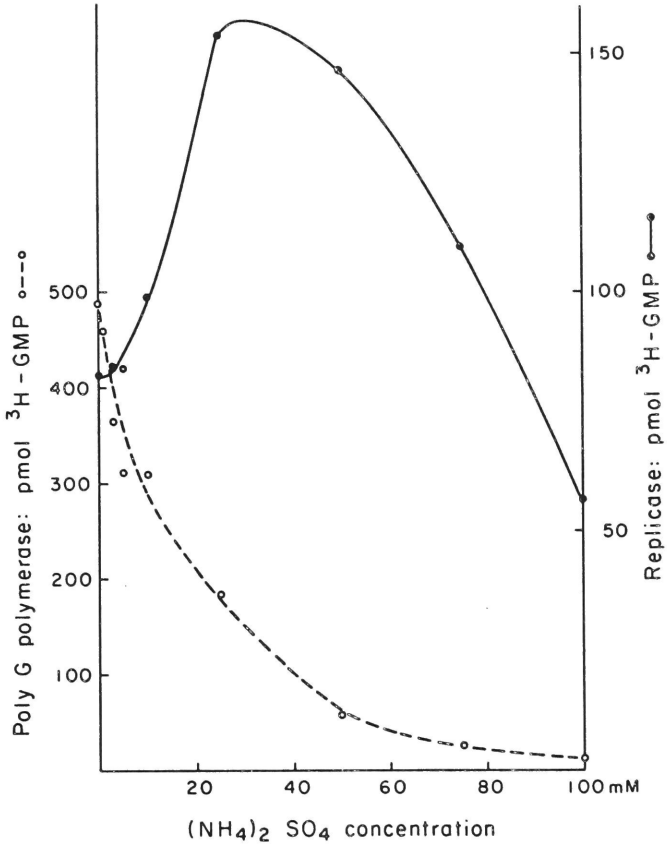

Figure 22. Effect of $(NH_4)_2SO_4$ concentration on f2 poly G polymerase and replicase activities

Assays were done under replicase assay I (low salt) conditions (Chapter III). Each 0.1 ml replicase assay mixture contained 20 µg enzyme protein, 2 µg denatured f2 RE template and $(NH_4)_2SO_4$ at the indicated concentration. Each 0.1 ml poly G polymerase assay mixture contained 20 µg enzyme protein, 2 µg poly C template and $(NH_4)_2SO_4$ at the indicated concentration. Acid-insoluble radioactivity was determined after a 20 min incubation at 30° C. Poly G polymerase (o----o); replicase (•——•).

70-80% inhibition in the presence of either salt.

2. Stability and temperature optimum. The stability of the replicase and poly G polymerase activities was tested by preincubating the enzyme at 30° in a buffer containing no added salt and then assaying for the surviving activity after various intervals of preincubation. Fig. 23 shows that under these conditions, both activities are highly unstable. Half of the activity is lost within 10 min regardless of which activity is measured. The curves appear to be bi-phasic, with an initial rapid inactivation, followed by a slower inactivation rate after the first 10 min.

Consonant with the low stability is the observation of a rather low temperature optimum for the two activities. Fig. 24 shows that the optimal incubation temperature is about 30° C. Activity falls off rapidly at higher temperatures, probably due to thermal inactivation of the enzyme. Similar curves of incorporation as a function of incubation temperature are obtained with both the replicase and poly G polymerase activities.

3. Magnesium optimum. Both replicase and poly G polymerase activities require the presence of a divalent cation for activity. However, it can be seen in Fig. 25 that when Mg^{++} is used as the divalent cation, the two activities are maximal at somewhat different magnesium concentrations. The replicase has a rather sharp Mg^{++} optimum in the 5-10 mM range, while the poly G polymerase has a broader optimum in the 20-40 mM Mg^{++} range.

4. Effect of phosphate ion. In view of early conflicting reports on the phosphate sensitivity of a Group I replicase obtained from MS2-infected cells (Weissmann et al., 1964b; Fiers et al., 1967), it was of some interest to determine the effect of phosphate ion on the f2 replicase. The phosphate concentration range tested (1-5 mM) was sufficiently high to inhibit the reversible polynucleotide phosphorylase reaction, but low enough to minimize the general inhibitory effect of ionic strength. Fig. 26 shows that replicase activity is completely unaffected

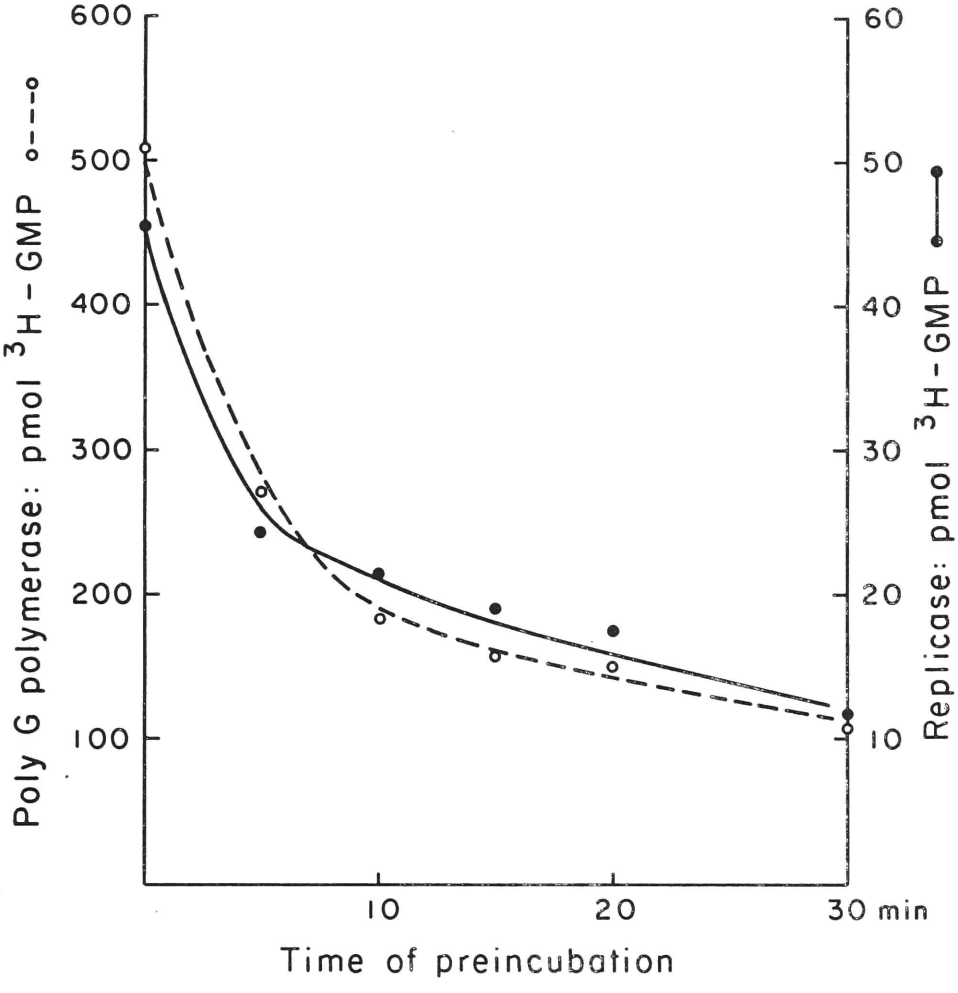

Figure 23. Inactivation of f2 poly G polymerase and replicase at 30° C

The enzyme (20 µg) was preincubated for the indicated times in 25 µl of SB20Mg; 75 µl of the complete reaction mixture was then added as described for replicase assay II (Chapter III) and incubation continued for 20 min at 30° C. Acid-insoluble radioactivity was determined after incubation. Poly G polymerase was assayed in the presence of 2 µg poly C template (o----o); replicase was assayed in the presence of 2 µg denatured f2 RE template (●——●).

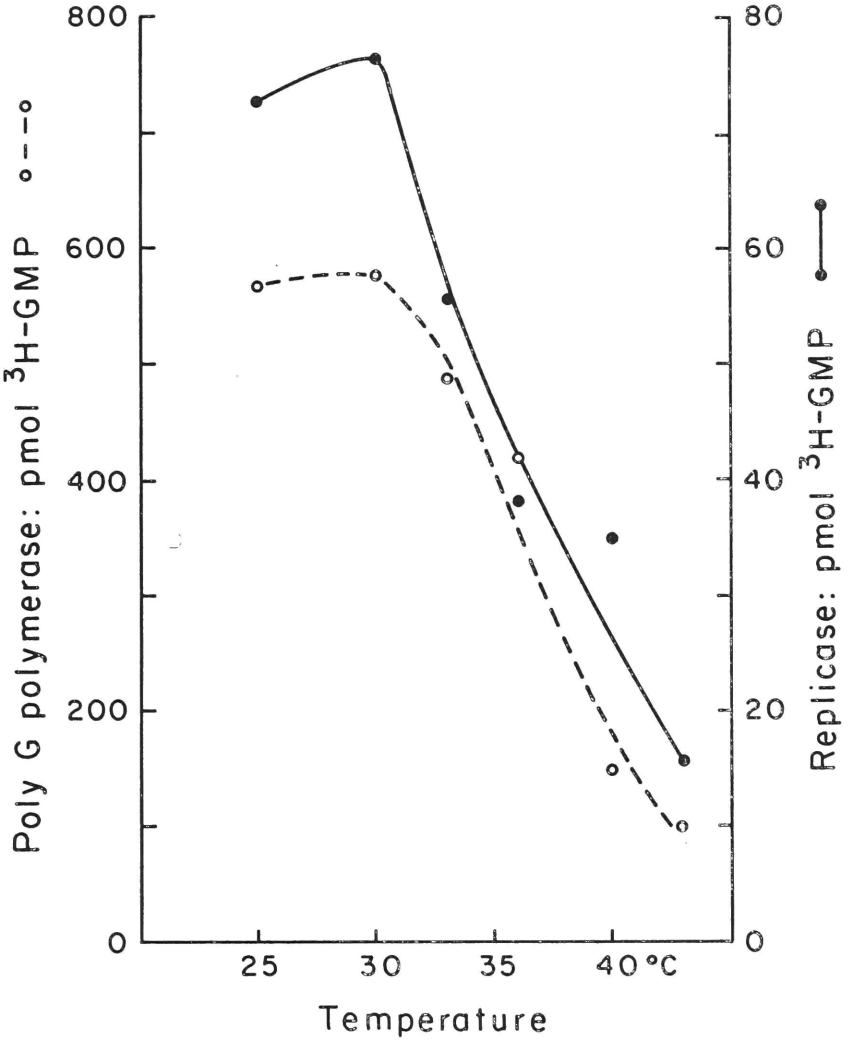

Figure 24. Effect of incubation temperature on poly G polymerase and replicase activities

Assays were done under replicase assay I (low salt) conditions (Chapter III). Each 0.1 ml replicase assay mixture contained 20 µg enzyme protein and 2 µg denatured f2 RE template. Each 0.1 ml poly G polymerase assay mixture contained 20 µg enzyme protein and 2 µg poly C template. Acid-insoluble radioactivity was determined after a 20 min incubation at the indicated temperature. Poly G polymerase (o----o); replicase (•——•).

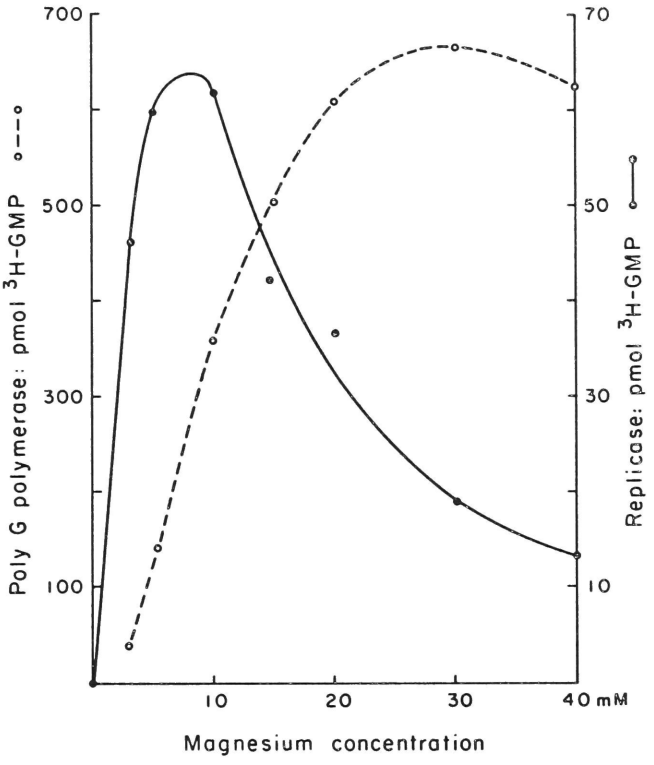

Figure 25. Effect of magnesium ion concentration on f2 poly G polymerase and replicase activities

Assays were done under the replicase assay I (low salt) conditions (Chapter III), except that EDTA was omitted from the reaction mixture and magnesium acetate was added at the indicated concentrations. Each 0.1 ml reaction mixture contained 20 µg enzyme protein and 2 µg denatured f2 RE (replicase assay) or 1 µg poly C (poly G polymerase assay). Acid-insoluble radioactivity was determined after a 20 min incubation at 30° C. Poly G polymerase (o----o); replicase (●——●).

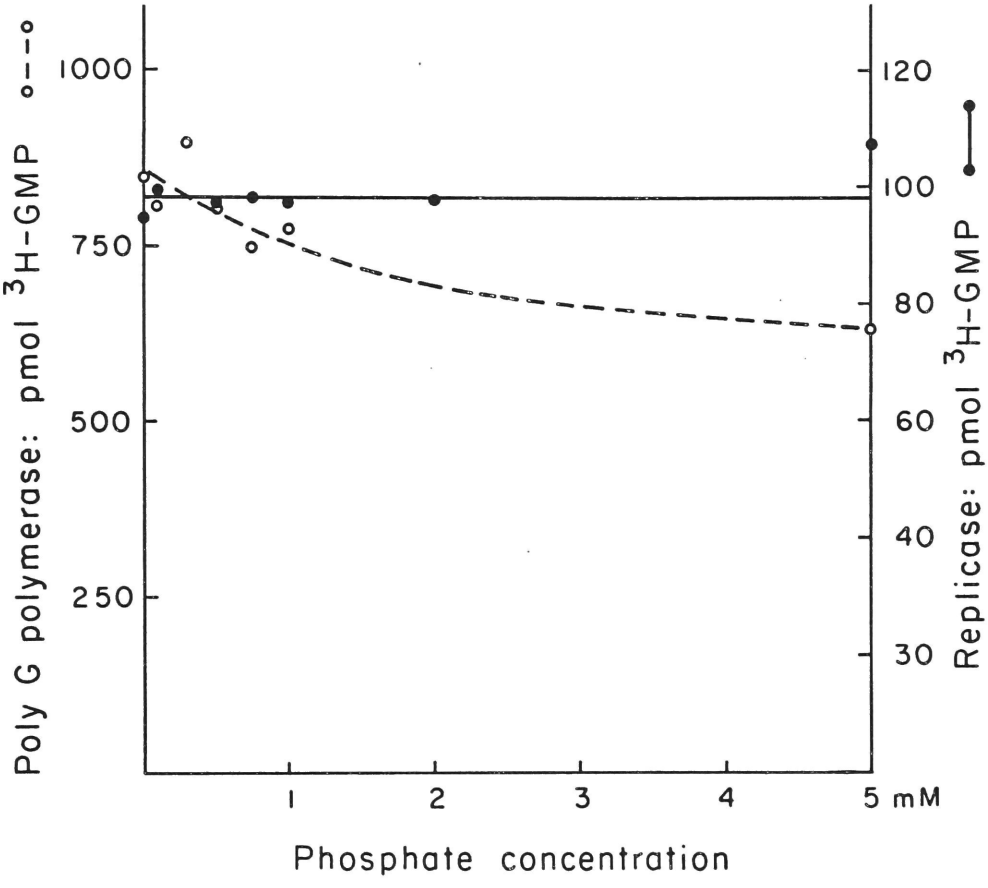

Figure 26. Effect of phosphate ion concentration on f2 poly G polymerase and replicase activities

Assays were done under replicase assay I (low salt) conditions (Chapter III). Each 0.1 ml reaction mixture contained 20 µg enzyme protein, 2 µg denatured f2 RE (replicase assay) or 2 µg poly C (poly G polymerase assay), and sodium phosphate buffer (pH 7.0) at the indicated concentration. Acid-insoluble radioactivity was determined after a 20 min incubation at 30° C. Poly G polymerase (o----o); replicase (•——•).

by phosphate ion in this range of concentrations. The poly G polymerase activity shows a slight inhibition in the 1-5 mM range of phosphate concentration. However, since the inhibition is no greater in the presence of phosphate than in the presence of ammonium sulfate at the same ionic strength, it appears likely that the inhibition is due only to the elevated ionic strength of the assay medium and not to a specific effect of phosphate ion.

5. Template saturation. The effect of template concentration on enzyme activity is shown in Fig. 27. Poly G polymerase activity increases linearly with template concentration up to about 1 μg of poly C per 20 μg of enzyme protein. About 2.5 μg of f2 RE are required to obtain maximal activity in the replicase reaction. However, it was shown in the previous section that the minus strands of the f2 RE are used almost exclusively as template under these conditions. Since only 1/3 of the RNA in RE is minus strand RNA, the actual amount of minus strand template required to obtain maximal activity is less than 1 μg per 20 μg of enzyme. Hence template saturation occurs at low and similar concentrations of minus strands and poly C template. As shown in the previous section, a comparable level of nucleotide polymerization under these conditions requires the presence of more than 10 times as much plus strand template (Table V).

6. Effect of non-template RNAs and synthetic polynucleotides on replicase and poly G polymerase activities. If the synthetic polymer poly C and the natural phage template RNA bind to the same site or sites on the enzyme, it should be possible to show that the presence of one interferes with the transcription of the other. Such an experiment is shown in Fig. 28. Replicase activity, as measured by the incorporation of [^{3}H]ATP (to screen out poly G polymerase activity), was assayed in the presence of increasing amounts of poly C. At the highest concentration of poly C, which is approximately equal to the amount of f2 minus strand template present, replicase activity is inhibited by 65%. Addition of E. coli rRNA or Qβ plus strand RNA, which do not serve as templates for the f2 enzyme (Table VI), has little or no effect on

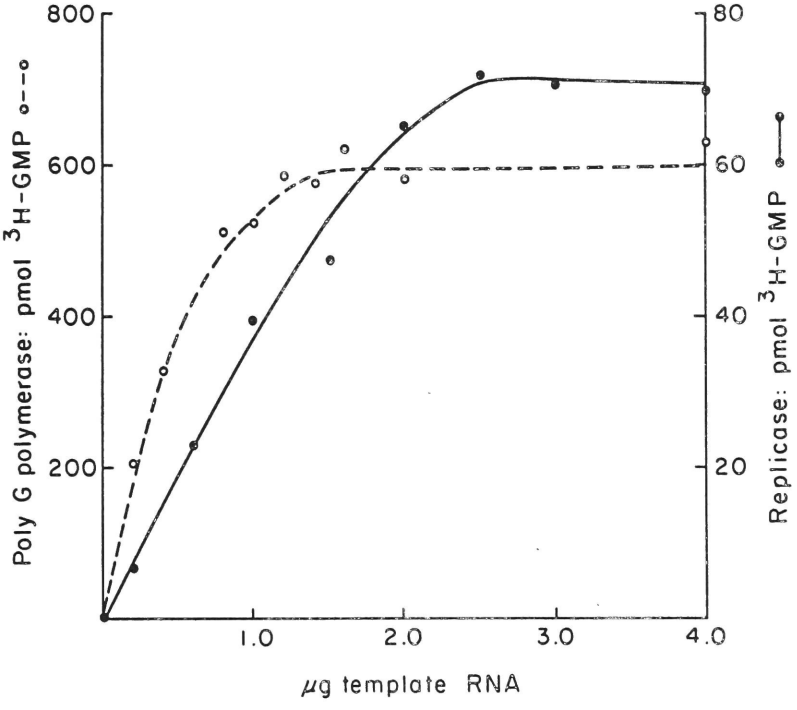

Figure 27. Template saturation curves

Assays were done under replicase assay I (low salt) conditions (Chapter III). Each 0.1 ml reaction mixture contained 20 μg of enzyme protein and the indicated amount of template RNA. Acid-insoluble radioactivity was determined after a 20 min incubation at 30° C. Poly C template (o----o); denatured f2 RE template (●——●).

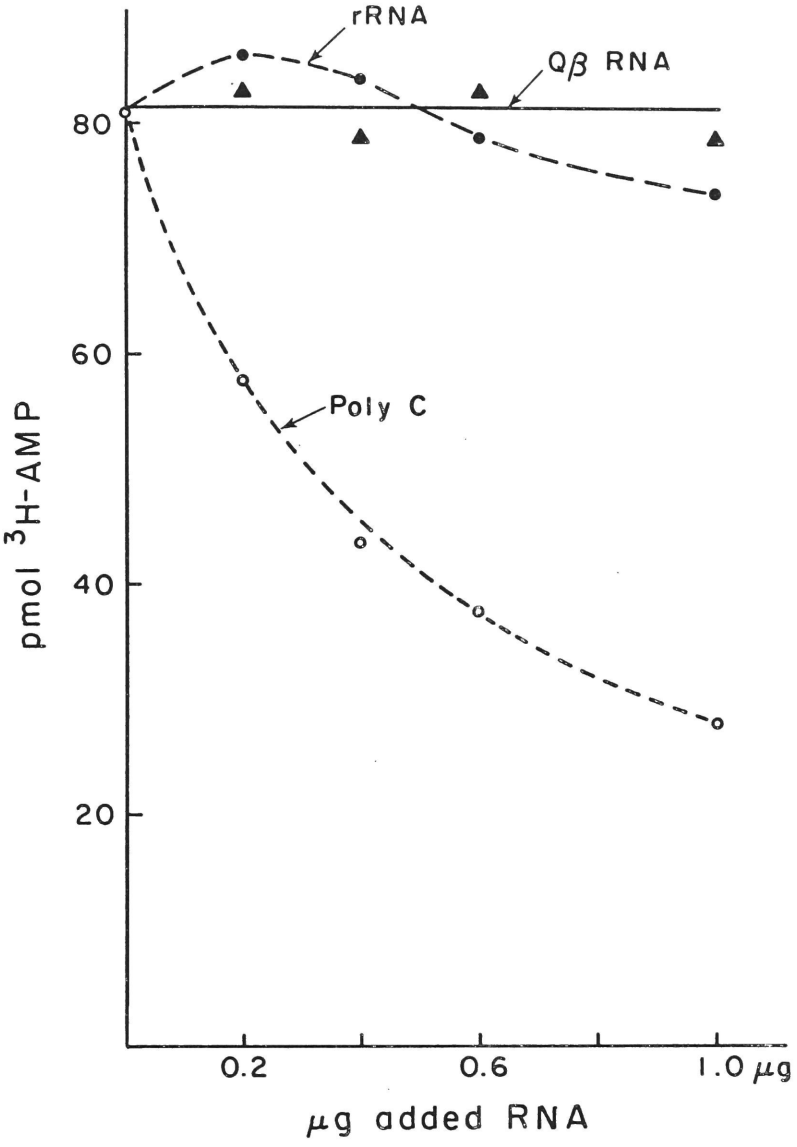

Figure 28. Effect of poly C, E. coli rRNA and Qβ plus strand RNA on f2 replicase activity

Assays were done under replicase assay I (low salt) conditions (Chapter III), except that [^{3}H]ATP was the labeled substrate (sp. act. 28 cpm/pmol). Each 0.1 ml reaction mixture contained 20 μg enzyme protein, 2 μg denatured f2 RE template and the indicated amounts of poly C (o----o), E. coli rRNA (●----●), or Qβ plus strand RNA (▲——▲). Acid-insoluble radioactivity was determined after a 20 min incubation at 30° C.

replicase activity. That poly C interferes specifically with the initial binding of enzyme to template is suggested by the experiment shown in Fig. 29. In this experiment the poly C was added either together with the denatured RE template, or later in the reaction. The inhibitory effect of the poly C decreases the later it is added. Addition of poly C later than 4-5 min has no effect on the overall incorporation.

While it would be tempting to assume a very specific and exclusive affinity of poly C and f2 minus strands for the same site or sites on the same enzyme, the inhibitory effect on replicase activity is not confined to poly C. As shown in Table X, poly U, which is not a template for the f2 enzyme, inhibits replicase activity about as well as does poly C. Table X further shows that while E. coli rRNA and Qβ plus strand RNA have no effect on the poly G polymerase activity, poly U inhibits the poly G polymerase very effectively. This result suggests that the interference with replicase activity by poly C must be interpreted with some caution, since the same results are obtained with a template-inactive polymer.

7. Substrate saturation. Replicase activity with denatured RE template was measured as a function of the nucleoside triphosphate concentration. To measure the effect of one NTP at a time, three of the four were not radioactive and present at a fixed concentration of 1 mM, and the variable substrate was labeled. The results are shown in Fig. 30. The substrate saturation curves for CTP, UTP and ATP are similar and of the simple Michaelis-Menten type for substrate concentrations up to about 0.1 mM. Substrate inhibition occurs at concentrations in excess of 0.1 mM. The substrate saturation curve with GTP as the variable substrate is quite different. Activity increases more slowly with substrate concentration and is optimal at about 0.5 mM. The curve is somewhat sigmoid at low concentrations of GTP. Substrate inhibition is observed at GTP concentrations in excess of 0.5 mM. When the reciprocal of the velocity is plotted against the reciprocal of the substrate concentration (Lineweaver-Burk plot), the contrast is even more apparent

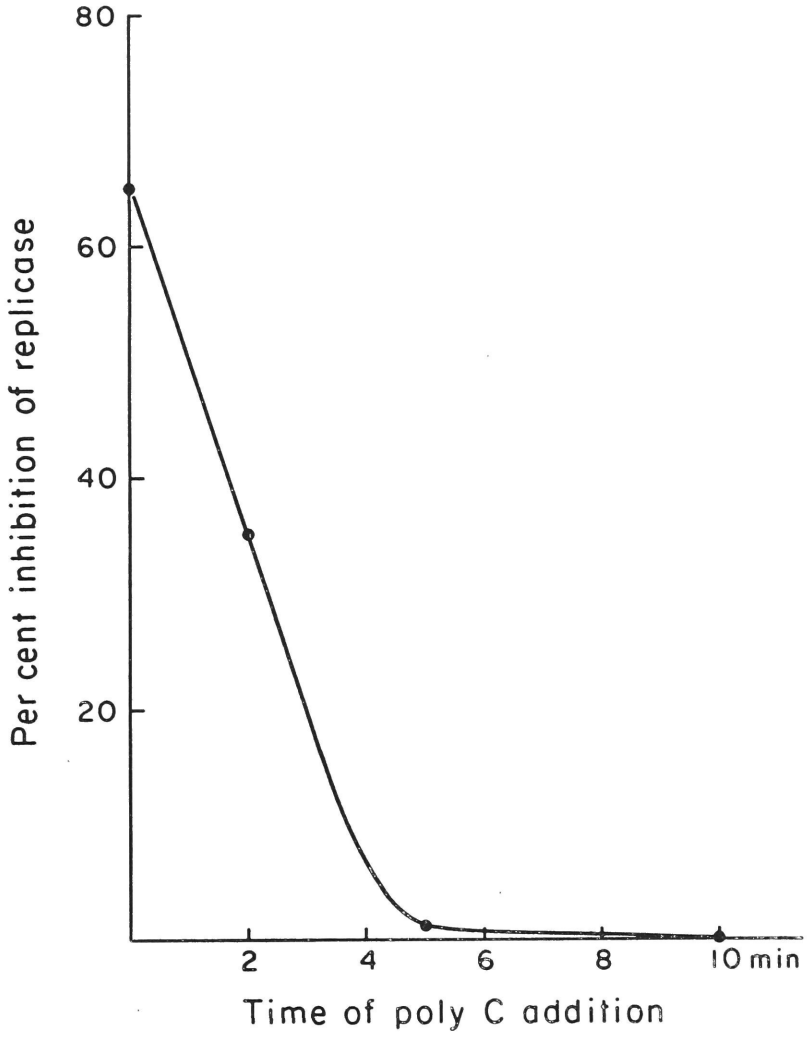

Figure 29. Inhibition of replicase activity as a function of the time of poly C addition

Assays were done under replicase assay I (low salt) conditions (Chapter III), except that [^{3}H]ATP (sp. act. 28 cpm/pmol) was used as the labeled substrate. Each 0.1 ml reaction mixture contained 20 µg enzyme protein and 2 µg denatured f2 RE. Poly C (10 µg) was added after the indicated time of incubation at 30° C. Acid-insoluble radioactivity was determined after 20 min incubation at 30° C. Results are given as % inhibition of replicase activity.

Table X

Effect of non-template RNAs and synthetic polynucleotides on replicase and poly G polymerase activities

Added RNA or homopolymer	% inhibition of [^{3}H]NMP incorporation	
	Replicase	Poly G polymerase
E. coli ribosomal RNA	6	0
Qβ plus strand RNA	2	3
Poly C	65	-
Poly U	67	97

Replicase and poly G polymerase assays were carried out as described in Chapter II (replicase assay I). Each 0.1 ml replicase assay mixture contained [^{3}H]ATP as the labeled substrate, 20 µg of replicase protein, 2 µg denatured f2 RE template and 1 µg of the indicated competing RNA or ribopolymer. Each 0.1 ml poly G polymerase assay contained [^{3}H]GTP as the labeled substrate, 10 µg replicase protein, 1 µg poly C template and 1 µg competing RNA or ribopolymer. Acid-precipitable radioactivity was determined after a 20 min incubation at 30° C.

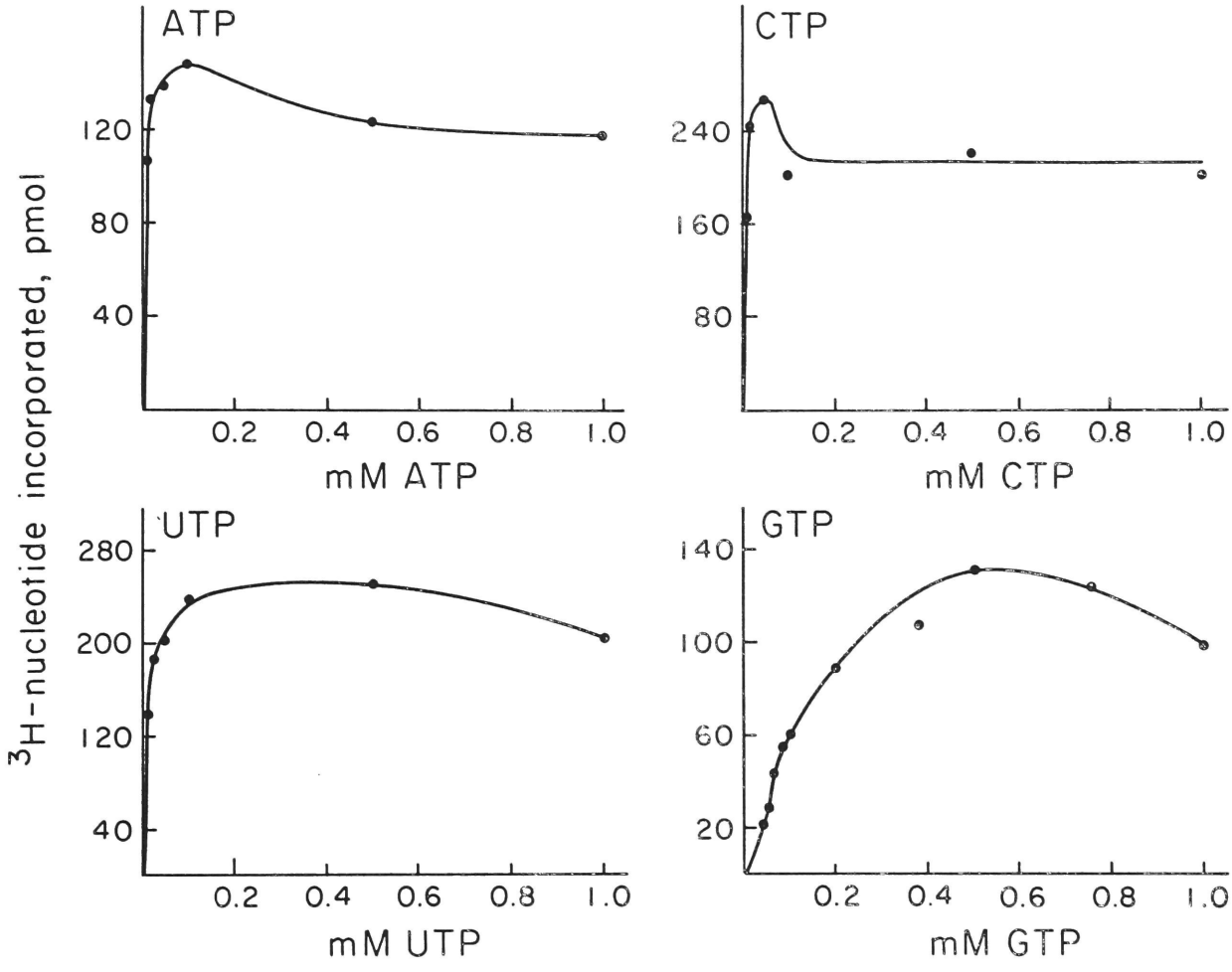

Figure 30. Replicase activity as a function of nucleoside triphosphate concentration

Assays were done under replicase assay I (low salt) conditions (Chapter III) except for nucleoside triphosphate concentrations. In each experiment, three unlabeled nucleoside triphosphates were present at a concentration of 1 mM. The variable NTP was present at the indicated concentration; each 0.1 ml reaction mixture contained 20 µg enzyme protein, 2 µg denatured f2 RE template and 1.1×10^6 cpm of [^{3}H]labeled variable substrate (specific activities: ATP, 22.7 Ci/mmole; CTP, 20.8 Ci/mmole; UTP, 13.0 Ci/mmole; GTP, 9.2 Ci/mmole).

(Fig. 31). In the low concentration range, the double-reciprocal plots for ATP, CTP and UTP are quite linear. Apparent K_m values extrapolated from the linear portion of these curves are all in the 0.01-0.02 mM range, suggesting a high affinity of the enzyme for ATP, CTP and UTP. The double-reciprocal plot for GTP is markedly non-linear in the low concentration range, rendering difficult the estimation of a Michaelis constant. However, extrapolation of the relatively linear portion at high substrate concentrations gives an estimate of 0.13-0.15 mM for the apparent K_m.

The substrate saturation curve for GTP in the poly G polymerase reaction is shown in Fig. 32a and is quite similar to the analogous curve for the replicase reaction. However, the activity optimum occurs at an even higher GTP concentration (about 1 mM) in this reaction than in the replicase reaction. It can be seen in Fig. 32b that the double-reciprocal plot of the GTP saturation data shows a marked upward concavity at low substrate concentrations. Extrapolation of the linear portion of the curve at high substrate concentrations gives an estimate of about 1 mM for the apparent K_m. Fig. 33 shows that when the GTP saturation data obtained in either the replicase or poly G polymerase reactions are plotted as a function of the square of the substrate concentration ($1/v$ vs. $1/s^2$), the curves become linear. Apparent K_m values in the 0.1-0.2 mM range are obtained for GTP in both reactions by extrapolation. However, the values obtained by extrapolation of higher order plots, although frequently referred to as apparent K_m's, are not true Michaelis constants (Atkinson et al., 1965).

E. Comparative Studies on the f2 and Qβ Replicases

The results of early studies on Group I replicases suggested the existence of rather marked differences between these enzymes and the well-studied Qβ replicase (Chapter I). The results of the studies described in Chapter IV, however, show that the poly G polymerase moieties of the two enzymes are extremely similar. Both the Qβ and f2 poly G polymerases consist of four polypeptides, three of which are

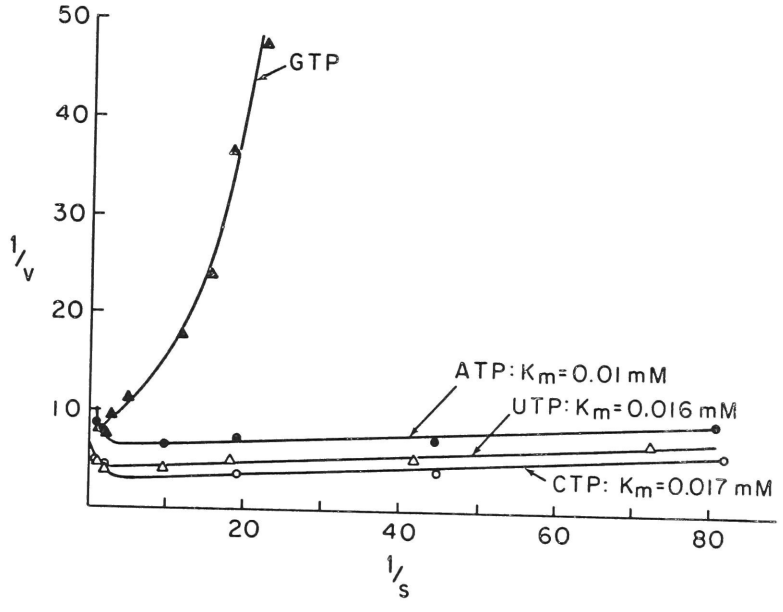

Figure 31. Reciprocal substrate concentration curves for the replicase reaction

Data from the experiment described in Fig. 30 are plotted in double-reciprocal form. GTP (▲——▲); ATP (●——●); UTP (Δ——Δ); and CTP (o——o).

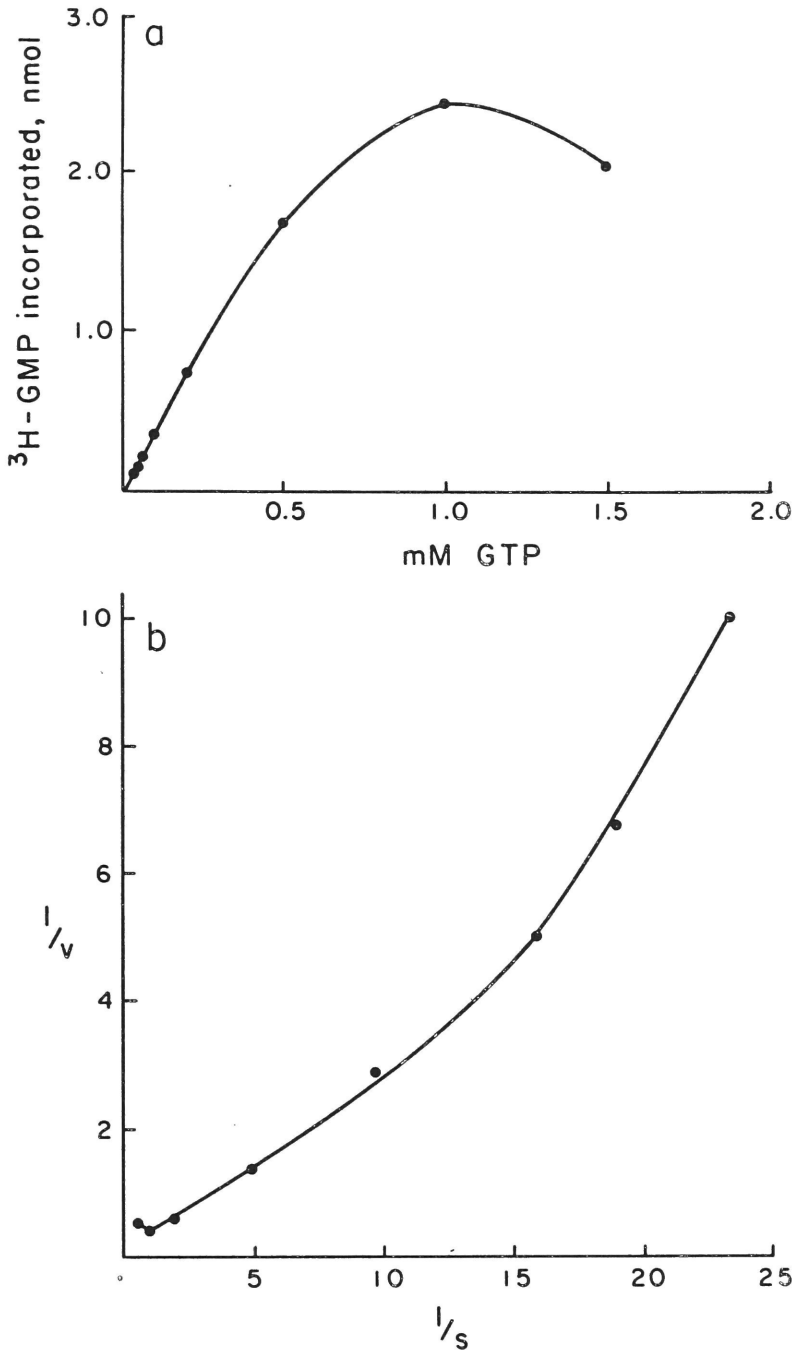

Figure 32. Substrate saturation curves for the f2 poly G polymerase

Assay conditions were those described in Fig. 30, except that ATP, CTP and UTP were omitted. Each tube received 5.5 x 10^5 cpm of [^{3}H]GTP and unlabeled GTP at the indicated concentration. Acid-insoluble radioactivity was determined after a 10 min incubation at 30° C. Part a: Poly G polymerase activity as a function of GTP concentration; part b: double-reciprocal substrate saturation curve.

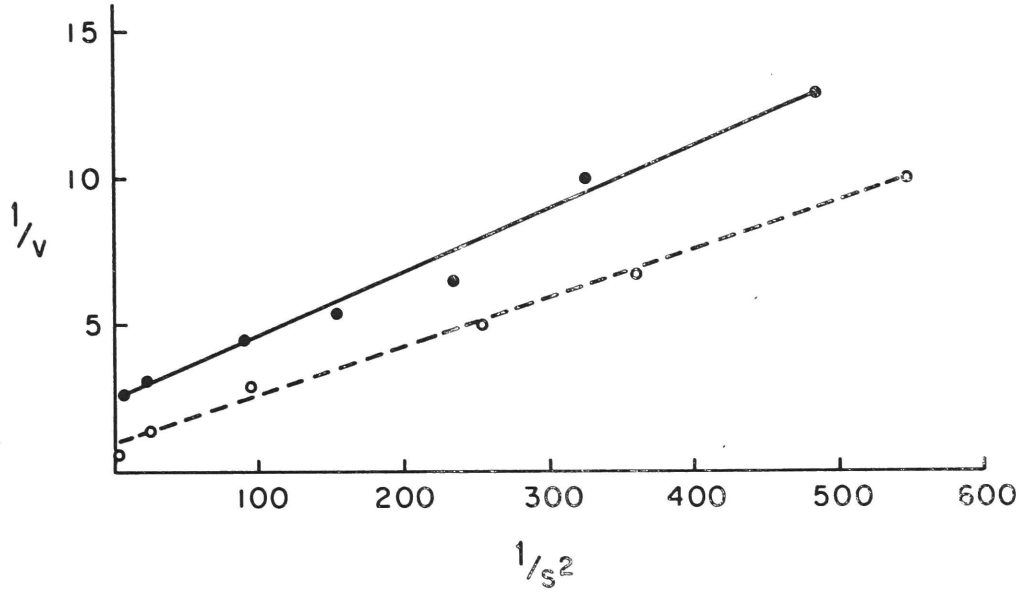

Figure 33. Reciprocal substrate concentration curves for poly G polymerase and replicase activities

GTP saturation data derived from Fig. 31 for the replicase and Fig. 32 for the poly G polymerase replotted as a function of $1/s^2$. Reaction velocities were adjusted for total nucleotide incorporation in the case of the replicase activity. Poly G polymerase (o----o); replicase (●——●).

electrophoretically indistinguishable between the two enzymes. The results presented in this chapter suggest that the replicase activities of the f2 and Qβ enzymes differ in some respects. The purification procedures used by others to prepare Qβ replicase differ somewhat from those used here for the f2 replicase (Haruna and Spiegelman, 1965a; Eoyang and August, 1968). In addition, the f2 replicase was isolated from cells infected with a mutant which hyperproduces the replicase protein, while most Qβ replicase preparations are obtained from wild-type Qβ-infected cells. To rule out the possibility of artifactual differences in properties arising from these differences in source material and purification methods, we purified Qβ replicase by our methods from E. coli Q13 infected with am 12, a replicase-hyperproducing coat mutant of Qβ (Horiuchi and Matsuhashi, 1970).

In Fig. 34, elution profiles of the Qβ and f2 poly G polymerases from DEAE cellulose have been superimposed. Enzyme activity is plotted as a function of the salt concentration of the eluant (measured by conductivity). As might be expected from the results described in Chapter IV, the two poly G polymerases show similar elution profiles. The Qβ poly G polymerase elutes at a slightly higher salt concentration than does the f2 poly G polymerase, but the difference is not profound. When these poly G polymerase preparations, purified by liquid-polymer phase partitioning and DEAE chromatography as described in Chapter III, were assayed for replicase activity under identical (low salt, low template) conditions, the results shown in Fig. 35 were obtained. Both Qβ am 12 and f2 sus 11 preparations are highly template dependent. The time course of nucleotide polymerization with homologous denatured RE template is similar for both enzymes. It is evident, however, that the enzyme preparations are very different in their response to homologous plus strand template. As described above, the activity of the f2 enzyme with low concentrations of plus strand RNA is almost undetectable under the standard low salt assay conditions. The Qβ replicase, on the other hand, is extremely active with Qβ plus strand RNA. In this experiment, net synthesis of RNA obtained after about 1 hour of incubation and the

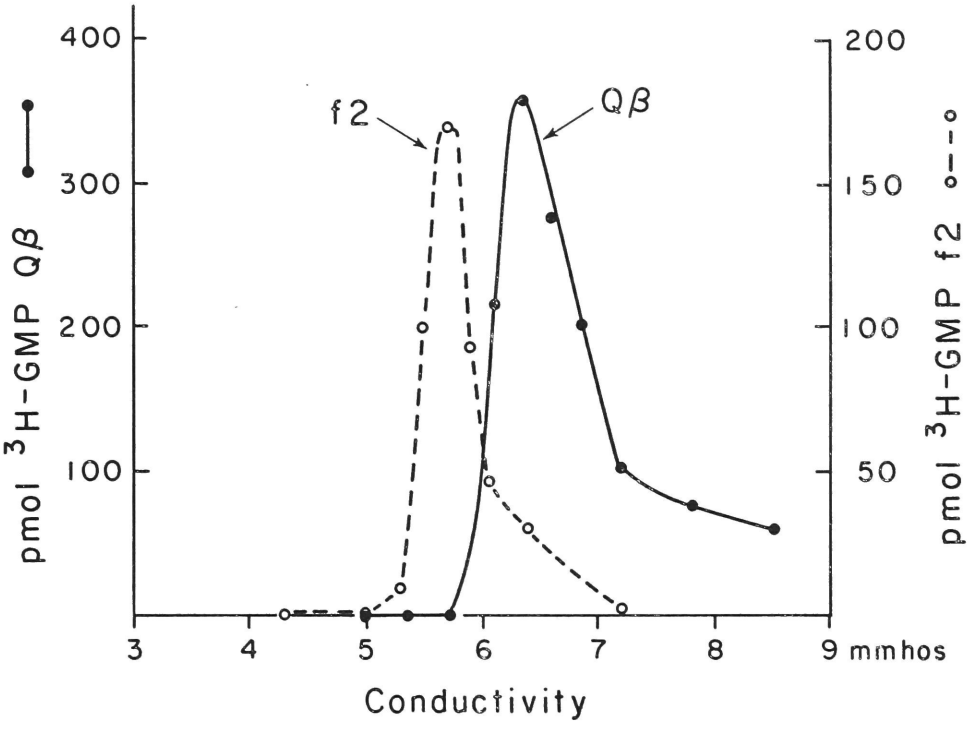

Figure 34. Elution of f2 and Qβ poly G polymerases from DEAE cellulose

Extracts of E. coli Q13 infected with f2 sus 11 and with Qβ am 12 mutants were purified by liquid-polymer phase partitioning and DEAE cellulose chromatography as described in Chapter III. The two preparations were purified on separate columns; enzyme activity is plotted as a function of the salt concentration of the eluant (measured by conductivity). Qβ poly G polymerase activity (●——●); f2 poly G polymerase activity (o----o).

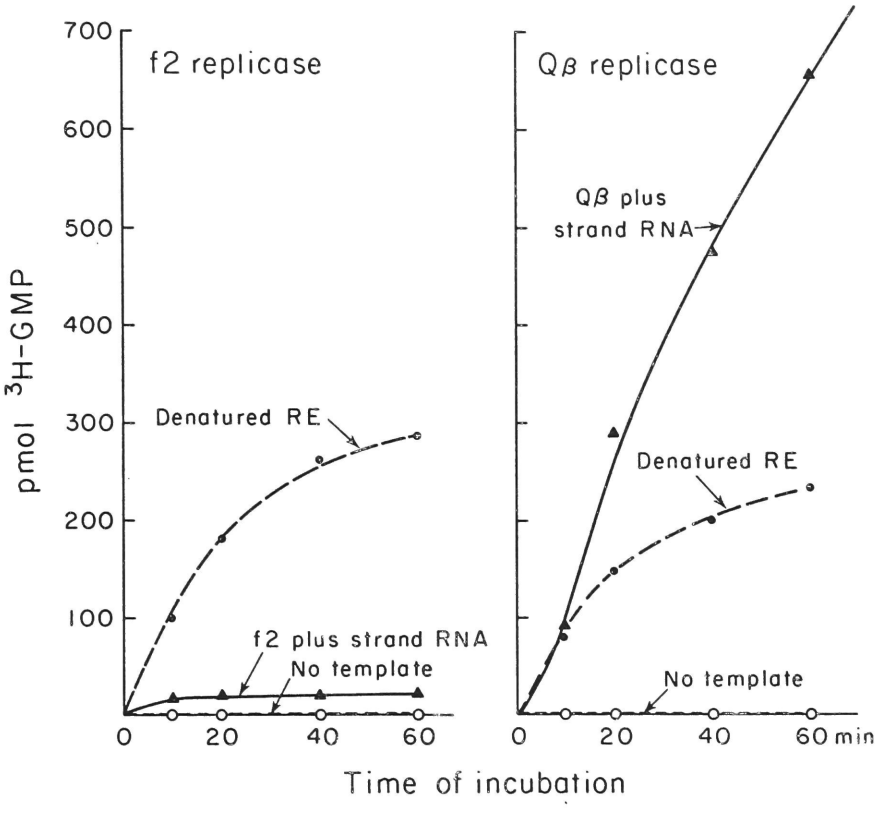

Figure 35. Qβ and f2 replicase activities as a function of time

Assays were done under replicase assay I (low salt) conditions (Chapter III). Each 0.5 ml reaction mixture contained 100 µg f2 enzyme protein and 10 µg denatured f2 RE, 10 µg f2 plus strand RNA or no template for the f2 replicase assay. Each 0.5 ml reaction mixture contained 50 µg Qβ enzyme protein, and 10 µg denatured Qβ RE, 5 µg Qβ plus strand RNA or no template. Incubation was at 30° C for the f2 replicase and 36° for the Qβ replicase. Aliquots of 100 µl were taken for determination of acid-insoluble radioactivity at the indicated times.

rate of RNA synthesis remained constant, even after 2 hr of incubation (not shown). Thus the differences between the f2 and Qβ enzymes observed in studies with plus strand template are not attributable to differences either in source material or preparative procedure.

In view of the differential effect of salt on the f2 replicase and poly G polymerase activities, it was of some interest to test the effects of salt concentration on the analogous activities of the Qβ enzyme. Table XI shows that salt stimulates Qβ replicase activity with both Qβ plus strands and denatured Qβ RE. The extent of stimulation is about the same for both templates (ca. 1.5-fold) and the optimal ionic strength is about the same as for the f2 replicase. However, the Qβ poly G polymerase, unlike the f2 poly G polymerase, is not inhibited by salt, but rather shows the same stimulation as the Qβ replicase.

Fig. 36 shows a reciprocal template specificity experiment with the partially purified f2 and Qβ enzymes. As expected from the results given in Table VI, the f2 replicase shows no incorporation with either Qβ plus strand RNA or Qβ denatured RE, even after prolonged incubation. Surprisingly, the Qβ replicase, although inactive with f2 plus strand RNA, shows substantial nucleotide polymerization in the presence of denatured f2 RE. Thus the Qβ replicase appears to be capable of using f2 minus strand RNA template, albeit at reduced efficiency.

F. Discussion

Experiments described in this chapter show that f2 replicase activity is present in partially purified preparations of the rifampicin-resistant poly G polymerase produced during infection with bacteriophage f2. The properties of this activity are quite characteristic for an enzyme of this type. The f2 replicase is template-dependent and shows a high degree of template specificity. Among the natural RNA templates tested, only f2 plus and f2 minus strands were found to be active as templates.

Analysis of the *in vitro* product of the replicase reaction shows that it consists of f2 RNAs. Although the f2 replicase does not

Table XI

Effect of salt on partially purified Qβ replicase

	Incorporation (pmol [^{3}H]GMP)		
Template:	Qβ plus strands	Qβ denatured RE	poly C
Added salt			
--	160	57	184
25 mM $(NH_4)_2SO_4$	204	80	232
50 mM $(NH_4)_2SO_4$	142	61	155
100 mM NaCl	---	--	320

Assays were done under replicase assay I (low salt) conditions (Chapter III). Each 0.1 ml reaction mixture contained 10 μg Qβ replicase (purified through DEAE chromatography), and 0.5 μg Qβ plus strand RNA, 1 μg Qβ denatured RE or 1 μg poly C. The salt was added, where indicated, prior to addition of enzyme. Acid-insoluble radioactivity was determined after a 20 min incubation at 36° C.

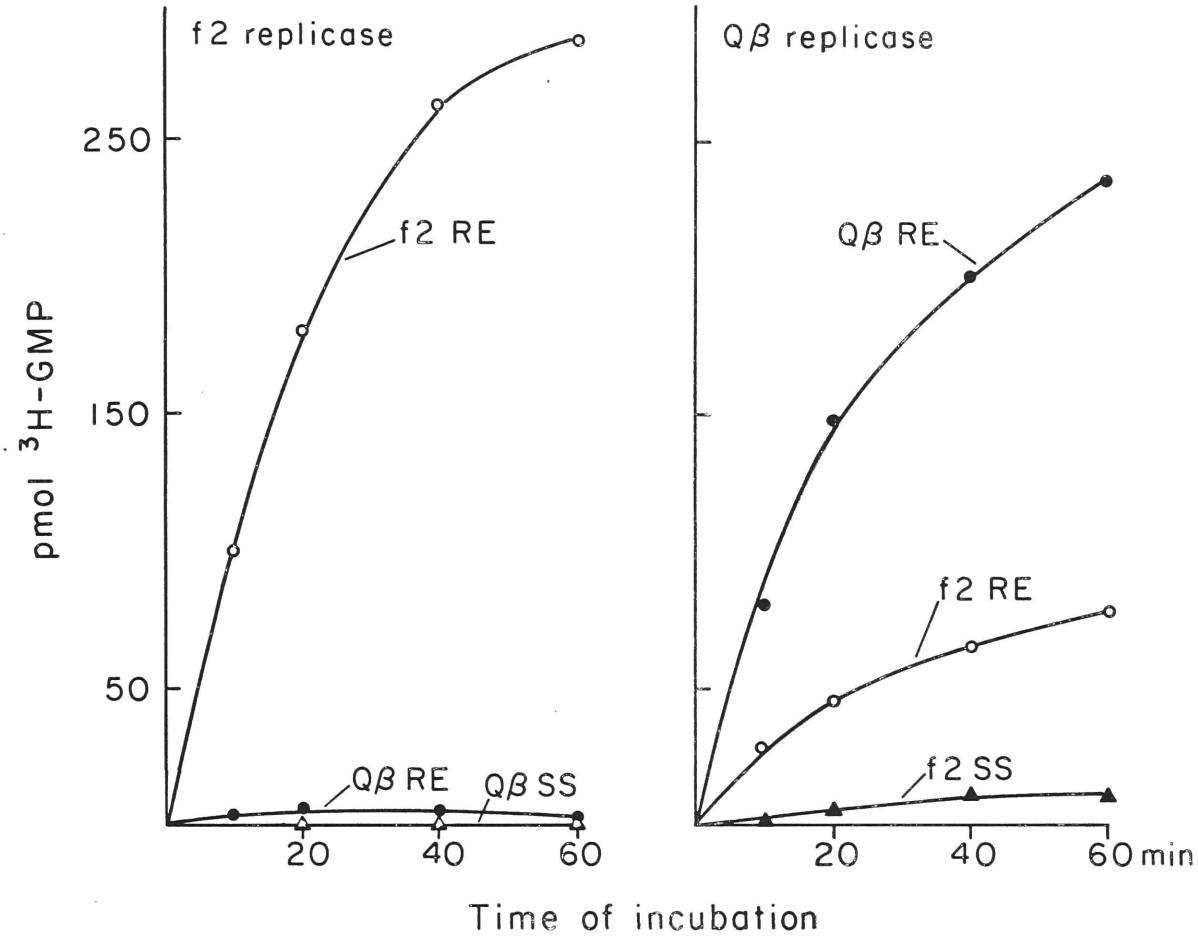

Figure 36. Template specificity of the f2 and Qβ replicases

Assay conditions were as described in Fig. 35. F2 replicase activity was assayed with f2 denatured RE template (o——o), Qβ denatured RE template (●——●), and Qβ plus strand template (Δ——Δ). Qβ replicase activity was assayed with denatured Qβ RE template (●——●), f2 denatured RE template (o——o), and f2 plus strand template (▲——▲).

synthesize more than the input template amount of RNA, it appears to be capable of carrying out all of the component reactions of phage RNA replication. In the presence of phage plus strand template, the replicase first synthesizes complementary minus strand RNA and then uses the newly-synthesized minus strand RNA for the synthesis of plus strand RNA. The absence of net RNA synthesis and the preponderance of partially double-stranded structures in the product RNA suggest that the f2 enzyme terminates RNA synthesis and releases finished single strands quite inefficiently. Comparative studies on the Qβ and f2 enzymes isolated from similar sources by the same procedure suggest that these differences are not artifacts of enzyme isolation and are not attributable to the use of mutant phage strains. The Qβ and f2 enzymes differ substantially in stability (Eoyang and August, 1968; Eoyang and August, 1971; Chapter V). However, it is not clear whether the inability of the f2 enzyme to replicate f2 RNA is due only to its lower stability. It is quite possible that as yet unidentified factors or conditions are required to facilitate net synthesis of RNA by the f2 replicase. Since the fidelity of *in vitro* transcription was judged only by annealing criteria, rather than by the biological competence of the product RNA, it is possible that the RNA synthesized *in vitro* is defective. Perhaps an error level too low to be detected by annealing criteria is sufficient to prevent release of completed RNAs and/or reinitiation of RNA synthesis.

Comparative studies on the poly G polymerase and replicase have revealed both similarities and differences in the responses of these activities to the manipulation of experimental conditions. Some knowledge of the structural relationship between the replicase and poly G polymerase is important for the evaluation of these results. This question is considered in the next chapter. Discussion of the results reported in this chapter is therefore deferred to Chapter VI.

CHAPTER VI

RELATIONSHIP BETWEEN THE REPLICASE AND THE POLY G POLYMERASE

In Chapter IV we discussed the purification of a phage-induced poly G polymerase from f2-infected cells. We presented evidence that the poly G polymerase consists of 4 polypeptides, one of which is the phage-coded replicase protein. In Chapter V we showed that the partially purified poly G polymerase enzyme displays replicase activity and discussed some properties of both the replicase and poly G polymerase activities. In the course of these studies it became apparent that the replicase and poly G polymerase activities differed markedly in some respects, raising an inevitable uncertainty as to their identity. In this chapter, we will discuss experiments designed to elucidate this question.

A. Evidence for Separation of Poly G Polymerase and Inactive Factor Fraction

To recapitulate briefly, it was our basic assumption that the f2 and Qβ replicases were organized in much the same way. As illustrated in Fig. 2, the Qβ replicase consists of the 'core' replicase moiety, capable of using either Qβ minus strands or synthetic polymers as template, and some macromolecular factors necessary for the replication of Qβ plus strand RNA. The structure of the f2 poly G polymerase and the properties of the partially purified enzyme proved to be quite similar to those observed for the Qβ replicase system in many respects, justifying our assumption in a general way. If the organization of the two enzymes is indeed identical, one would expect activity with f2 minus strand template to remain proportional to f2 poly G polymerase activity from preparation to preparation and during the course of purification, even though activity with plus strands might be lost.

One of the first indications that this might not be the case for the f2 replicase system came from comparing the replicase activities of

different poly G polymerase preparations. Different preparations tend to vary with respect to the specific activity of the phage-induced poly G polymerase. This is in part due to differences in the efficiency of infection by the phage. Another somewhat variable parameter is the efficiency of dissociation of the enzyme-template complex early in the purification (Chapter III). As a result, preparations differing as much as tenfold in the specific activity of the poly G polymerase have been obtained after DEAE chromatography. Two such markedly different preparations are compared in Fig. 37. Replicase activity was measured using denatured RE template. In Fig. 37a, the incorporation of GTP in the presence of denatured RE has been plotted as a function of the amount of poly G polymerase added to the assay for the two preparations. The two curves are far from coincident, suggesting that replicase activity with minus strands is not necessarily proportional to poly G polymerase activity. When the same replicase parameter is plotted as a function of total protein added (Fig. 37b), a single curve is obtained. Most of the protein present in these preparations, which have been purified through DEAE chromatography, is bacterial protein. Thus the single curve in Fig. 37b suggests that replicase activity is proportional to some protein other than the poly G polymerase, whose concentration is in turn proportional to the total bacterial protein background.

A slight disparity is noted also during purification of the f2 enzyme by DEAE chromatography. When a fairly steep salt gradient is used to elute the column, replicase and poly G polymerase activities are recovered as a single peak. However, a slight displacement of the activities is noted when a shallower gradient is employed. This is illustrated in Fig. 38. The poly G polymerase elutes as a single sharp peak. A single peak of replicase activity is also observed, both when measured with plus strand template and when measured with minus strand template. The replicase peak, however, elutes at a slightly higher salt concentration than does the poly G polymerase peak. The amount of replicase activity with plus strands in a given fraction appears to

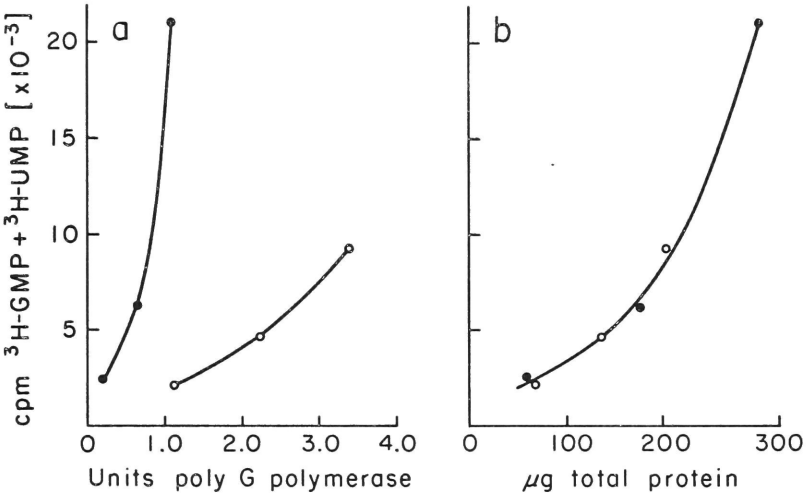

Figure 37. Replicase and poly G polymerase activities in different preparations purified by DEAE cellulose chromatography

Assays were done under replicase assay I (low salt) conditions. The amount of acid-insoluble radioactivity after a 20 min incubation at 30° C with denatured f2 RE template is plotted as a function of the amount of poly G polymerase (1 unit = 1 nmol GMP/10 min) added to each incubation mixture in part a and as a function of the amount of protein added to each incubation mixture in part b. Preparation I (●——●); preparation II (o——o).

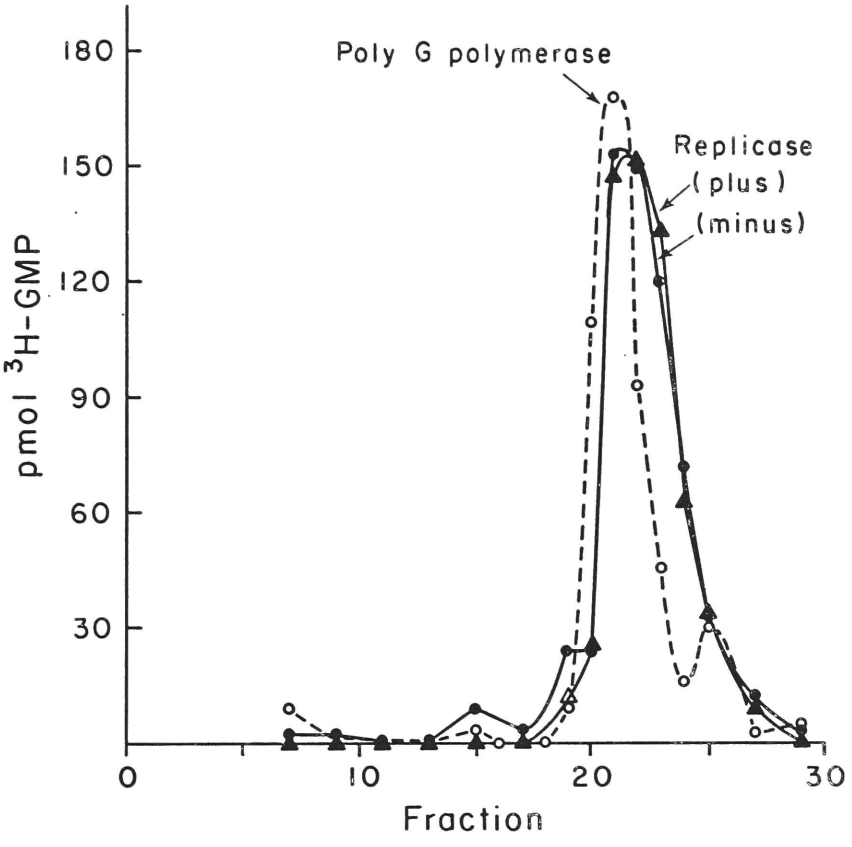

Figure 38. Elution of poly G polymerase and replicase activities from DEAE cellulose

F2 enzyme was purified from E. coli Q13 by liquid-polymer phase partitioning and applied to a DEAE cellulose column as described in Chapter III. The column was eluted with a 0.05-0.20 M NaCl gradient in SB20. Fractions were assayed for poly G polymerase (o----o), replicase activity with denatured RE template (●——●) and replicase activity with f2 plus strand template (▲——▲) as described in Chapter III.

be proportional to the amount of replicase activity with minus strands. Neither replicase activity shows a constant proportionality with poly G polymerase activity.

Finally, the highly purified poly G polymerase preparations obtained by RNA cellulose chromatography are quite inactive with either f2 plus or f2 minus strand template. This is shown in Table XII. Thus the highly purified f2 poly G polymerase, unlike its Qβ counterpart, is not able to synthesize f2 plus strand RNA from minus strand template. This does not appear to be due simply to the fractionation of separable poly G polymerase and replicase activities during RNA cellulose chromatography. No replicase activity has been recovered after RNA cellulose chromatography in either low or high salt eluates of the column.

The behavior of poly G polymerase and replicase activities during glycerol gradient centrifugation suggests the existence of a dissociable component required for replicase activity. Fig. 39 shows such a gradient. In this experiment, partially purified enzyme having both poly G polymerase and replicase activities was sedimented through a glycerol gradient as described in Chapter III. The same gradient was then assayed for both replicase and poly G polymerase activities. The poly G polymerase sediments as a rather broad peak at 6.5 S. Replicase activity, as measured with denatured RE template, forms a much narrower band at the trailing edge of the poly G polymerase peak. The position and narrowness of the replicase peak suggest that it represents a region of overlap between the poly G polymerase and some more slowly-sedimenting, inactive component. To test this hypothesis, inactive fractions from the top of the gradient were combined with the leading fractions of the poly G polymerase peak and tested for replicase activity with denatured RE template. Fractions from the top of the gradient did indeed exert a stimulating effect on replicase activity when combined with fractions rich in poly G polymerase activity. The effective stimulation obtained by adding fractions 19-23 to fraction 14 is illustrated by the open squares. Thus there appears to be an easily

Table XII

Activity of RNA cellulose enzyme

Template	Incorporation (pmol [^{3}H]GMP)
None	0.4
Poly C	364
Native f2 RE	0.0
Denatured f2 RE	0.4

Assays for replicase and poly G polymerase activity were carried out as described in Chapter III. Each 0.1 ml reaction mixture contained 1 μg of RNA cellulose-purified enzyme; the replicase assay mixture contained 1.75 μg native or denatured f2 RE and the poly G polymerase assay mixture contained 1 μg poly C template. Acid-insoluble radioactivity was determined after a 20 min incubation for the replicase assay and a 10 min incubation at 30° C for the poly G polymerase assay.

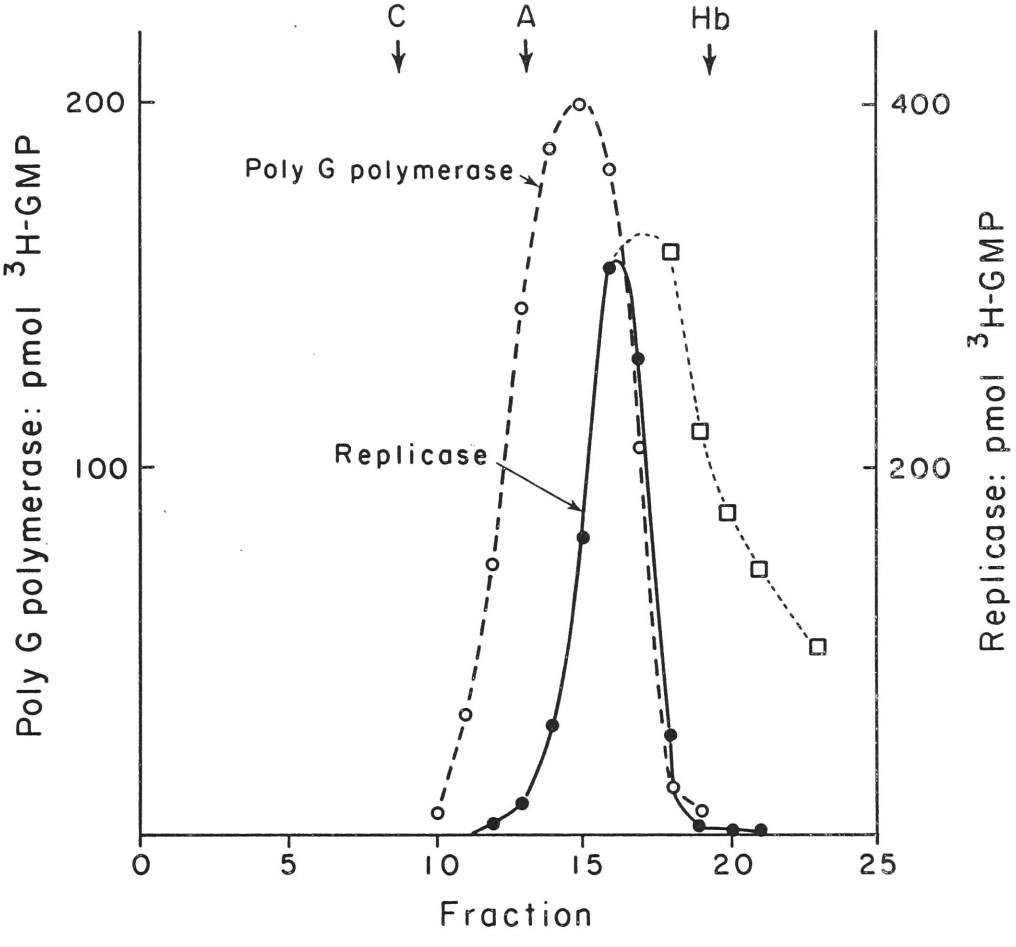

Figure 39. Glycerol gradient centrifugation of DEAE cellulose-purified f2 enzyme

Peak poly G polymerase fractions from a DEAE cellulose column were precipitated with $(NH_4)_2SO_4$, dissolved in SB4Mg containing 1 M NaCl and centrifuged through 14-ml 7-25% glycerol gradients at 40,000 rpm for 24 hr (Chapter III). Commercial preparations of hemoglobin (Hb), aldolase (A), and catalase (C) were used as sedimentation markers in separate tubes. Fractions were assayed for poly G polymerase and replicase activities as described in Chapter III. Fractions 19-23 (10 µl aliquots) were reassayed for replicase activity with denatured f2 RE template in the presence of 10 µl aliquots of fraction 14 (□----□). Poly G polymerase (o----o); replicase activity with denatured f2 RE (●——●).

dissociable smaller component which is required, in addition to the poly G polymerase, for replicase activity.

To further confirm this observation, pooled inactive fractions (19-22) from the top of the glycerol gradient were tested for their ability to stimulate replicase activity in poly G polymerase preparations obtained by RNA cellulose chromatography. This is shown in Fig. 40. Replicase activity was measured as a function of the amount of RNA cellulose-purified poly G polymerase added in the presence and absence of a constant amount of pooled glycerol gradient factor. The specific activity of the poly G polymerase used in this experiment was about 150 nmol GMP/mg/10 min. No activity with either denatured RE (minus strand) template or with plus strand template is detectable in the absence of the glycerol gradient factor. The poly G polymerase is quite active with both templates in the presence of the glycerol gradient factor. Stimulation with minus strand template is greater than stimulation of activity with plus strand template. Thus the f2 poly G polymerase appears to require additional macromolecular factors for activity with either f2 plus or f2 minus strands.

We know nothing of the nature, number, or function of the stimulating factor which separates from the poly G polymerase during glycerol gradient centrifugation. However, the region of the gradient from which it is derived also contains a preponderance of the total bacterial protein present in partially purified enzyme preparations, suggesting that it might be (or contain) a bacterial protein. The experiment described in Fig. 37 also implicates bacterial proteins. The difference in the extent to which the factor (or factors) stimulate activity with plus and minus strand templates probably reflects the differential affinity of the replicase for the complementary strands (Chapter V). No further fractionation has been carried out and we must assume that the stimulating factor is a complex mixture of proteins (and perhaps other macromolecules as well). We have observed, however, that the same factor preparation which stimulates f2 replicase activity also stimulates Qβ replicase activity. When f2 glycerol gradient factor was incubated

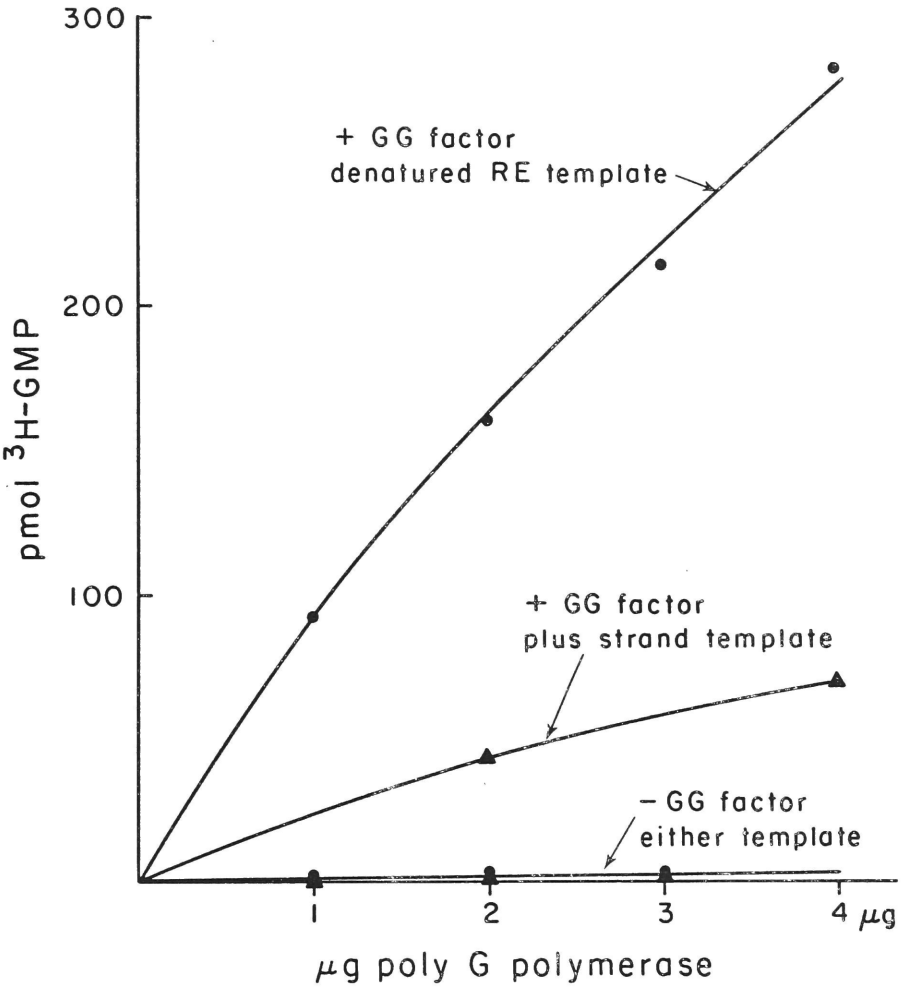

Figure 40. Stimulation of RNA cellulose-purified poly G polymerase by glycerol gradient fractions 19-22

Replicase assays were carried out under high salt conditions (0.1 M NaCl) but with no preincubation. Each 0.1 ml reaction mixture contained 2 μg denatured RE or f2 plus strand RNA and the indicated amount of RNA cellulose-purified poly G polymerase. Glycerol gradient fractions 19-22 (Fig. 39) were pooled and dialysed against SB50Mg; 10 μl aliquots of this preparation were added as indicated. Acid-insoluble radioactivity was determined after a 20 min incubation at 30° C. Template RNA: denatured RE (●——●); f2 plus strands (▲——▲).

with highly purified Qβ 'core' replicase, a fivefold stimulation of activity with Qβ plus strand RNA was observed. It is likely, then, that the stimulating factor is of bacterial origin and that the same bacterial component or components serve both the f2 and Qβ replicases.

B. Discussion

1. Comparison of f2 replicase and Qβ replicase. In this chapter we have shown that a macromolecular factor separates from the f2 poly G polymerase during purification and that f2 replicase activity with both plus and minus strands requires the presence of the factor, as well as the poly G polymerase itself. We have detected Qβ factor activity in an f2 factor preparation, suggesting the possible identity of these factors. As described in Chapter I, Qβ factor activity, referred to as 'light component' by Spiegelman and coworkers, is obtained by prolonged sucrose density gradient centrifugation, much as we have described for f2 factor activity (Eikhom and Spiegelman, 1967; Eikhom et al., 1968). Several observations suggest that the prolonged controversy over whether the Qβ factors obtained in different laboratories are the same or different is largely academic (Tooze, 1968). Kamen reports that replicase activity can be restored to Qβ poly G polymerase preparations upon addition of Spiegelman's 'light component' (Kamen, 1970). August's highly purified replicase preparations require the addition of Factors I and II for activity with Qβ plus strands (August, 1969; August et al., 1969). Gels of August's highly purified replicase contain the same four subunits as do Kamen's poly G polymerase preparations (compare Kamen, 1970, and Fedoroff and Zinder, 1971, Fig. 4). Qβ poly G polymerase preparations of the same composition have been obtained in our laboratory (Fig. 14). As described above, f2 glycerol gradient factor, which resembles Spiegelman's 'light component', stimulates replicase activity by both f2 and Qβ poly G polymerase preparations with their homologous phage templates.

August's group has substantially purified Factors I and II of the Qβ replicase system. Factor I activity appears to be associated with a protein having a molecular weight of about 75,000 and consisting of six identical subunits (Hayward and Franze de Fernandez, 1971). Factor II activity, on the other hand, does not appear to be associated with a unique protein (Kuo, 1971). A number of basic bacterial proteins will satisfy the requirement of the replicase for Factor II (Kuo, 1971). In addition, basic proteins from other sources, including mammalian histones, will satisfy this requirement (Kuo and August, 1971). Both Factor I protein and Factor II-type proteins bind strongly to nucleic acids, and the factor requirement for replicase activity is proportional to the template RNA concentration, rather than to the concentration of enzyme (August et al., 1969; Hayward and Franze de Fernandez, 1971; Kuo and August, 1971). Although neither Spiegelman's 'light component' nor the f2 'factor' have been purified, it seems likely that the active ingredients are either the same or interchangeable for all replicase factor preparations.

The evidence presented in this and preceding chapters suggests that the components of the Group I f2 replicase and the Group III Qβ replicase are very similar. The fundamental element of both replicase complexes is the poly G polymerase or 'core' replicase. Both f2 and Qβ poly G polymerases consist of four protein subunits. Of these four proteins, three appear to be the same, based on electrophoretic mobility. They are bacterial proteins in the Qβ poly G polymerase and are probably the same proteins in the f2 poly G polymerase. The fourth protein is unique to each enzyme and represents the replicase protein encoded by the respective phage RNAs (Kamen, 1970; Kondo et al., 1970; Fedoroff and Zinder, 1971). The complete replicase complex contains, in addition to the poly G polymerase moiety, certain bacterial macromolecular factors. As discussed above, these appear to be interchangeable between the f2 and Qβ replicase systems. Similar or identical bacterial macromolecules probably fulfill this function in both replicase systems. The data, while not yet conclusive, suggest that the only component

that differentiates the two enzyme complexes is the phage-coded replicase protein itself.

Functionally the enzymes are also very similar. Both display a high degree of template specificity, although perhaps the specificity is somewhat less stringent in the case of the Qβ enzyme than in the case of the f2 enzyme (Haruna and Spiegelman, 1965a; also Chapter V). Both enzymes show a marked preference for homologous RNA templates. Template preference exists both with respect to phage plus strand RNA and phage minus strand RNA (Chapter V). This template specificity is a quite predictable property for enzymes which must replicate a specific type of RNA in the presence of competing bacterial RNAs.

Partially purified Qβ and f2 replicases carry out all of the reactions characteristic of in vivo RNA replication. Under appropriate conditions, both will synthesize complementary minus strand RNA in response to plus strand template and then make plus strand RNA from the newly-synthesized minus strands. The Qβ replicase is a great deal more efficient in this respect than is the f2 replicase. It will accomplish manyfold net synthesis of biologically competent RNA in vitro (Spiegelman et al., 1965). The f2 replicase reaction ceases before net synthesis is achieved. The profound difference in the stabilities of the two enzymes may in part account for the early cessation of the f2 reaction. However, we have also suggested that the synthesis may terminate prematurely due to a deficiency of some unidentified replicase component, suboptimal assay conditions, or a high error level during transcription (Chapter V).

There is a major functional difference between the f2 and Qβ poly G polymerases. The f2 poly G polymerase has no detectable replicase activity. It is dependent on the presence of bacterial factors for the utilization of both f2 plus and f2 minus strand templates. The Qβ poly G polymerase requires bacterial factors only for the utilization of Qβ plus strand RNA. There appears to be no factor requirement for synthesis of plus strand RNA from minus strand template by the Qβ enzyme (August et al., 1968; Kamen, 1970).

2. Comparison of f2 replicase with other Group I phage replicases. In general, the properties of the f2 replicase resemble those of the Group III Qβ replicase much more closely than they do the properties of other Group I replicase preparations described in the literature. Several reports on MS2 and f2 phage-specific enzymes were summarized in Chapter I (see also Weissmann and Ochoa, 1967). The observation by Fiers et al. (1967) that autocatalytic synthesis of MS2 RNA was associated with a 40 S particle containing both protein and about 5% RNA is of some interest in relation to the f2 replicase. The f2 enzyme sediments as a low molecular weight complex (Fig. 39), but does not replicate RNA, as defined either by autocatalytic kinetics of nucleotide polymerization or net synthesis of phage RNA. It is possible that the aggregate or large complex isolated by Fiers et al. (1967) contains a structural element, missing in the soluble f2 preparation, necessary for efficient replication of RNA.

It is difficult to explain the differences in properties between the present f2 replicase and the enzyme isolated by August et al. (1965) from f2 sus 11-infected cells. However, the requirement of their enzyme preparation for extremely large quantities of template RNA and its lack of template specificity have not been observed for any other phage RNA replicase (Haruna and Spiegelman, 1965a; Miyaki et al., 1971). The high template specificity and the requirement for small amounts of template characteristic of the present f2 replicase, on the other hand, are not at all exceptional for this type of enzyme.

3. Enzyme-template interactions. We showed in Chapter V that the f2 replicase will use both f2 plus strand and f2 minus strand RNAs as template. However, the affinity of the enzyme appears to be greater for f2 minus than for f2 plus strands. Comparable levels of nucleotide polymerization with both templates require the presence of about 10 times as much plus strand as minus strand RNA. Replicase activity with both plus and minus strands is stimulated by increasing the ionic strength of the incubation mixture and including a brief preincubation of the enzyme at high ionic strength in the assay. The salt effect

appears to be related to the affinity of the enzyme for template RNA. Similar levels of nucleotide polymerization obtain at much lower plus strand template concentrations in the presence of salt than in the absence of salt. Thus it appears likely that salt stimulates replicase activity by increasing the affinity of the enzyme for template RNA.

A preference of the Group III Qβ replicase for minus strand template has been reported by Feix et al. (1968). They found that with partially purified Qβ replicase, the initial nucleotide polymerization rates were much higher with minus strand template than with plus strand template, but that template saturation occurred at lower concentrations of plus than of minus strands. They further showed that the initial rates were additive at saturating concentrations of plus strand RNA and increasing concentrations of minus strand RNA. On the basis of these results, Feix et al. (1968) proposed the existence of a small number of binding sites for plus strand RNA and a larger number of binding sites for minus strand RNA on the enzyme. August et al. (1968) showed, however, that differential rates of nucleotide polymerization obtain only when the Qβ replicase preparation is deficient in the bacterial protein factors (Factors I and II) required for replicase activity with plus strand RNA, but not for activity with minus strand RNA. In the presence of a sufficient factor concentration, no difference could be detected in the activity of the Qβ replicase with plus and with minus strands (August et al., 1968). These authors therefore suggest that the results obtained by Feix et al. (1968) are attributable to their use of factor-deficient Qβ replicase preparations. And indeed, the results of Feix et al. (1968) are quite consistent with the presence of a limited number of complete enzyme complexes capable of binding plus strands and an excess of 'core' replicase, capable only of binding Qβ minus strands.

The results of the present study are more consistent with a true difference in the affinity of the f2 replicase for plus and minus strands. The data given in Fig. 27 and Table V suggest that saturation of the enzyme requires at least 10 times more plus strand than minus

strand RNA. The almost exclusive synthesis of plus strand product under both low and high salt assay conditions from a mixture of f2 plus and minus strand RNA templates (Table VIII) further suggests that the binding sites for plus and minus strands are not entirely independent. If synthesis from plus and minus strands were additive, as in the Qβ experiments of Feix et al. (1968), the fraction of minus strand product made under the high salt conditions at the template concentration used in these experiments (Table VIII) should be at least 20-30% after 20 minutes of incubation. These observations suggest that plus and minus strands probably bind to the same site or sites on the replicase complex, but that the affinity of the enzyme is greater for minus than for plus strand RNA. The affinity of the enzyme for both plus and minus strands appears to be enhanced in the presence of salt. Preincubation of the enzyme at a high salt concentration stimulates replicase activity and preincubation in low salt inactivates the replicase; both stimulation and inactivation occur regardless of the presence or absence of template RNA. It is likely, therefore, that at least some of the salt effect is exerted directly on the enzyme complex.

The structural basis of template recognition is poorly understood. It is fairly well established in the case of the Qβ replicase that the enzyme interacts with the 3' terminal end of the template RNA (August et al., 1968). The 3' terminal nucleotide sequence of both Qβ plus and Qβ minus strands is -CCA. Removal of the terminal adenylate residue from the plus strand affects neither the infectivity of the RNA in a spheroplast assay (Kamen, 1969), nor its ability to be replicated by the enzyme in vitro (August et al., 1969). Removal of the penultimate cytidylate residue, however, drastically reduces both infectivity and template efficiency (Kamen, 1969; August et al., 1969). These observations show that, predictably, the 3' terminal sequence of the template RNA is an important element in the template recognition mechanism.

However, the recognition of phage template RNAs by the Qβ replicase appears to be more complex than a simple interaction with the 3'

end of the RNA. All of the phage RNAs so far examined appear to have very similar 3' terminal sequences in both plus and minus strands. This includes plus and minus strand RNAs of both Group I and Group III phages (Cory et al., 1970; Goodman et al., 1970). Furthermore, it has been found that when phage RNA interactions are measured by simple binding criteria, such as the retention of an RNA-enzyme complex on Millipore filters, the Qβ replicase will bind to a variety of non-homologous RNAs (August et al., 1968; Kolakofsky and Weissmann, 1971). In addition, the bacterial factors which must be present for the replication of Qβ plus strand RNA have no effect on the formation of the enzyme-template complex (August et al., 1969). The enzyme will bind equally well to plus strand RNA in the presence or absence of factors, but binding does not lead to replication in the absence of factors.

Bacteriophage RNAs appear to have a considerable degree of secondary structure (Strauss and Sinsheimer, 1963; Mitra et al., 1963; Gesteland and Boedtker, 1964). At physiological salt concentrations, the RNA has a very high sedimentation coefficient and a small radius of gyration for its molecular weight (Strauss and Sinsheimer, 1963). Limited digestion of Qβ RNA with ribonuclease results in the production of defined fragments, suggesting that certain regions of the RNA are consistently accessible to attack, which, in turn, implies a non-random secondary and perhaps even tertiary structure (Bassel and Spiegelman, 1967). The secondary structure has been implicated as a possible basis for translational control (Lodish, 1970). Recent nucleotide sequence studies have revealed the existence of many regions of the molecule capable of internal hydrogen bonding (Cory et al., 1970; Jeppesen et al., 1970).

The available evidence suggests that the secondary structure of the RNA is an important element in determining template specificity. Early experiments of Haruna and Spiegelman (1965b) showed that fragmented Qβ RNA is an extremely inefficient template for the Qβ replicase, suggesting that the recognition mechanism requires more than the initial sequence in order for the RNA to be replicated. Recent experiments by

Kolakofsky and Weissmann (1971) show that binding of the Qβ replicase to Qβ RNA renders internal regions of the RNA inaccessible to ribosomes. When the Qβ replicase is bound to a phage RNA which it cannot replicate (R17 RNA), no such secondary interaction occurs and ribosomes can bind internally on the RNA.

In the present study we have shown that the f2 replicase complex has a higher affinity for f2 minus strands than for f2 plus strands. If the enzyme recognizes and binds to an internal sequence on the RNA molecule, as implied by the Qβ experiments described above, then the difference in affinity may be attributable to the fact that the internal recognition sites have complementary sequences in the plus and minus strands. Another possibility is that some element of the secondary (or perhaps even tertiary) structure itself is critical to the recognition mechanism. The structure of the complementary plus and minus strands might be sufficiently different to account for differences in relative affinity. Both of these mechanisms are consistent with the observation that the replicase reaction (both f2 and Qβ) is optimal at a physiological salt concentration. Some element of the salt effect may well be attributable to its effect on the secondary structure of the RNA, which undergoes a transition from an extended to a compact structure at ionic strengths approaching physiological (Strauss and Sinsheimer, 1963).

4. The poly G polymerase reaction. The polymerization of GTP in the presence of the synthetic polymer poly C differs in a number of ways from the replication of phage RNAs. This reaction requires only the 4-polypeptide poly G polymerase and responds quite differently to the salt concentration of the incubation medium. The synthetic polymer-dependent reaction of the Qβ replicase has been studied in some detail (Hori et al., 1967). It has been shown that the Qβ enzyme will use copolymers as template, as well as poly C, so long as the polymers contain cytidylate residues. Synthetic polymers are not replicated. The average chain length of the polynucleotide product made from synthetic polymer template is less than 10% of the 3600 nucleotide polymer

synthesized in the replicase reaction. Furthermore, the base composition of the product does not necessarily reflect the base composition of the template polymer (Hori et al., 1967). Regardless of the base composition of the template polymer, the product contains a high proportion of guanylate residues and invariably has a 5' pppG residue. When a co-polymer containing all four nucleotides is used as template, the polymerization reaction is relatively insensitive to the omission of any substrate except GTP and proceeds relatively well in the presence of GTP alone (Hori et al., 1967).

All phage RNAs so far examined contain 5' terminal guanylate residues (Cory et al., 1970; Goodman et al., 1970) and, as mentioned earlier, the penultimate 3' cytidylate residues of phage RNAs are indispensable, both for infectivity of the phage RNA and for template activity in vitro (Kamen, 1969; August et al., 1969). In addition, all polynucleotide products synthesized by the Qβ replicase in vitro contain a 5' guanylate residue, regardless of the template used. Thus the synthetic polymer reaction resembles an aberrant chain-initiation reaction: nucleotides other than GTP are polymerized reluctantly and the template efficiency of a given polymer depends strongly on its cytidylate content.

In Chapter V we presented evidence that the synthetic polymer poly C and phage f2 minus strand RNA compete for binding to the f2 enzyme. We showed that the replicase reaction was inhibited by 65% in the presence of an amount of poly C approximately equal to the amount of phage minus strand RNA. We further showed that the replicase reaction was inhibited by poly C only when the synthetic polymer was added early in the reaction, suggesting that poly C cannot displace the replicase once it has bound to phage RNA. In analogous studies on the Qβ replicase, Haruna and Spiegelman (1966) reported that poly C did not inhibit the replication of Qβ plus strand RNA. However, in view of the results obtained by Feix et al. (1968), it may be that the replicase preparation used by Haruna and Spiegelman (1966) contained a sufficient excess of Qβ poly G polymerase so that no competition could be demonstrated. Alternatively, the relative affinity of the

Qβ replicase for poly C or C-containing polymers may be significantly lower than its affinity for Qβ RNA. If this is the case, then inhibition by poly C would not be apparent at the RNA concentrations used.

Poly U inhibits the f2 replicase, the f2 poly G polymerase (Chapter V) and the Qβ replicase (Haruna and Spiegelman, 1966). The pattern of poly U inhibition of the Qβ replicase suggests that the poly U inhibits binding of the enzyme to Qβ RNA. Neither Qβ plus strand RNA, nor E. coli rRNA have any effect on either the f2 replicase or the f2 poly G polymerase reactions (Chapter V). While these studies are hardly exhaustive, they suggest that a common site on the f2 enzyme interacts with both the synthetic polymers and the phage RNA.

In experiments with partially purified f2 enzyme (Chapter V), we showed that the poly G polymerase reaction is substantially inhibited at salt concentrations which stimulate replicase activity. Partially purified Qβ replicase preparations showed a similar salt stimulation of replicase activity, but no salt inhibition of poly G polymerase activity. However, an inhibitory effect of salt on Qβ poly G polymerase, analogous to that observed with the f2 poly G polymerase, has been found in studies with highly purified Qβ enzyme (T. Blumenthal, personal communication). We have no explanation for this disparity. Blumenthal's further studies suggest that the inhibitory effect of salt is related to chain initiation. Salt inhibits the Qβ poly G polymerase reaction if it is added to the template-containing reaction mixture before the enzyme, but not if added after the enzyme. Finally, in binding studies with isolated Qβ poly G polymerase subunits, Blumenthal has demonstrated that subunit III will bind GTP, but not ATP, CTP, or UTP (T.B., personal communication).

5. Chain initiation vs. polymerization. The arguments presented so far show that the f2 poly G polymerase is central to the f2 replicase reaction and that the synthetic polymer-dependent reaction is related to RNA chain initiation. It is also quite clear that the replicase reaction is more complex than the synthetic polymer reaction. It requires the presence of additional macromolecular factors and responds quite differently to the manipulation of certain experimental parameters.

A possible basis for these differences is suggested by the results of the substrate saturation studies described in Chapter V.

Simple Michaelis-Menten saturation kinetics are observed at low concentrations of ATP, CTP, and UTP in the replicase reaction. Double-reciprocal plots are linear for low substrate concentrations and apparent K_m's in the 0.01-0.02 mM range are obtained from such plots, suggesting that the affinity of the replicase for these nucleotides is quite high. Complex saturation kinetics are observed for GTP when either denatured f2 RE or poly C templates are used. Linear double-reciprocal plots are obtained only as a function of the square of GTP concentration, suggesting the interaction of more than one molecule of GTP with the enzyme (Atkinson et al., 1965). Furthermore, K_m values estimated by extrapolation of the relatively linear portion of double-reciprocal plots at high substrate concentrations suggest that the affinity of the enzyme is much lower for the initiator nucleotide GTP than for the other nucleotides.

A kinetic distinction between nucleotides which initiate RNA chains and those which appear only internally in the polymer product of the reaction has been observed with other ribonucleotide polymerizing enzymes (Chamberlin and McGrath, 1970; Goldthwait et al., 1970). A rather extensive analysis of such effects has been carried out by Anthony and coworkers (Anthony et al., 1969; Goldthwait et al., 1970) on the E. coli DNA-dependent RNA polymerase. Based on equilibrium dialysis studies, these authors suggest that the kinetic distinction between initiator and internal nucleotides resides in the existence of different initiation and polymerization sites on the enzyme (Goldthwait et al., 1970).

While many models have been proposed to explain complex enzyme-substrate interactions of the type observed with the f2 enzyme (Atkinson, 1966; Cleland, 1967) and we do not wish to overinterpret kinetic data obtained with so complex an enzyme as the replicase, we believe that a model of the type proposed by Goldthwait et al. (1970)

is appropriate for the f2 replicase. The observation of second order kinetics for GTP in both the replicase and poly G polymerase reactions suggests that initiation involves the simultaneous binding of two molecules of GTP to the poly G polymerase moiety of the enzyme complex. The substantial difference in the apparent affinity of the enzyme for GTP and for other nucleoside triphosphates further suggests the existence of both a low-affinity initiation site and a second, high-affinity polymerization site on the enzyme complex.

6. The two-site model. In the foregoing discussion we have drawn a clear distinction between RNA replication reactions and synthetic polymer-dependent nucleotide polymerization reactions carried out by bacteriophage RNA replicases. We have also suggested that the active enzyme complex may contain different active sites for chain-initiation and chain-elongation (polymerization). We now suggest possible roles for the different active sites in the replicase-catalysed synthetic reactions.

Chain initiation by RNA replicases requires the presence of cytidylate residues in the template polymer and GTP as one of the substrates. These conditions are necessary, but not sufficient, for phage RNA replication (August et al., 1969; Kolakofsky and Weissmann, 1971). We suggest that the chain-initiation site on the enzyme normally recognizes the 3' cytidylate residues in template RNAs and directs the polymerization of the first few guanylate residues, perhaps to serve as an oligonucleotide primer for further polymerization. A requirement for such a primer has been demonstrated for a number of nucleotide-polymerizing enzymes (Jovin et al., 1968; Goulian, 1968; Klee, 1971). Since the presence of C-containing template and GTP substrate are both necessary and sufficient for the synthetic polymer-dependent reaction, we suggest that this reaction occurs primarily or exclusively at the initiation site. The observation by De Wachter and Fiers (1969) that Qβ RNA has two 5' terminal sequences, differing only in the number of terminal guanylate residues, suggests a low transcriptional fidelity for in vivo chain initiation. This is quite consistent with the characteristics of the synthetic polymer reaction and suggests that the chain-initiation

site has a strong preference for GTP. Perhaps the GTP-specific binding site which is present in subunit III of the Qβ poly G polymerase is a part of the postulated initiation site (T. Blumenthal, personal communication).

RNA replication, unlike the synthetic polymer-dependent reaction, proceeds only when certain additional conditions are satisfied. Not only must the RNA be from the correct source (i.e. from the same family of RNA bacteriophage) and contain the normal complement of 3' cytidylate residues, but it must also be completely single-stranded and intact (Haruna and Spiegelman, 1965a and b; Weissmann et al., 1967; August et al., 1968). The highly specific replication reaction also requires the presence of some additional bacterial proteins, which appear to interact with the template RNAs (August et al., 1969). We suggest that these additional conditions necessary for RNA replication govern the availability and/or substrate affinity of the postulated polymerization site on the enzyme. Thus, for example, the enzyme may contain a specific recognition site which binds an internal sequence or structure in the RNA template; the correct secondary binding might then activate the polymerization site, either by making it available to substrate or by enhancing its substrate affinity. The interaction of the bacterial factors with the RNA may promote the secondary binding by altering the secondary (and/or tertiary) structure of the RNA. Since the Qβ and f2 'core' replicases appear to differ only in the phage-coded replicase protein, it is this protein which is likely to contain such a specific binding site.

In the preceding discussion we have equated all replicase reactions, neglecting the fact that the Qβ 'core' replicase is active with Qβ minus strands in the absence of bacterial factors. However, the assigned role of the bacterial factors in the present scheme is simply to promote the correct alignment of the enzyme with respect to the RNA. Thus the absence of a factor requirement with a given template suggests that the internal binding site of the RNA is normally available.

According to the model proposed here, the binding and initiation reactions with natural RNAs and with synthetic polymers are assumed to be similar and to occur at the same site on the enzyme. If this is correct, then the effect of the ionic strength of the incubation medium must be similar for both normal initiation and the synthetic polymer-dependent reaction. The overall stimulation of the replicase reaction at high ionic strength may therefore represent the sum of a negative effect on initiation and a large positive effect on polymerization. It has already been suggested that salt may affect the structure of the RNA (and thereby facilitate binding of the internal region to the enzyme). It is also possible that salt has some effect on the structure of the enzyme complex or on the activity of the proposed polymerization site. The observation that salt enhances the apparent affinity of the enzyme for template RNA is consistent with any of these interpretations, since replicase activity can be measured only when the correct enzyme-template complex is formed.

In conclusion, the two-site model constitutes an attempt to understand how RNA replicases can carry out the rather low-fidelity nucleotide polymerization observed with synthetic polymer templates and at the same time exhibit a very high degree of specificity for natural template RNAs. We have suggested that different sites on the enzyme function in RNA chain initiation and subsequent polymerization reactions and that the discrimination between natural templates involves the polymerization site. At present, the evidence for the existence of two active sites on the enzyme is quite tenuous. It is our hope that subsequent research efforts will provide a better definition of the role played by the various components of this complex enzyme system and permit a direct test of the present hypothesis.

REFERENCES

Adams, J. M., and Cory, S., 1970, Nature, 227, 570.

Alberts, B. M., 1967, in S. P. Colowick and N. O. Kaplan (Editors), Methods in Enzymology, Vol XII part A, Academic Press, New York, p. 566.

Alberts, B., and Herrick, G., 1971, in S. P. Colowick and N. O. Kaplan (Editors), Methods in Enzymology, Vol. XXI part D, Academic Press, New York, p. 198.

Anthony, D. D., Wu, C. W., and Goldthwait, D. A., 1969, Biochemistry, 8, 246.

Atkinson, D. E., 1966, Ann. Rev. Biochem., 35, 85.

Atkinson, D. E., Hathaway, J. A., and Smith, E. C., 1965, J. Biol. Chem., 240, 2682.

August, J. T., 1969, Nature, 222, 121.

August, J. T., Banerjee, A. K., Eoyang, L., Franze de Fernandez, M. T., Hori, K., Kuo, C.-H., Rensing, U., and Shapiro, L., 1968, Cold Spring Harbor Symp. Quant. Biol., 33, 73.

August, J. T., Cooper, S., Shapiro, L., and Zinder, N. D., 1963, Cold Spring Harbor Symp. Quant. Biol., 28, 95.

August, J. T., Eoyang, L., Franze de Fernandez, M. T., Hasegawa, S., Kuo, C.-H., Rensing, U., and Shapiro, L., 1969, J. Cell. Physiol., 74: sup. 1, 187.

August, J. T., Ortiz, P. J., and Hurwitz, J., 1962, J. Biol. Chem., 237, 3786.

August, J. T., Shapiro, L., and Eoyang, L., 1965, J. Mol. Biol., 11, 257.

Banerjee, A. K., Rensing, U., and August, J. T., 1969, J. Mol. Biol., 45, 181.

Bassel, B. A., and Spiegelman, S., 1967, Proc. Nat. Acad. Sci. USA, 58, 1155.

Beers, R. F., and Sizer, I. W., 1952, J. Biol. Chem., 195, 133.

Billeter, M. A., Weissmann, C., and Warner, R. C., 1966a, J. Mol. Biol., 17, 145.

Billeter, M. A., Libonati, M., Viñuela, E., and Weissmann, C., 1966b, J. Biol. Chem., 241, 4750.

Burgess, R. R., 1969, J. Biol. Chem., 244, 6160.

Chamberlin, M., and Berg, P., 1962, Proc. Nat. Acad. Sci. USA, 48, 81.

Chamberlin, M., and McGrath, J., 1970, Cold Spring Harbor Symp. Quant. Biol. 35, 250.

Cleland, W. W., 1967, Ann. Rev. Biochem., 36, 77.

Cooper, S., and Zinder, N. D., 1963, Virology, 20, 605.

Cory, S., Spahr, P. F., and Adams, J. M., 1970, Cold Spring Harbor Symp. Quant. Biol., 35, 1.

De Wachter, R., and Fiers, W., 1969, Nature, 221, 233.

Eikhom, T. S., and Spiegelman, S., 1967, Proc. Nat. Acad. Sci. USA, 57, 1833.

Eikhom, T. S., Stockley, D. J., and Spiegelman, S., 1968, Proc. Nat. Acad. Sci. USA, 59, 506.

Engelhardt, D. L., Robertson, H. D., and Zinder, N. D., 1968, Proc. Nat. Acad. Sci. USA, 59, 972.

Eoyang, L., and August, J. T., 1968, in S. P. Colowick and N. O. Kaplan (Editors), Methods in Enzymology, Vol. XII part B, Academic Press, New York, p. 530.

Eoyang, L., and August, J. T., 1971, in G. L. Cantoni and D. R. Davies (Editors), Procedures in Nucleic Acid Research, Vol. 2, Harper and Row, New York, p. 829.

Erikson, R. L., 1968, Ann. Rev. Microbiol., 22, 305.

Erikson, R.L., and Franklin, R. M., 1966, Bacteriol. Rev., 30, 267.

Fedoroff, N. V., and Zinder, N. D., 1971, Proc. Nat. Acad. Sci. USA, 68, 1838.

Feix, G., Pollet, R., and Weissmann, C., 1968, Proc. Nat. Acad. Sci. USA, 59, 145.

Feix, G., Slor, H., and Weissmann, C., 1967, Proc. Nat. Acad. Sci. USA, 57, 1401.

Fiers, W., Verplancke, H., and Van Styvendaele, B., 1967, in V. V. Koningsberger and L. Bosch (Editors), Regulation of Nucleic Acid and Protein Biosynthesis, Vol. 10, Elsevier Publishing Co., New York, p. 154.

Fox, C. F., Robinson, W. S., Haselkorn, R., and Weiss, S. B., 1964, J. Biol. Chem., 239, 186.

Franze de Fernandez, M. T., Eoyang, L., and August, J. T., 1968, Nature, 219, 588.

Fromageot, H.P.M., and Zinder, N. D., 1968, Proc. Nat. Acad. Sci. USA, 61, 184.

Gesteland, R. F., and Boedtker, H., 1964, J. Mol. Biol., 8, 496.

Goldthwait, D. A., Anthony, D. D., and Wu, C.-W., 1970, in L. Silvestri (Editor), RNA-Polymerase and Transcription, North-Holland Publishing Co., London, p. 10.

Goodman, H. M., Billeter, M. A., Hindley, J., and Weissmann, C., 1970, Proc. Nat. Acad. Sci. USA, 67, 921.

Goulian, M., 1968, Cold Spring Harbor Symp. Quant. Biol., 33, 11.

Granboulan, N., and Franklin, R. M., 1968, J. Virol., 2, 129.

Haruna, I., Nozu, K., Ohtaka, Y., and Spiegelman, S., 1963, Proc. Nat. Acad. Sci. USA, 50, 905.

Haruna, I., and Spiegelman, S., 1965a, Proc. Nat. Acad. Sci. USA, 54, 579.

Haruna, I., and Spiegelman, S., 1965b, Proc. Nat. Acad. Sci. USA, 54, 1189.

Haruna, I., and Spiegelman, S., 1966, Proc. Nat. Acad. Sci. USA, 56, 1333.

Hayes, W., 1968, The Genetics of Bacteria and Their Viruses, Ed. 2, John Wiley and Sons, Inc., New York, p. 480.

Hayward, W. S., and Franze de Fernandez, M. T., 1971, in G. L. Cantoni and D. R. Davies (Editors), Procedures in Nucleic Acid Research, Vol. 2, Harper and Row, New York, p. 840.

Hori, K., Eoyang, L., Banerjee, A. K., and August, J. T., 1967, Proc. Nat. Acad. Sci. USA, 57, 1790.

Horiuchi, K., Lodish, H. F., and Zinder, N. D., 1966, Virology, 28, 438.

Horiuchi, K., and Matsuhashi, S., 1970, Virology, 42, 49.

Jeppesen, P. G. N., Nichols, J. L., Sanger, F., and Barrell, B. G., 1970, Cold Spring Harbor Symp. Quant. Biol., 35, 13.

Jovin, T. M., and Kornberg, A., 1968, J. Biol. Chem., 243, 250.

Kamen, R., 1969, Nature, 221, 321.

Kamen, R., 1970, Nature, 228, 527.

Kimhi, Y., and Littauer, U. Z., 1968, J. Biol. Chem., 243, 231.

Klee, C. B., 1971, in G. L. Cantoni and D. R. Davies (Editors), Procedures in Nucleic Acid Research, Vol. 2, Harper and Row, New York, p. 896.

Kolakofsky, D., and Weissmann, C., 1971, Nature New Biology, 231, 42.

Kondo, M., Gallerani, R., and Weissmann, C., 1970, Nature, 228, 525.

Konigsberg, W., Maita, T., Katze, J., and Weber, K., 1970, Nature, 227, 271.

Kuo, C.-H., 1971, in G. L. Cantoni and D. R. Davies (Editors), Procedures in Nucleic Acid Research, Vol. 2, Harper and Row, New York, p. 846.

Kuo, C.-H., and August, J. T., 1971, Fed. Proc. 30, 1316.

Litman, R. M., 1968, J. Biol. Chem., 243, 6222.

Littauer, U. Z., and Kornberg, A., 1957, J. Biol. Chem., 226, 1077.

Lodish, H. F., 1970, J. Mol. Biol., 50, 689.

Lodish, H. F., Cooper, S., and Zinder, N. D., 1964, Virology, 24, 60.

Lodish, H. F., and Zinder, N. D., 1966a, Science, 152, 372.

Lodish, H. F., and Zinder, N. D., 1966b, J. Mol. Biol., 19, 333.

Loeb, T., and Zinder, N. D., 1961, Proc. Nat. Acad. Sci. USA, 47, 282.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., 1951, J. Biol. Chem., 193, 265.

Mitra, S., Enger, M. D., and Kaesberg, P., 1963, Proc. Nat. Acad. Sci. USA, 50, 68.

Miyake, T., Haruna, I., Shiba, T., Itoh, Y. H., Yamane, K., and Watanabe, I., 1971, Proc. Nat. Acad. Sci. USA, 68, 2022.

Nathans, D., Oeschger, M. P., Eggen, K., and Shimura, Y., 1966, Proc. Nat. Acad. Sci. USA, 56, 1844.

Nathans, D., Oeschger, M. P., Polmar, S. K., and Eggen, K., 1969, J. Mol. Biol., 39, 279.

Niyogi, S. K., and Stevens, A., 1965, J. Biol. Chem., 240, 2587.

Overby, L. R., Barlow, G. H., Doi, R. H., Jacob, M., and Spiegelman, S., 1966a, J. Bacteriol., 91, 442.

Overby, L. R., Barlow, G. H., Doi, R. H., Jacob, M., and Spiegelman, S., 1966b, J. Bacteriol., 92, 739.

Phillips, L. A., Hotham-Iglewski, B., and Franklin, R. M., 1969, J. Mol. Biol., 45, 23.

Prives, C., 1971, Fed. Proc., 30, 1316.

Robertson, H. D., Webster, R. E., and Zinder, N. D., 1968, Nature, 218, 533.

Robertson, H. D., and Zinder, N. D., 1969, J. Biol. Chem., 244, 5790.

Schneider, W. C., 1957, in S. P. Colowick and N. O. Kaplan (Editors), Methods in Enzymology, Vol. III, Academic Press, New York, p. 680.

Schonberg, M., Silverstein, S. C., Levin, D. H., and Acs, G., 1971, Proc. Nat. Acad. Sci. USA, 68, 505.

Shapiro, L., and August, J. T., 1965, J. Mol. Biol., 11, 272.

Shapiro, L., Franze de Fernandez, M. T., and August, J. T., 1968, Nature, 220, 478.

Sippel, A., and Hartmann, G., 1968, Biochim. Biophys. Acta, 157, 218.

Spiegelman, S., 1970, The Harvey Lectures, 64, 1.

Spiegelman, S., Haruna, I., Holland, I. B., Beaudreau, G., Mills, D., 1965, Proc. Nat. Acad. Sci. USA, 54, 919.

Stavis, R. L., and August, J. T., 1970, Ann. Rev. Biochem., 39, 527.

Strauss, J. H., and Sinsheimer, R. L., 1963, J. Mol. Biol., 7, 43.

Sugiyama, T., Stone, H. O., and Nakada, D., 1969, J. Mol. Biol., 42, 97.

Tooze, J., 1968, Nature, 219, 675.

Vandenberghe, A., Van Styvendaele, B., and Fiers, W., 1969, Eur. J. Biochem., 7, 174.

Viñuela, E., Algranati, I. D., Feix, G., Garwes, D., Weissmann, C., and Ochoa, S., 1968, Biochim. Biophys. Acta, 155, 558.

Viñuela, E., Algranati, I.D., and Ochoa, S., 1967, Eur. J. Biochem., 1, 3.

Weber, K., and Osborn, M., 1969, J. Biol. Chem., 244, 4406.

Webster, R. E., Engelhardt, D. L., Zinder, N. D., and Konigsberg, W., 1967, J. Mol. Biol., 29, 27.

Weissmann, C., Borst, P., Burdon, R. H., Billeter, M. A., and Ochoa, S., 1964a, Proc. Nat. Acad. Sci. USA, 51, 682.

Weissmann, C., Borst, P., Burdon, R. H., Billeter, M. A., and Ochoa, S., 1964b, Proc. Nat. Acad. Sci. USA, 51, 890.

Weissmann, C., Colthart, L., and Libonati, M., 1968, Biochemistry, 7, 865.

Weissmann, C., Feix, G., Slor, H., and Pollet, R., 1967, Proc. Nat. Acad. Sci. USA, 57, 1870.

Weissmann, C., and Ochoa, S., 1967, in C. J. N. Davidson and W. E. Cohn (Editors), Progress in Nucleic Acids Research and Molecular Biology, Vol. 6, Academic Press, New York, p. 353.

Weissmann, C., Simon, L., Borst, P., and Ochoa, S., 1963, Cold Spring Harbor Symp. Quant. Biol., 28, 99.

Zinder, N.D., 1965, Ann. Rev. Microbiol., 19, 455.

Zinder, N. D., and Cooper, S., 1964, Virology, 23, 152.

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