THE REPLICATION OF BACTERIOPHAGE MS2

Thesis by

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ABSTRACT

The genetic information of the small bacteriophage MS2 is stored in a single-stranded RNA molecule. This thesis is an analysis of the mechanism whereby the MS2 genetic material is replicated.

In Part I, the presence of phage-specific double-stranded RNA in MS2-infected cells is demonstrated. The kinetics of conversion of the input parental strand to a duplex form, and of the formation of progeny duplexes, is also described.

In Part II, the replication of phage-specific RNA duplexes is studied by means of density labeling and CsCl equilibrium density sedimentation. Neither progeny nor parental duplexes are conserved, which is strong evidence that the duplex plays some role in replication.

The above experiments were performed on material isolated after ribonuclease digestion of the nucleic acids from infected cells. In Part III, two other procedures are described which permit identification of phage-specific replicative intermediates (RI) without use of ribonuclease: the host cell RNA synthesis can be preferentially blocked by use of the antibiotic, Actinomycin D; or RI can be separated from the other infected cell components by a centrifugal method based on studies of RNA denaturation with the organic solvent DMSO. These procedures lead to the conclusion that the true RI is a heterogeneous, approximately 15S component which is only partially double-stranded.

In Part IV, use is made of this partial double-stranded nature to purify and fractionate the RI on columns of benzoylated, naphthoylated DEAE cellulose, which seem to fractionate nucleic acids on the basis of secondary structure. From measurements of the ribonuclease-resistance and the buoyant densities of purified RI fractions it was concluded that the RI consists of a duplex of constant length to which is attached one single-stranded "tail" of length less than the viral RNA. The RI is only infective in the spheroplast assay when denatured.

This structure for the RI is interpreted to mean that the nascent strand is still attached after purification of the RI. It was therefore possible to determine the mode of replication by determining whether the "tail" consisted of the nascent strand (conservative replication) or the displaced portion of the viral strand (semi-conservative replication). Appropriate labeling experiments indicated that the tail could arise from either origin, equally often.

It was concluded that double-stranded RNA is involved in phage RNA replication and that replication is both conservative and semi-conservative, equally often.

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INTRODUCTION

This thesis is a study of the small E. coli bacteriophages which have RNA as their genetic material (instead of the conventional DNA). To scrutinize the anomolous bacteriophages of a harmless micro-organism is justifiable if the properties of such bacteriophages are relevant to the general problems of biology. In consequence, before describing the work done, some of the theoretical and actual advantages of the RNA bacteriophage as an experimental system are presented.

There are several problems to which the RNA bacteriophages are clearly capable of contributing. For example, the reason that DNA is normally used as the genetic material might become clear by studying the behaviour of an organism which does not use DNA. In addition, since RNA bacteriophages are the smallest bacteriophages known, their replication should present an extremely simple system to study. The genome also acts as a polycistronic messenger RNA and, consequently, could hopefully yield information about the mechanism and control of protein synthesis. Finally, the fact that one RNA molecule can direct both protein and RNA synthesis poses some rather interesting questions.

The RNA bacteriophage system has proved useful in several diverse ways. For example, the first protein synthesized *in vitro*

used the RNA from the phage f2* as template (Nathans et al, 1962). Using the same in vitro system it has been possible to demonstrate the universality of the genetic code (Schwartz et al, 1965), the mechanism of genetic suppression (Notani et al, 1965) and the way in which the synthesis of a peptide chain is initiated (Adams & Capecchi, 1966). The observations on the nature of f2 polarity mutants and on the stringent and relaxed control of RNA phage replication (Friesen, 1965; Lodish, 1966) have contributed to an understanding of the control of nucleic acid and protein synthesis. The usefulness of the RNA bacteriophage as a tool for studying nucleic acid replication will be the subject for the remainder of this thesis. It does indeed appear that the replication mechanism is more elementary than that of the small DNA bacteriophages, for example. In consequence, the first biologically active nucleic acid synthesised in vitro was the RNA of the RNA bacteriophage Qβ (Spiegelman et al, 1965).

The experiments described in this thesis attempt to clarify the mechanism of replication of the bacteriophage MS2. It is known that the RNA enters the host cell and directs the synthesis of some enzymes. One of these enzymes then catalyses the conversion of single-stranded RNA to a double-stranded form. In Part I of this thesis the discovery of a double-stranded form is documented and data given

* Many RNA phages have now been isolated. The ones most commonly mentioned in the literature and their countries of origin are f2, MS2, R17,(U.S.A.) Qβ, (Japan), fr and M12(Germany). All of these isolates have very similar biological and physical properties (although Qβ has a slightly unusual base ratio) and, in this thesis, are considered identical.

on the kinetics of its synthesis. In Part II it is shown that these duplexes are not conserved during replication and, in consequence, must play some role in replication. Single-stranded MS2 RNA is synthesized on the double-stranded RNA template. The duplex to which the growing single-strand is attached can be purified from infected cells and fractionated on the basis of the length of the growing strand (Part IV). Use is made of these procedures in Part V to determine if the growing strand is transcribed conservatively or semi-conservatively.

A great deal of the data relevant to the experiments in this thesis has been acquired in other laboratories. Some of the conclusions, if not the data, in the literature have been quite controversial. In order to give a proper background to this thesis a brief summary is now given of what seem the most likely events in RNA phage replication, based on available evidence.

(a) Physical properties of the bacteriophage MS2

The physical properties of the phage MS2 have recently been described in some detail (Strauss, 1966). In brief, MS2 is a small spherical virus of molecular weight 3.6×10^6. Its nucleic acid is a single-stranded RNA molecule of 1.05×10^6 daltons. Having, therefore, about 3,000 nucleotides it contains information for the synthesis of 10 proteins at the most. There are 129 amino acids in the major coat protein of f2 and their sequence is known (Weber et al, 1966).

(b) Adsorption and Penetration

RNA phages were first isolated on the basis of their specificity for male strains of E. coli (Loeb & Zinder, 1961). It has since been shown that other bacteria such as Salmonella, Shigella and Proteus can act as hosts if they contain the coli F factor (Brinton, et al. 1964). Replication of RNA can, however, take place in spheroplasts of female strains (Davis et al., 1961). It seems that the male specificity of RNA phages is based on the requirement for F pili as an adsorption site (Brinton et al, 1964; Crawford & Gesteland 1964).

The first step in infection is the adsorption of the phage to the F pili. The coat now remains outside the bacterium (Edgell & Ginoza, 1965) while the RNA penetrates. At some point, the infecting RNA is sensitive to RNase (Zinder, 1963). Since F pili can be removed by blending, the RNA is considered to have penetrated when the infective centre is no longer destroyed by blending (Brinton et al., 1964). The role of the pili and the molecular mechanism of penetration are, like all nucleic acid transport phenomena, very difficult to explain.

(c) Early protein synthesis

The first function of the parental RNA strand on entering the cell is to direct protein synthesis. Thus, if infection takes place in the presence of a block to protein synthesis, such as chloramphenicol (CAM), no further phage functions occur (Kelly & Sinsheimer, 1964).

It is interesting to know if all the phage specific proteins

are being made, or only those proteins which are required for the early functions, such as polymerase. The latter situation is known to be the case in infection with T- even phage and λ (Epstein et al., 1963; Skalka, 1966). This is not true, however, for RNA phages since infection with a polar mutant (sus-3) which is defective in coat protein synthesis does not produce detectable polymerase (Lodish & Zinder, 1966b). The existence of a polar coat protein mutant implies that translation of a late function (coat protein) is required for translation of the early protein, polymerase. An amber coat protein mutant which is not polar, sus-11, permits polymerase synthesis. Thus, the injected RNA strand must act, at least initially, as a polycistronic messenger, directing the synthesis of late and early proteins.

The phage RNA can act as a polycistronic message but with a difference. The typical messenger RNA is degraded only a few minutes after synthesis (Levinthal, 1962) whereas it is essential that this does not happen to the phage RNA if a successful infection is to occur. In all other respects the phage RNA appears to act as a normal messenger, first attaching to the 30 S ribosome sub-units which then acquire 50 S particles to produce polysomes (Godson & Sinsheimer, 1967). Some degradation of the parental material must occur, however, since late in infection, after many copies of the parental strand have been made, parental label is found in ribosomal RNA and transfer RNA (Godson & Sinsheimer, 1967). This must

be caused by breakdown of the input RNA to nucleotides which are then re-incorporated into RNA. The significance of these observations is not clear, however, since only 10 to 30% of any phage preparation are plaque formers, and it is not known at what stage in infection the defective particles are aberrant. If it is the penetration step which is blocked then all of the radioactivity studied by Godson & Sinsheimer is relevant to infection (since phage which had not penetrated were removed by blending.) If the block is after penetration (for example, some RNA molecules are damaged before they can produce an infection) then the properties of the majority of the radioactivity need not reflect those of the infective material. This point obviously requires clarification.

(d) Formation of a complementary strand

The input parental RNA directs one or more rounds of protein synthesis and then changes its role to start producing copies of itself. Can the mechanism of this duplication be predicted? Probably, so, for one of the few general laws of molecular biology is that information is passed from a parental strand of nucleic acid to a progeny strand through the synthesis of a meaningless strand, complementary to the parental, which acts as a template. Since this is such a satisfying principle it is an obvious prediction that the first step the parental RNA will perform in order to duplicate itself is the synthesis of a complementary strand to give a double-stranded molecule.

About 5 to 8 minutes after infection the parental molecule acquires some resistance to RNase digestion, a characteristic of a double-stranded RNA (Kelly & Sinsheimer, 1964; Weissmann et al., 1964a). The fraction of the input radioactivity which becomes resistant varies from 2 to 30% and usually reflects the fraction of the labeled particles which are plaque-formers. This RNase-resistant material has, in addition, the sedimentation, density and melting characteristics of a double-stranded RNA. Direct evidence that the parental RNA enters a duplex without degradation was obtained in two ways. In the first, a dense radioactive phage was used to infect cells and density measurements made of the RNase-resistant material to show that it was indeed a double-stranded molecule containing intact parental material (Kelly & Sinsheimer, 1967a). In the second type of experiment it was shown that careful denaturation could release an intact parental strand from the duplex (Erikson et al., 1965). These results justify the conclusion that step 1 in RNA replication is the synthesis of a complementary strand.

The enzyme which catalyzes the synthesis of the complementary strand is coded for by the phage RNA. If protein synthesis is blocked with CAM before infection no complementary strand synthesis is observed (Kelly & Sinsheimer, 1964). More direct evidence has come from the discovery of temperature sensitive mutants of f2 which synthesize complementary strand at low temperatures but not at high

(Lodish & Zinder, 1966a). This enzyme which catalyses Step 1 has been unimaginatively called Enzyme 1. An enzyme which catalyses complementary strand synthesis has indeed been found in cells infected with a coat protein amber mutant (sus-11) of f2 (Shapiro & August, 1965) but this enzyme has not the specificity expected for the natural enzyme (vide infra) and its relevance to the normal infection process is doubtful.

At 5 minutes after infection there are one or more Enzyme 1 molecules per cell and one strand of RNA. Since these represent an infinitesimal fraction of the bacterial cytoplasm it is necessary to ask how an Enzyme 1 molecule finds the correct RNA and initiates replication. A possible explanation could be that after the translation of the parental RNA into an Enzyme 1 molecule the template and product remain bound together, and no enzyme is released into the cytoplasm. Alternatively, the enzyme may be released but have extremely high specificity and affinity for the phage RNA to the exclusion of other RNAs. It will become clear that the latter is the more likely hypothesis.

If an Enzyme 1 molecule is always firmly bound to RNA then in a complementation experiment it would be impossible to recover a mutant defective in Enzyme 1 function. When a temperature sensitive (ts) Enzyme 1 mutant is crossed with a coat protein mutant (sus-11) of f2, rescue of the temperature sensitive mutant is observed (Lodish &

Zinder, 1966). A similar result in which an early function ts mutant is rescued by a late function ts mutant has been described (Pfeifer et al., 1965). Evidence has also been obtained that normal amounts of polymerase are made even if RNA synthesis has been prevented (Lodish, Cooper & Zinder, 1964; Lodish, 1966) implying that the parental RNA can itself synthesize the normal number of polymerase molecules. Finally, addition of CAM 10 to 15 minutes after infection blocks polymerase synthesis but allows double strand synthesis to continue (Lodish, 1966) which suggests the presence of more polymerase molecules in the cell at 15 minutes than there are double-stranded molecules.

Additional evidence for the release of the Enzyme 1 molecule from the RNA comes from the discovery of the great specificity in vitro of the "replicase" for its own RNA (Haruna & Spiegelman, 1965a) since it would be superfluous for an enzyme to be specific if it was never released from its own template. The specificity of replicase for Qβ RNA, to the exclusion of MS2 RNA presumably acts at the Enzyme 1, rather than the Enzyme 2 (vide infra) level, since it would be uneconomical if all the single-stranded RNAs in a cell were made into duplexes. Although the specificity of the Qβ replicase is clear, the nature of this specificity is not. A claim has been made that fragmented RNA is only poorly recognised but the evidence is weak (Haruna & Spiegelman, 1965b). Experiments on the inhibition

(Haruna & Spiegelman, 1966) and the priming (Hori, 1967) of replicase by polynucleotides are difficult to understand (e.g. poly C primes, but does not inhibit; poly U inhibits, but does not prime). Whether the specificity is on the level of base sequence or secondary structure remains to be determined.

The complex consisting of the enzyme bound to the parental strand has been tentatively identified in infected cells (Godson & Sinsheimer, 1967). It has a sedimentation coefficient of about 40S. Since double-stranded RNA first appears in this region, it is possible that this complex synthesizes the complementary strand. Recently, using the Qβ -replicase, a 40S component containing the complementary strand has been observed in vitro (Feix & Weissmann, 1967).

In summary, the parental RNA phage probably directs the synthesis of several Enzyme 1 molecules. Eventually one of these forms a complex (40S) with the parental molecule and starts synthesizing the complementary strand.

(d) Is double-stranded RNA a red herring?

The major argument so far proposed for the relevance of double-stranded RNA to infection is that it is a satisfying hypothesis. An observation by Haruna & Spiegelman (1966) that RNA fragments can be converted to duplexes suggested, however, that the double-stranded RNA molecules are enzymatic by-products, produced by action of the replicase on damaged input strands. In the light of these

observations and the suggestion that complementarity might not be as satisfying as other replication schemes (Spiegelman & Haruna, 1966) it is necessary to review the evidence that the double-stranded RNA plays a precursor role in infection.

If RNA duplexes are an inert by-product of the infection they would be conserved during replication; i.e. if it is a random irrelevant process that makes double strands there is not likely to be another such process for taking the strands apart again. There is a classical test to determine if duplexes are conserved (Meselson & Stahl, 1958). When this test is adapted to RNA duplexes it has been shown that in fact duplexes are not conserved during replication (Kelly & Sinsheimer, 1967a; Part II of this thesis).

A study of the kinetics of synthesis of double-stranded and infective RNA shows that both begin to be synthesized about 8 minutes after infection, at an approximately exponential rate. Thus it is not possible to establish a product-precursor relationship between those two types of RNA on the basis of kinetics. A claim that "replicative form" accumulates late in infection (Weissmann et al., 1964a) must be discounted, for the authors failed to distinguish between the 14S "core" RNA which is involved in replication and a small 6-7S fragment which apparently is not (Kelly, Gould & Sinsheimer, 1965).

A common approach to the study of phage-specific RNA involved the depression of host cell RNA synthesis by agents such as actinomycin D (Kelly et al., 1965; Part III of this thesis), or ultra-

violet light (Fenwick, et al., 1964) which permit phage RNA synthesis to continue. On analysing the RNA by sucrose gradient sedimentation several phage specific components were observed, but the one which, on the basis of short pulse labeling experiments, appeared to be the precursor, was heterogeneous and had a sedimentation coefficient of about 15S. Although this approach was initially very useful, interpretation of experiments was complicated by the indirect effects of the actinomycin D on phage biosynthesis (Haywood & Harris, 1966; Lunt & Sinsheimer, 1966).

Other experiments of a similar type but avoiding antibiotics have confirmed a precursor role for the heterogeneous 15S component. The 15S component can be identified in infected cells growing under normal conditions either by centrifuging the RNA under partially denaturing conditions (Kelly & Sinsheimer, 1967b; Part III) or by use of the specific dilution assay (Weissmann et al., 1964b). In both cases, short pulses of radioactivity appeared in the 15S component before it appeared in the viral RNA (Kelly & Sinsheimer, 1967c; Part V; Billeter et al, 1966).

The remaining argument in favour of a precursor role for this component is that its structure is compatible with its supposed function. The heterogeneous 15S component yields, after gentle RNase digestion, a homogeneous double-stranded 14S peak and some small fragments of single-stranded RNA (Erikson et al., 1965). Careful measurements of RNase resistance and density of the purified

15S component indicate that its structure consists of a double-stranded RNA, twice the molecular weight of the viral strand, to which is attached a single-stranded tail of length less than the viral strand. Denaturation of this material either by heat (Ammann et al., 1964; Erikson et al., 1966) or by dimethylsulfoxide (Kelly & Sinsheimer, 1967b; Part III) yields an infective strand of RNA; it also yields a molecule smaller than a viral RNA which, on the basis of pulse-labeling experiments, appears to be the nascent strand. These results clearly imply that the heterogeneous 15S component is a replicative intermediate (RI) and that the heterogeneity arises because the nascent strand remains attached during purification.

(e) The Mechanism of Replication

In the previous discussion two types of replication have been discussed. Step 1 involves the synthesis of complementary strand on a single-stranded template; and Step 2 has a single-stranded RNA synthesized on a double-stranded template. Experiments both in vitro (Weissmann, 1965) and in vivo (Billeter et al., 1966) indicate that at least late in infection the viral strand is synthesized in much greater amounts than the complementary strand. The study of phage mutants, and the behaviour of "stringent" infected cells deprived of an amino acid, have provided evidence for two separable events in RNA replication, the synthesis of complementary strands and the synthesis of viral strands. It is thus satisfying to believe that complementary strands are made only by Step 1 and viral strands by Step 2, and that Step 2 occurs much more frequently than Step 1 late in infection.

If only viral strands are transcribed from the duplex then the replication is "asymmetric". Two types of asymmetric replication have been described in experiments in which a double-stranded nucleic acid has been used as a template for DNA-dependent RNA polymerase *in vitro*. If a double-stranded DNA is used (Hayashi, 1965) the nascent RNA strand is bound to the duplex by only a few nucleotides at the growing point. Such a mode of transcription is usually termed conservative, since the duplex is conserved. If a synthetic DNA-RNA hybrid is used as template, the nascent strand displaces the parental RNA from the duplex, in some cases, at least, (Sinsheimer & Lawrence, 1964; Chamberlin & Berg, 1964) in an asymmetric semi-conservative mode of replication. These two types of asymmetric replication are diagrammed in Figure 1.

It is now possible to diagram the complete replication scheme involving viral and complementary strand synthesis. For example, in Figure 2, a replication scheme is illustrated in which Step 2 is asymmetric and semi-conservative. It is possible on this scheme to produce viral and complementary strands at different rates. An analogous replication scheme can be drawn in which Step 2 is still asymmetric but is conservative. (Fig. 3). However, to reconcile such a scheme to the fact that duplexes are not conserved (Kelly & Sinsheimer, 1967a, Part II) it would be necessary to introduce yet a third step, involving presumably a third enzyme, to replicate the duplexes semi-conservatively.

FIGURE LEGENDS

Fig. 1 A schematic diagram of possible structure for the replicative intermediate (RI) of RNA phages. v denotes the viral strand identical to that in the phage. c is the strand complementary to the viral strand. n is the nascent strand, complementary to c, with the growing point indicated by an arrowhead. The single-stranded portion of the RI is referred to as the tail, and the double-stranded portion as the core or RNase-resistant duplex. The structures can be distinguished experimentally since for the semi-conservative case, the tail is part of the viral strand and in the conservative case, it is part of the nascent strand.

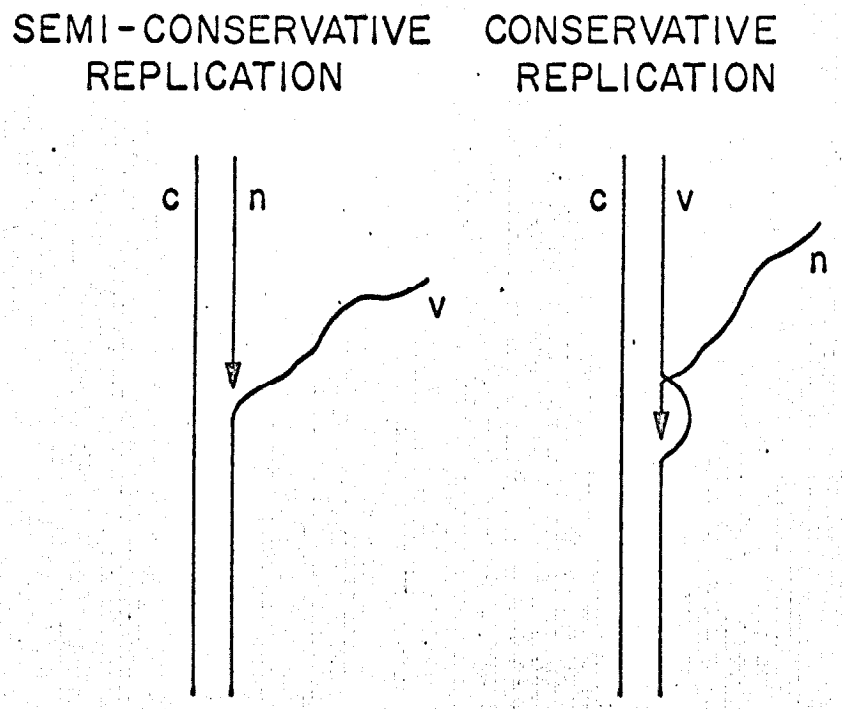
SEMI-CONSERVATIVE REPLICATION
CONSERVATIVE REPLICATION
c
n
v
c
v
n

Fig. 1.

Fig. 2 and 3. A replication scheme for RNA phages is described. Starting at the top left hand side the viral strand (v) synthesizes a complementary strand to give a duplex. This duplex is then, in this example, transcribed in a semi-conservative, asymmetric fashion (Fig. 1), giving a duplex which can be transcribed once again, and a viral strand which can be incorporated into phage, into polysomes or can synthesize a new s complementary strand. Fig. 3 is identical except that Step 2 is conservative and asymmetric.

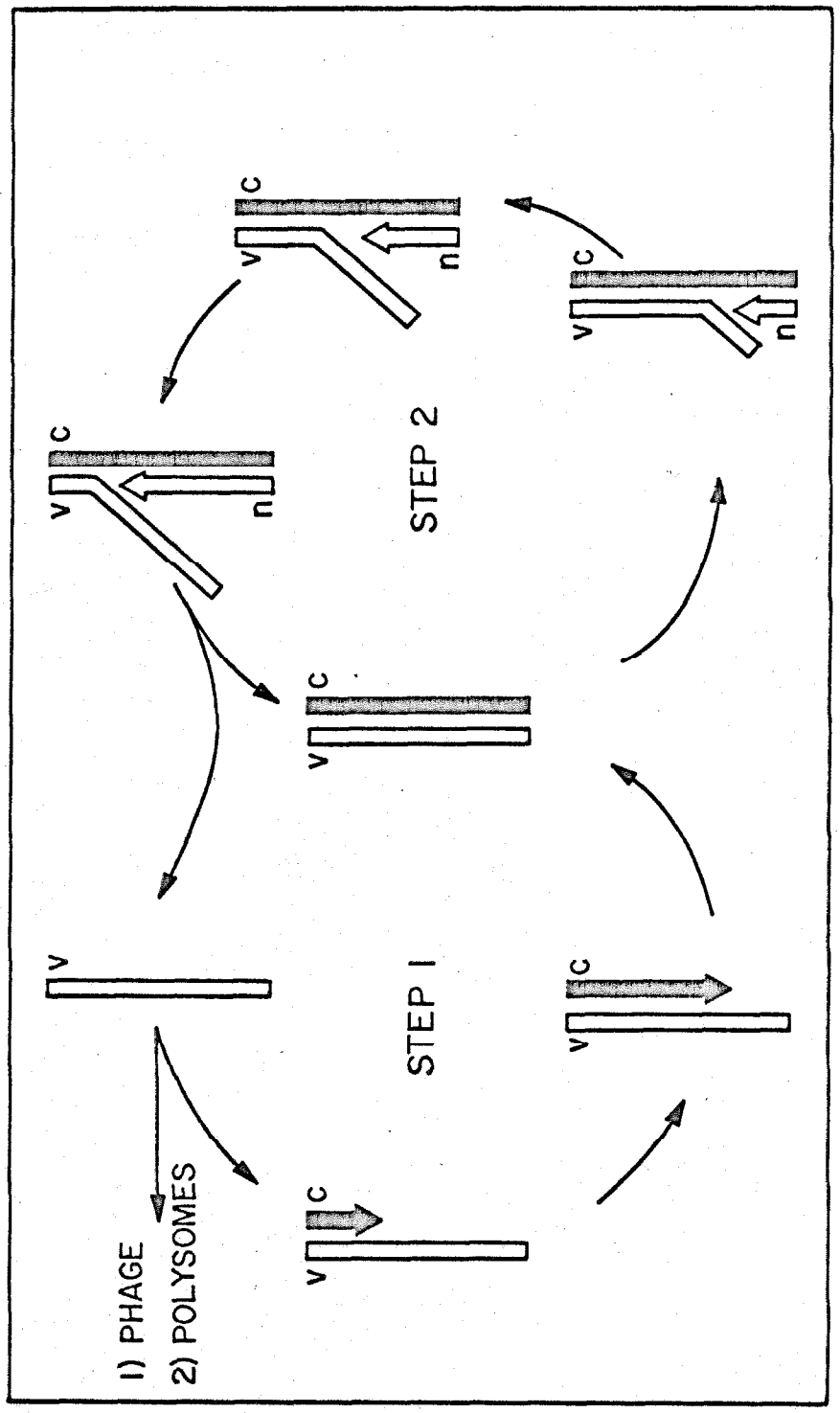

Fig. 2.

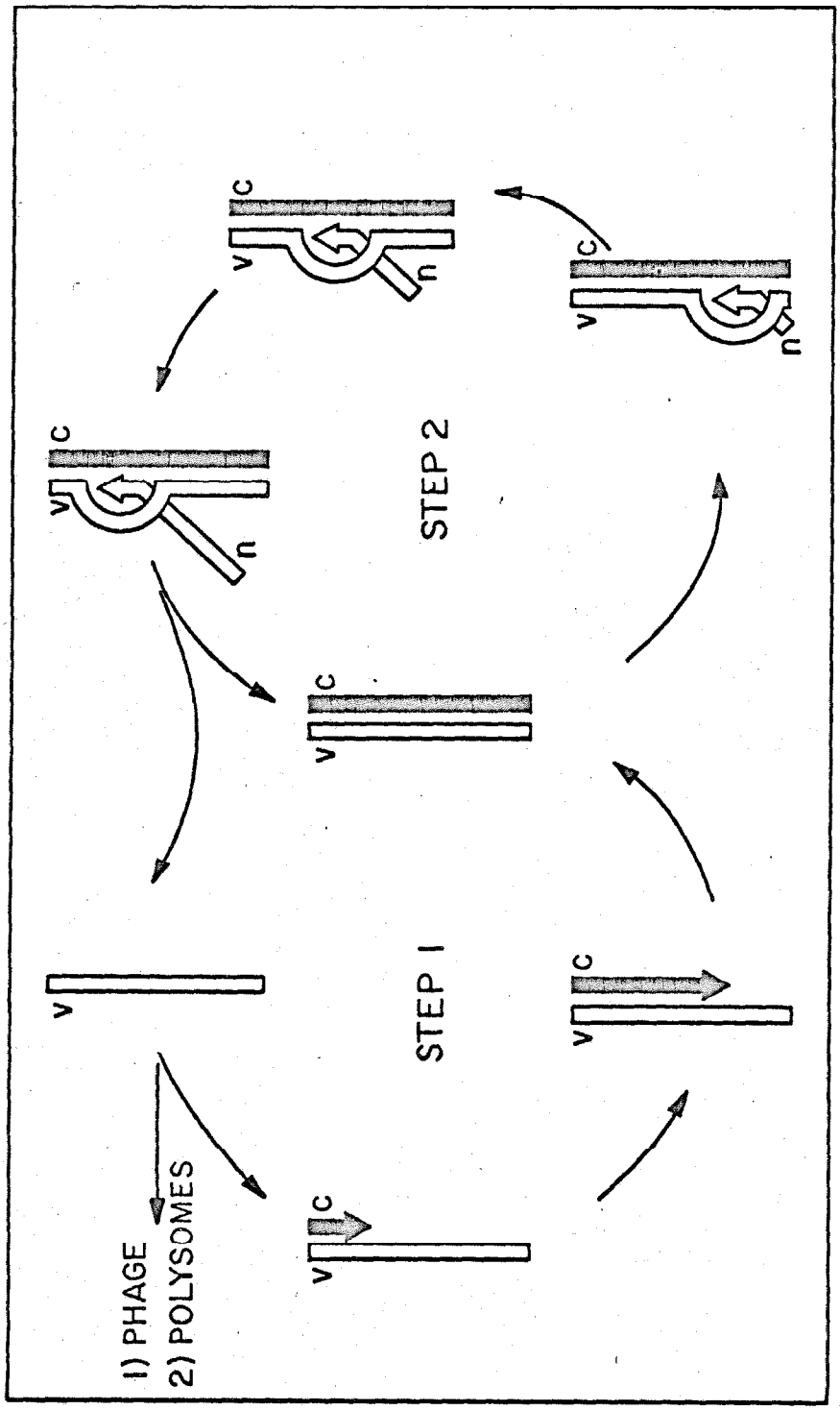

Fig. 3.

From an examination of the structure of the RI molecule it was concluded that both of the structures shown in Figure 1 are present in infected cells (Kelly & Sinsheimer, 1967c, Part V) and therefore both mechanisms of replication are in operation. This was an unexpected result and its possible significance will be discussed later (Part V(b)).

(f) Are there one or two polymerases?

Since the replication scheme in figure 2 seems to involve two quite distinct steps, the question arises whether one or two enzymes are required. It is clearly possible to isolate phage mutants defective in Step 1 but not in Step 2 (Lodish & Zinder, 1966a). However, both the synthetase enzyme purified by Weissmann et al (1964b) and the replicase purified by Haruna & Spiegelman (1965) synthesize both viral and complementary strands (Weissmann & Feix, 1966; Mills et al., 1966). There are several possible ways of interpreting this. The assumption made above that complementary strands are never transcribed from a duplex in a Step 2 process may be invalid, but this is the least likely possibility. Alternatively, there is either one enzyme with two genetically separable functions or two enzymes with a strong tendency to form an aggregate. The latter seems the more likely since an enzyme can be isolated which has only Enzyme 1 function (Shapiro & August, 1965). No mutant has yet been found defective in Step 2, however, and there is no evidence for the synthesis of a fourth phage-specific protein essential to infection. (Nathans et al., 1966). It is thus tempting to speculate that the

phage specific Enzyme 1 forms a stable complex with a host enzyme, perhaps DNA-dependent RNA polymerase, altering the specificity of the latter and changing its characteristics so that it can be purified from the original RNA polymerase. The only, very indirect, supporting evidence is that single-stranded viral RNA production also obeys stringent control (Friesen, 1965) in the same way as messenger RNA production, whereas complementary strand synthesis does not (Lodish, 1966).

In this regard it is interesting that, in contrast to the phage-bacterial system, the double-stranded RNA produced by animal RNA viruses is infective to the host cell (Montagnier & Sanders, 1963; Pons, 1964). This implies the existence in the host cell of an enzyme capable of producing a single-stranded RNA.

It is clear from arguments similar to those presented for Enzyme 1 synthesis, that there is a pool of Enzyme 2 in the cytoplasm which does not have an RNA primer attached. It is still possible, however, that the binding of a primer RNA to Enzyme 2 is irreversible. Double-stranded RNA does not act as a primer for the replicase (Mills et al., 1966) and polynucleotide inhibitors are much less effective against an in vitro synthesizing system if the template is first bound to the enzyme (Haruna & Speigelman, 1966). These results, and the strong attachment of the template to the purified

synthetase (Weissmann et al., 1964b) suggest that once the enzyme is bound to the primer it might not be dissociated; but this is by no means certain.

(g) Packaging the product

The major coat protein of the RNA phage f2 has been purified and the amino acid sequence determined (Weber et al., 1966). The presence of a minor protein in whole phage has been deduced from the discovery of a class of amber mutants which complement coat protein mutants, but yield a defective phage particle (Heisenberg, 1966; Argetsinger & Gussin, 1966). There are four histidine molecules in normal phage but none in the defective particles (Argetsinger, personal communication). Thus in addition to the 100 or so coat protein subunits there are one to four "maturation proteins" in whole phage. In infected cells before the end of the eclipse period there are a large number of non-infectious phage-like particles (80S) containing infective RNA (G.N. Godson, personal communication). It would be pleasing if these were analogous to the defective particles described previously and that the maturation event involves supplying a histidine-containing adsorption organ. Such an event could perhaps be performed in vitro, analogous to in vitro maturation of T4 (Edgar & Wood, 1966). It is also possible, however, that the histidine-containing protein is an internal protein.

Little is known about the mechanism of lysis except that no phage-induced lysozyme is made (Zinder, unpublished data). No

mutants defective in lysis have yet been found.

(h) Checks and balances

The RNA phage is a simple, unsophisticated virus but even so it has developed control mechanisms in the interests of economy. During an infection about 10^7 coat protein molecules must be synthesized. It would be wasteful to synthesize more polymerase molecules than there are RI molecules, i.e. 100 to 1,000 per cell. Some way of controlling protein synthesis seems necessary, but, since polymerase is needed early and coat protein late this control should not become effective until near the end of the eclipse period.

In the previous discussion experimental evidence was presented that all phage proteins are synthesized on the parental strand, and so the control process does not involve switching on a hyper-active coat protein gene late in infection. Histidine containing proteins are synthesized earlier than the majority of the phage specific proteins (Oeschger & Nathans, 1966) and polymerase synthesis appears to shut off quite early in infection (Lodish, Cooper & Zinder, 1964). The crucial observation is, however, that polymerase synthesis is not shut-off if the cell is infected with an amber mutant defective in coat protein synthesis.

It is thus clear that all proteins are made initially, and that the concentration of each of these increases until the coat protein

pool reaches an appropriate level. The coat protein then acts as a repressor of the synthesis of polymerase. In a mutant which lacks coat no control will be possible. Recently, weak repression of histidine-containg protein synthesis was observed if coat protein was added to an *in vitro* protein synthesizing system (Sugiyama & Nakada, 1967; Eggen & Nathans, 1967). This does not exclude the possibility that coat protein also acts to stimulate the production of its own messenger RNA and thus that control is transcriptional as well as translational.

Lodish et al. (1964) have presented evidence that the parental RNA can synthesize all the necessary polymerase and have suggested that this may be a mechanism of control. There are no grounds for believing such a control exists normally, nor is it obvious how a cell distinguishes parental from progeny single strands.

In the previous discussion it was pointed out that single-stranded phage RNA synthesis stops if infected stringent cells are deprived of an amino acid. This can be explained in terms of coupling protein synthesis with transcription of the RNA as has been postulated for the stringent control of messenger RNA production (Shih, *et al.*, 1966). This control is not operative *in vitro* either for phage RNA synthesis (Spiegelman *et al.*, 1966). or for messenger RNA synthesis (Hayashi, 1965) which emphasizes the difference between *in vivo* and *in vitro* systems.

SUMMARY

An RNA phage adsorbs to the pilus of a male bacterium and infiltrates RNA into the cell cytoplasm by an unknown mechanism. The free RNA then produces a polysome on which coat protein and polymerase are synthesized. When a polymerase finds the correct starting point on the parental RNA (3' end?) it synthesizes a complementary strand. Either the same enzyme, or another, begins synthesis of a new viral strand on the duplex as template. It is immaterial whether the new or the old viral strand is displaced. The released single-strand can direct the synthesis of more proteins while the duplex (with the same enzyme?) produces more single strand. In addition, some of the single strands encounter a polymerase and synthesize new complementary strands.

When the concentration of coat protein reaches a critical level it represses the further production of polymerase to permit production of large amounts of coat protein. The coat protein and RNA combine to give an uninfective particle which is made infective by the addition of a histidine containing adsorption protein. When 10^5 particles have accumulated (10% of which are plaque formers) the cells lyse by an unknown mechanism.

On the basis of present knowledge, including the results presented herein, this seems the most reasonable sequence of events in the life cycle of an RNA phage. Data are given in this thesis to justify only two aspects of this scheme; the production of double-stranded

RNA and its role in single-stranded RNA production. The other parts of the life-cycle are, however, very pertinent to the discussion of these observations.

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Part I

THE KINETICS OF SYNTHESIS OF SINGLE- AND DOUBLE-STRANDED RNA.

INTRODUCTION

This part of the thesis is in two main sections. The first is a paper describing the original observation of double-stranded RNA. In the second section, information is given on the synchronization of infection and on the amounts of single and double-stranded RNA in infected cells at various times after infection. Efforts are also described to determine the kinetics of polymerase synthesis by using CAM to block protein synthesis. This description is brief since Lodish (1966) arrived at essentially identical conclusions using the same methods and confirmed his results by direct assay of the polymerase.

MATERIALS AND METHODS

All the procedures used in this section have been published. TPG medium and the preparation of ^{32}P labeled MS2 were described in Kelly & Sinsheimer (1967a). The methods for determining the adsorption of phage to bacteria and the penetration were described by Valentine & Wedel (1965). The use of CAM to synchronize infections was discussed by Kelly et al., (1965). The assay for 14S ribonuclease-resistant duplex is described in several papers, the most recent of which is Godson & Sinsheimer (1967). To assay the amount of infectious RNA in cells, they were lysed and their nucleic acids extracted with phenol (Kelly et al., 1965). Samples of the nucleic acids were assayed for infectious RNA content using the procedure of Strauss (1964).

RESULTS

(a) The discovery of double-stranded RNA in MS2 infected cells.

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LETTERS TO THE EDITOR

A New RNA Component in MS2-infected Cells

The replication of the phage ϕX174, a single-stranded DNA-containing phage, involves the formation of a double-stranded component, the "replicative form" (Sinsheimer, Starman, Nagler & Guthrie, 1962). Therefore in studying the mechanism of replication of the single-stranded RNA-containing phage, MS2, an obvious approach is to look for an analogous double-stranded material.

Since it is known that the RNA of the parental phage is not incorporated into the progeny phage (Davis & Sinsheimer, 1963; Doi & Spiegelman, 1963), it might be found in a replicating component in the infected cell. The following experiments were therefore designed to observe the fate of labeled parental phage RNA after infection. A culture of *Escherichia coli* 3000 (2 to 4×10^8 cells/ml.) was infected with ^{32}P-labeled MS2 at a multiplicity of infection of about 0·2, chilled at 7 minutes after infection, and spun down. The phenol-extracted RNA was analysed on a sucrose density gradient and the results are given in Fig. 1. The single-stranded MS2 RNA appears as a 27 s peak as expected (Strauss & Sinsheimer, 1963), but a considerable fraction of the counts appear in a slower component of approximately 18 s.

The slower sedimenting peak is not produced when protein synthesis is prevented by addition of chloramphenicol (25 μg/ml.), 10 minutes prior to infection (Fig. 2), and would appear therefore to play a significant role in the replication process.

Material was isolated from the two peaks of radioactivity in Fig. 1 and treated with ribonuclease (1 μg/ml. in 0·01 M-NaCl, 0·05 M-tris, pH 7·4, and 0·005 M-$MgCl_2$; 60 min at 37°C) as in the experiments of Gomatos & Tamm (1963) on reovirus. By comparison of the counts of the two fractions obtained by precipitation with trichloroacetic acid, it was found that the slower sedimenting component was more than 5 times as resistant to ribonuclease digestion as the 27 s component. The ribonuclease-resistant fraction was not demonstrable in the total RNA extracted from the cells infected in chloramphenicol.

When the RNA extracted from infected cells was given a milder ribonuclease treatment (0·5 μg/ml. in 0·1 M-tris, pH 7·3, 0·02 M-EDTA; 20 min at 22°C) and analysed on a sucrose gradient (Fig. 3), the ribosomal and viral RNA was hydrolysed and the ribonuclease-resistant material could be seen to sediment as a single component.

In addition to parental RNA, the 18 s component contains RNA synthesized during the replication of the phage. This has been demonstrated by an analysis of the uptake of [^{14}C]uracil into infected cells which had been converted to protoplasts and treated with actinomycin D (Haywood & Sinsheimer, 1963), to minimize the synthesis of cellular RNA. Such infected protoplasts were incubated in warm nutrient medium containing [^{14}C]uracil (18 mc/m-mole, 12 μmole/l.) and actinomycin D (10 μg/ml.). After 15 minutes the protoplasts were spun down on to a sucrose pad (Guthrie & Sinsheimer, 1963) and the RNA extracted by phenol. This RNA was centrifuged in a sucrose density gradient as before and the results, similar to those of Fig. 1, are shown in Fig. 4.

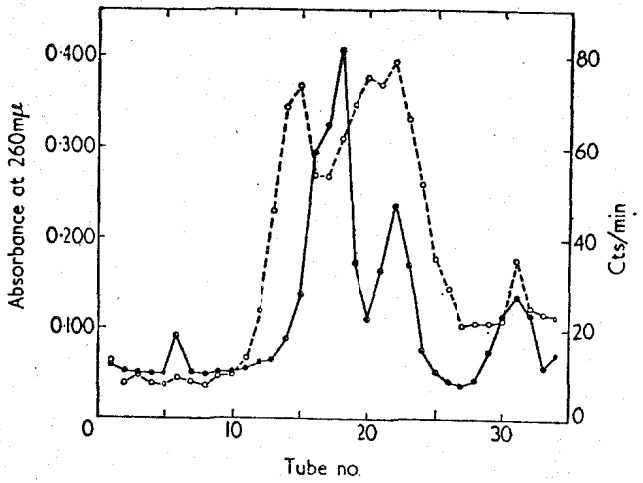

FIGURE 1

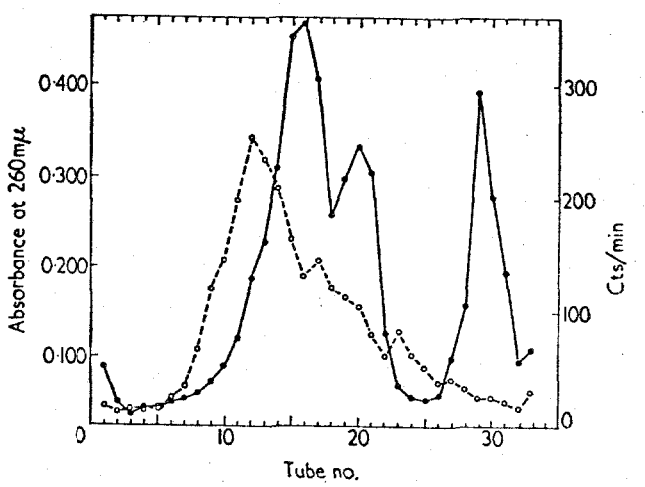

FIGURE 2

FIG. 1. Distribution of ^{32}P (– – ○ – – – ○ – –) and u.v. absorption (— ● —— ● —) after sucrose gradient sedimentation of RNA from cells infected with ^{32}P-labeled MS2 phage. In all experiments, a 5 to 20% w/v sucrose gradient was used (0·1 M-tris, pH 7·3, 5°C). Samples were centrifuged for 6 hr at 37,000 rev./min in a Spinco SW39 rotor.

FIG. 2. Distribution of ^{32}P (– – ○ – – – ○ – –) and u.v. absorption (— ● —— ● —) after sucrose gradient sedimentation of RNA from cells incubated with chloramphenicol for 10 min, then infected for 10 min with ^{32}P-labeled MS2 phage.

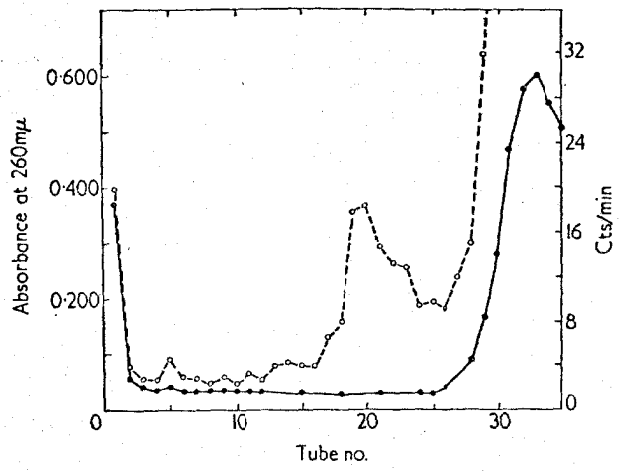

FIGURE 3

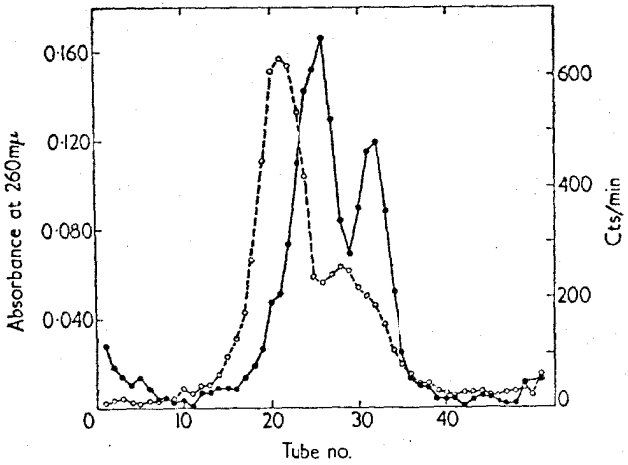

FIGURE 4

FIG. 3. Distribution of ^{32}P (– – ○ – – – ○ – –) and u.v. absorption (— ● —— ● —) after sucrose gradient sedimentation of RNA extracted from cells infected with ^{32}P-labeled MS2 phage and then digested with ribonuclease.

FIG. 4. Distribution of ^{14}C (– – ○ – – – ○ – –) and u.v. absorption (— ● —— ● —) after sucrose gradient sedimentation of RNA from infected protoplasts, incubated for 15 min in a medium containing [^{14}C]uracil and actinomycin D.

Buoyant density experiments in cesium sulfate have shown that the density of the ribonuclease-resistant material is lower than that of the single-stranded MS2 RNA by about 0·08 g/cm^3.

As yet no infectivity has been found associated with the ribonuclease-resistant component or the slow-sedimenting component before ribonuclease treatment (Davis, Strauss & Sinsheimer, 1961).

These experiments suggest the existence of a distinct RNA component—probably a double-stranded RNA—in MS2 infected cells. Although this result is in some disagreement with the investigation of Doi & Spiegelman (1963), it does support the *in vitro* studies of Weissman & Borst (1963) on MS2 and is analogous to the results obtained by Montagnier & Sanders (1963) with encephalomyocarditis virus.

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(b) Synchronization of Infection

To characterize the growth cycle of a phage-infected cell it is useful if the infection is synchronous, i.e. if all phage initiate their infection simultaneously (e.g. Denhardt & Sinsheimer, 1965). To synchronize an infection it is necessary to arrest the development of the phage after the steps in the infection process which are the major causes of the asynchrony (e.g. adsorption, injection) have been completed. The cells are infected in the presence of such a blocking agent then, after a suitable time, the block is removed to produce a synchronized infection.

If the major source of asynchrony is the adsorption event, synchrony should be achieved by pre-adsorption (Erikson et al., 1965). When RNA phage are incubated with bacteria for about ten minutes at $0^{\circ}C$, the phage adsorb but the RNA does not penetrate. Synchrony might then be achieved by rapidly diluting into pre-warmed medium. However, if the rate of penetration at $25^{\circ}C$ is compared for two cultures, one pre-adsorbed, the other not, (Figure I - 5) the spread in penetration time is only marginally less for the pre-adsorbed culture.

This spread in penetration does not mean that an individual RNA strand takes 10 minutes at $25^{\circ}C$ to enter the bacterium. It

Figure I - 5

The effect of pre-adsorption on the kinetics of penetration of ^{32}P MS2. E. coli C 3000 was grown to 2 x 10^8 cells/ml. in broth at 37°C. One half of the culture was chilled to zero degrees and infected with ^{32}P MS2 phage at a multiplicity of one. 10 minutes later (t = 0) the cells were warmed to 25°C rapidly and the kinetics of penetration determined by blending the cells, sedimenting them and then counting the pellet. (-x---x-). The other half of the culture was chilled to 25°C, infected, and the kinetics of penetration determined in the same way (-•——•-).

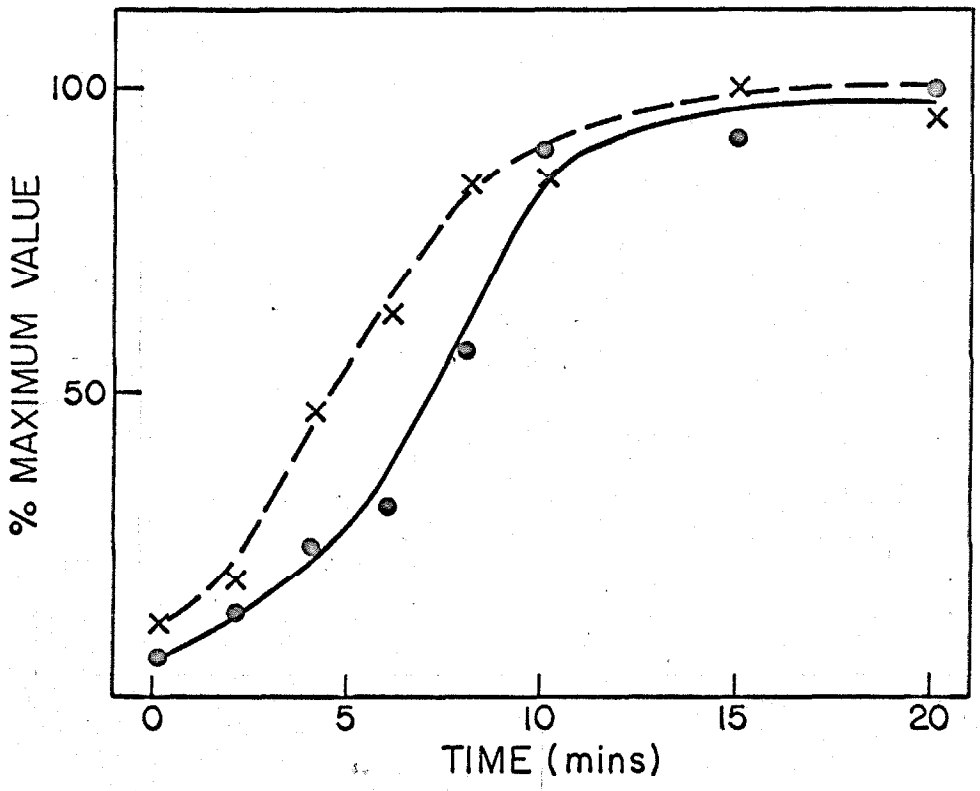

Fig. I - 5.

is found that the kinetics of appearance of infective centres (i.e. infected bacteria after blending) almost exactly co-incides with the kinetics of penetration of the RNA (i.e. radioactivity in the cell after blending). If an RNA strand requires 10 minutes to enter the bacterium, no infective centres should appear until all the radioactivity has penetrated. It can be concluded that the time for penetration at 25°C is short (less than 30 seconds) and that the asynchrony derives from a spread in the times that a penetration event is initiated.

To achieve synchrony, the RNA must therefore be allowed to penetrate the host, but not start protein synthesis. This has been done by infecting in the presence of CAM, and then removing it 5 minutes after infection.

(c) Kinetics of RNA synthesis

In section 1(a) it was shown that double-stranded RNA can be isolated from infected cells as an approximately 14S peak, if the nucleic acids of infected cells are digested to a very limited extent with RNase. If ^{32}P labeled MS2 phage are used to infect cells growing in medium containing ^{3}H-uracil it is possible to follow the kinetics of appearance of both the parental duplexes (i.e. ^{32}P-labeled 14S material) and the progeny duplexes (i.e. ^{3}H-labeled 14S material). The results are shown in Figure I - 6.

On the basis of experiments of this type it is concluded that the parental RNA is first detected in a double-stranded form at about five minutes after infection and reaches a maximum value 3 minutes

FIGURE LEGENDS

Figure I - 6

The kinetics of RNA synthesis in an MS2 infected cell. E. coli C 3000 was grown in TPG medium to 2 x 10^8 cells/ml. and 25 γ/ml. CAM added to permit the infection to be synchronized (Kelly et al., 1965). Five minutes later the cells were infected with MS2 at a multiplicity of one. After a further five minutes the CAM was removed, ^{3}H-uracil (10 μc/ml., 2 γ/ml.) added and the cells incubated at 37°C. Samples were removed at intervals and assayed for whole phage, infective RNA and ^{32}P and ^{3}H-labeled ribonuclease-resistant (14S) duplexes. ^{32}P cts/min (—●———●—); ^{3}H cts/min. (-O----O-); infectious RNA (-x----x-).

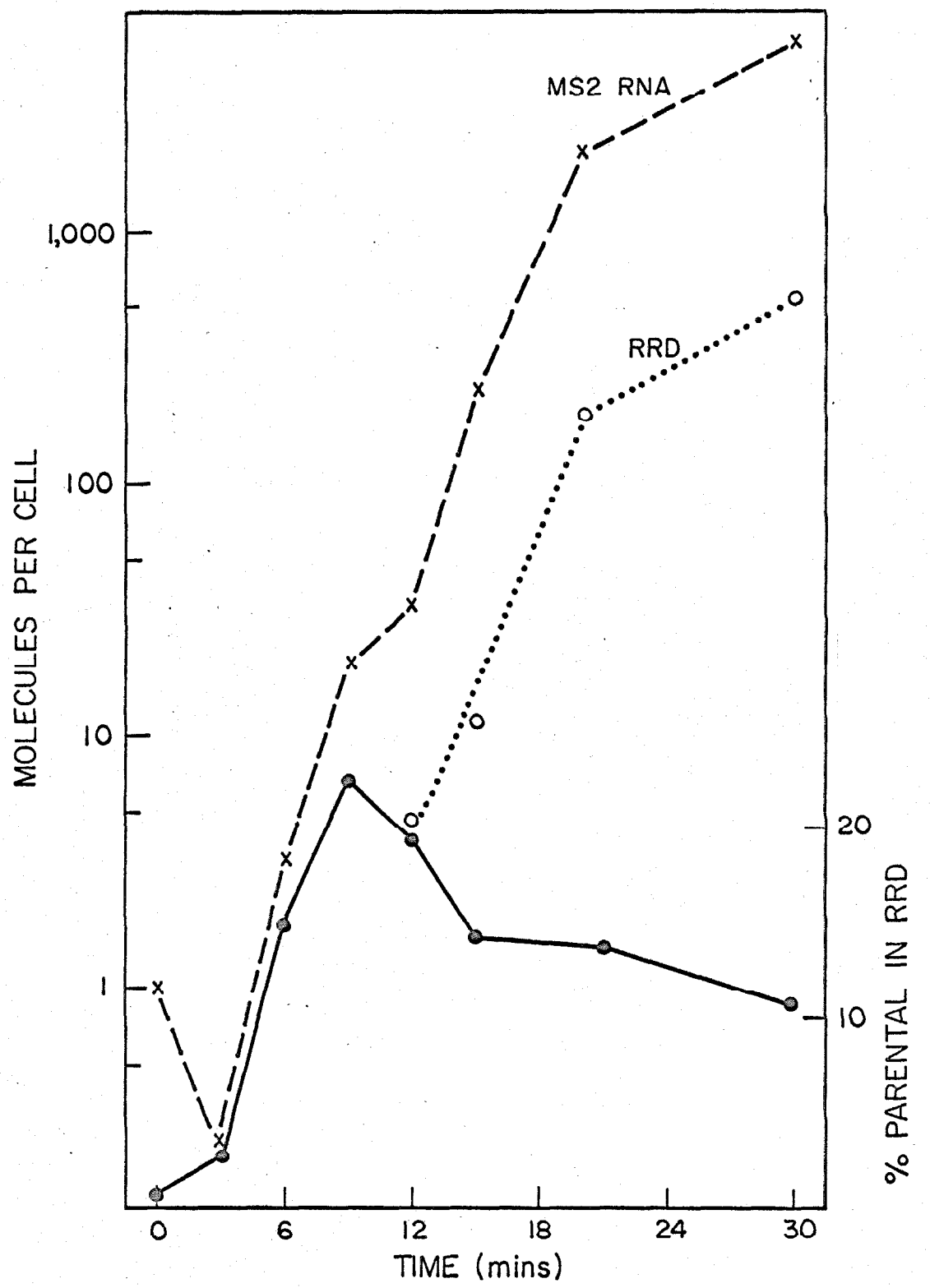
MS2 RNA
RRD
1,000
100
10
1
MOLECULES PER CELL
20
10
% PARENTAL IN RRD
0
6
12
18
24
30
TIME (mins)

Fig. I - 6

later. A possible reason for this lack of synchrony will be discussed later. The amount of parental material in duplex decreases after 8 minutes but the significance of this is obscure because of the extensive breakdown and re-incorporation of parental material which occurs. The maximum conversion to duplex is 20% of the input material (the infected culture was not blended) but this is likewise difficult to interpret because of the lack of synchrony, and because only 30% of the input particles were plaque-formers. The maximum obtained is, however, proportional to the percentage of plaque-formers in a preparation.

Infective RNA begins to appear almost simultaneously with the conversion of the parental RNA to a double-stranded form. This result, which is due to asynchrony, does not permit a product-precursor relationship to be established. The initial decrease ("eclipse") in infectivity has been observed on several occasions but because of the presence of relatively large amounts of the inhibiting ribosomal RNA (Strauss, 1966) these results are on the limit of statistical significance. The synthesis of RNA infectivity precedes the appearance of whole phage (not shown) by about 10 minutes, which indicates the presence of a large pool of free infectious RNA. Such a pool is expected if RNA must act as a messenger before becoming encapsulated into a phage.

The sensitivity of the assay is such that ^{3}H-labeled progeny duplexes are only detected 12 minutes after infection. Thereafter, the kinetics of synthesis of progeny duplexes can be considered exponential up to 20 minutes, after which the rate of synthesis drops. Since the intracellular specific activity of the ^{3}H-uracil was not known (a prototroph was used), the number of progeny duplexes at 15 minutes, given by the accurate method described in Part II of this thesis, was used to normalize the data. With this assumption there are ten times more infective single strands than duplexes, which is in good agreement with published data (Billeter et al., 1966).

(d) The effect of CAM on phage-specific RNA synthesis

The synthesis of phage-specific RNA was also examined in the presence of CAM to determine whether RNA synthesis requires concomitant protein synthesis. Samples were removed from an infected culture at various times after infection. To one half of the sample, CAM (25 γ/ml.) was added and the incubation continued; the other half was assayed for infectious RNA. At 40 minutes after infection, the incubation in CAM was stopped and the samples assayed for infectious RNA.

The results of an experiment are given in Table I-1. Clearly, infectious RNA can be synthesized in CAM indicating that protein synthesis is not necessary for RNA synthesis. By 18 minutes

TABLE I - 1.

Effect of CAM addition on infective RNA production at 30°C

Time of sampling or adding CAM (t) (minutes).	Infectivity at t / Infectivity at 40'.	Infectivity at 40'; CAM from t to 40'. / Infectivity at 40'; no CAM.
3'	0.1%	6.5%
6'	0.2%	17%
9'	0.6%	28%
12'	0.2%	50%
15'	1.4%	64%
18'	5.2%	170%
23'	7.5%	175%
40'	100%	100%

Cells were grown at 37°C in TPG medium to 2 x 10^8 cells per ml., and infected in CAM (Kelly et al., 1965). After removing the CAM the cells were resuspended in TPG at 30°C. Samples were removed at intervals to determine the amount of infectious RNA; at the same time CAM (25 γ/ml.) was added to another sample which was incubated further At 40 min. after infection all the incubations were stopped and the infectious RNA determined. All infectivities are given as a ratio to the infectivity at 40 min.

after infection enough polymerase has been synthesized to give a normal yield of infectivity whereas infective phage are observed only after 20 minutes of infection. It is not possible by this technique to determine if the synthesis of polymerase ceases at 20 minutes, but other experiments by more direct techniques indicate that it does (Lodish et al., 1964).

Complementary strand can also be synthesized in CAM. If a culture is infected, synchronously, with ^{32}P MS2 phage and samples assayed for RNase-resistant (14S) duplexes as described above, the results obtained (Table I - 2) are identical to those in Figure I - 6. If CAM is added at various times after infection and the amount of duplex at 8 minutes determined (Table I- 2) it is clear that complementary strand synthesis can occur in the presence of CAM when the drug is added later than 3 minutes after infection.

The results in Table I-2 are somewhat surprising, however. Normally all parental duplexes can replicate semi-conservatively (Lodish & Zinder, 1966c; Kelly & Sinsheimer, 1967c) and the approximately constant amount of parental material in duplex form implies that the parental strand cycles between a single-stranded and a double-stranded form. Assuming RNA replication remains semi-conservative in the presence of CAM, the results in Table I - 2 suggest that this cycling can take place in CAM. This was verified by adding CAM at 8 minutes and determining the amount of

TABLE 1 - 2

The effect of CAM addition on the synthesis of parental duplex

Time of sampling or adding CAM (t) (minutes).	14S at t. / 14S at 8 min.	14S at 8 min; CAM from t to 8 min. / 14S at 8 min., no CAM.
0	0	0
1.5	0	0
3.0	0	0
4.5	27%	53%
6.0	80%	100%
8.0	100%	100%

The experimental design was identical to that of Table I - 1, except that the amount of the input ^{32}P-labeled RNA in parental duplex was determined and referred to that at 8 min. after infection. Also, the incubation was at 37°C, not 30°C.

^{32}P in double-stranded RNA at various times after this. No striking decrease is observed on blocking protein synthesis. (Table I - 3). To explain this observation it could be postulated that the parental RNA takes the polymerase when it leaves the duplex but this would predict an accumulation of double-stranded RNA and a block of infectious RNA synthesis, neither of which are observed. Alternatively, if the infected cell contains many polymerase molecules free in the cytoplasm, such a result would be expected.

This second hypothesis is supported by the observation that adequate amounts of polymerase are made by 18 minutes (at 30°C) whereas double-stranded RNA synthesis continues much longer. It is also supported by the experiments of others quoted in the Introduction. Finally, such a scheme would explain the asynchrony that is observed even in a supposedly synchronized culture, as a result of the random nature of the combination of polymerase and template to initiate an infection. True synchrony might be better achieved by infecting an auxotroph in the absence of a required RNA precursor.

Though indirect, the results presented in this section suggest several conclusions. It is clear that when protein synthesis is blocked, viral and complementary strand synthesis continues. The polymerase appears in the first 4 minutes of infection and all the polymerase necessary for viral RNA synthesis is made by 18 minutes (at 30°C). The data also favour the conclusion that polymerase molecules are free in the cytoplasm rather than always bound to RNA templates.

TABLE I - 3.

Effect of CAM addition on the turnover of the parental duplex.

Time of sampling. (minutes).	% of parental in double-strand, CAM added at 8 min.	% of parental in double-strand, no CAM.
8'	25%	25%
10'	22%	-
12'	16%	-
16'	16%	-
20'	17%	20%

Cells were synchronously infected with ^{32}P MS2. After 8 min. of infection at 37°C, a control sample was removed and CAM added to the remainder. At intervals, samples were removed and assayed for double-stranded RNA (in this case, RNA resistant to digestion with 25 ɣ/ml. RNase, for 30 min. at 37°C.

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Part II

NON-CONSERVATIVE REPLICATION OF DUPLEXES

INTRODUCTION

In Part I, the discovery of double-stranded RNA and some observations on its rate of synthesis were described. Because of asynchrony, it was not possible to deduce a product-precursor relationship from the kinetics. Indeed, since the parental material was rapidly broken down and re-incorporated, and the fraction of the input material which became double-stranded was so small, the "parental duplex" was possibly irrelevant to infection. This possibility was strengthened by the in vitro observation of Haruna & Spiegelman (1965) that "damaged" single-stranded RNA became double-stranded when incubated with replicase.

Clearly, if the parental strand were labeled with both dense and radioactive isotopes, whether or not it was intact when it entered a duplex could be determined. In addition, by use of dense isotopes, it could be determined if the duplexes are conserved during replication. If double-stranded RNA is not conserved, then it must play some role in infection; if it is conserved, then either replication is conservative or it does not involve double-stranded RNA.

A paper submitted to the Journal of Molecular Biology is first presented, followed by some additional data to justify the claim that the kinetics observed in chase experiments reflect a nucleotide pool effect. Finally, another experiment is briefly described which confirms that parental duplexes are not conserved.

RESULTS

(a) The non-conservative replication of double-stranded RNA

This part of the thesis has been accepted for publication in the Journal of Molecular Biology. It is included in the form in which it was submitted.

SUMMARY

The replication of phage-specific RNA duplexes in cells infected with the bacteriophage MS2 has been studied by means of density labeling and CsCl equilibrium density sedimentation. It was found that the input parental RNA strand enters a duplex and that progeny duplexes appear soon afterwards with approximately exponential kinetics. Chase experiments indicate that most of the parental and progeny duplexes are not conserved.

1. INTRODUCTION

There is now good evidence showing that double-stranded RNA molecules, or "duplexes," are present in cells infected with bacteriophages and viruses containing single-stranded RNA (see review Levintow, 1965). Evidence has been presented indicating that the double-stranded RNA is part of an intermediate involved in the replication process (Fenwick, Erikson & Franklin, 1964; Lodish & Zinder, 1966a; Billeter, Libonati, Vinuela & Weissmann, 1966). It has been postulated that the single-stranded RNA is made using a double-stranded template by an asymmetric process, either semi-conservative or conservative (Martin, 1966; Erikson & Franklin, 1966). Recently, evidence for an asymmetric semi-conservative replication process for the parental RNA has been presented (Lodish & Zinder, 1966b). However, ribonuclease-resistant RNA can also arise in vitro when the purified replicase functions abnormally (Haruna & Spiegelman, 1966).

It has been shown previously that RNA can be sedimented to equilibrium in a CsCl density gradient at elevated temperatures (Bruner & Vinograd, 1965; Kelly, Gould & Sinsheimer, 1965). The availability of the heavy isotopes ^{13}C and ^{15}N in chemically suitable form encouraged us to study the replication of double-stranded RNA by a chase procedure similar to that of Meselson & Stahl (1958). The results to be presented here show that both the parental duplexes (the double-stranded RNA molecules containing the input viral RNA

single strands) and the progeny duplexes formed later during the infection are not conserved.

2. MATERIALS AND METHODS

(a) Media

"Minimal" medium refers to a solution containing salts but no carbon or nitrogen source. It has per liter 12.1 g tris; 0.5 g NaCl, 8.0 g KCl, 0.046 g KH_2PO_4, 0.2 g $MgCl_2.6\ H_2O$ and 0.023 g Na_2SO_4. The pH is adjusted to 7.4. After autoclaving, 4 ml. of 1 M $CaCl_2$ and 1 ml. of thiamin hydrochloride (10 mg/ml.) are added.

"Heavy minimal" medium consists of minimal medium with 1 mg/ml. ^{13}C glucose (Merck, Sharp & Dohme of Canada Ltd., 43-47% ^{13}C) and 1.1 mg/ml. $^{15}NH_4Cl$ (Biorad Laboratories, 99% ^{15}N). "Light minimal" medium contains equal concentrations of ^{12}C glucose and $^{14}NH_4Cl$.

TPA medium has been described (Kelly et al., 1965). The chase medium consists of TPA medium supplemented with 50 µg/ml. of each of the nucleic acid bases and ribonucleosides (except guanine and guanosine), 20 mg/ml. glucose, 5 mg/ml. ribose, 10 mg/ml. KH_2PO_4 and 10 mg/ml. NH_4Cl.

(b) Preparation of ^{32}P MS2

E. coli C3000 was grown for over 7 generations in 10 ml. of heavy minimal medium to make $^{13}C^{15}N^{32}P$ MS2, or in 10 ml. of light minimal medium to make $^{12}C^{14}N^{32}P$ phage. In both cases the doubling time of the bacteria was 40 to 60 min. MS2 phage were added when the final cell concentration reached 5×10^7 cells/ml. at a m.o.i. = 2. Five min later, 4 mc of ^{32}P orthophosphate (carrier free) were added.

Lysis was complete after 5 hr.

The debris was sedimented and the supernatant extensively dialyzed. After dialysis CsCl was added to bring the density of the phage solution to 1.4 g/cm^3 and the lysate was sedimented to equilibrium at 45,000 rev./min for 20 hr in an SW50 Spinco rotor. The peak fractions were pooled and the sedimentation to density equilibrium repeated.

The total yield of phage obtained varied from about 5×10^{10} to 10^{12} plaque-forming units, less than is obtained using TPA as the growth medium. The ratio of plaque-formers to particles was 0.15 to 0.3 and the density difference between light phage and heavy phage, 0.028 g/cm^{-3}.

(c) Isolation of Ribonuclease-Resistant Duplexes

Because of the difficulty of interpretation of the density of a molecule which might be partly double- and partly single-stranded all density measurements were made on RNase-resistant duplexes. To isolate such resistant duplexes, nucleic acid was extracted from infected cells by lysis, treatment with phenol, and precipitation with alcohol as we have described previously (Kelly et al., 1965). The precipitate was resuspended and treated with 0.1 µg/ml. RNase, in 0.1 M NaCl and 0.01 M $MgCl_2$ for 10 min at 37°C. After chilling the samples were immediately sedimented in a 5 to 20% sucrose gradient in 0.1 M tris, pH 7.4, for 5 hours at 60,000 rev./min and at 5°C, in the SW65 rotor of the Spinco L2-65. The resistant duplex could be isolated as a peak of 13 to 14S (Kelly & Sinsheimer, 1964).

This material was either alcohol-precipitated or used directly in a CsCl gradient without removal of the sucrose.

(d) Preparation of Density Marker

^{3}H-labeled RNase-resistant duplexes were used as a density marker. E. coli C3000 was grown to 2 x 10^{8} cells/ml. in TPA medium and infected with MS2 phage at a multiplicity of 5 to 10. Twenty min after infection ^{3}H-uracil was added (6 μc/ml.; 15 c/mM). Five min later the culture was chilled and centrifuged.

In experiments such as that shown in Fig. 4, equal aliquots of the ^{3}H-labeled infected cells were added to infected cells containing heavy ^{32}P RNA, prior to phenol extraction, to provide both an estimate of ^{32}P recovery and, after the resistant duplexes have been isolated, a density marker in the CsCl density gradient. In other experiments ^{3}H-labeled resistant duplexes were isolated as described above and added prior to centrifugation to the material whose density was to be characterized.

(e) CsCl Equilibrium Density Gradients

As has been pointed out before (Bruner & Vinograd, 1965; Kelly et al., 1965) RNA will form a band in CsCl if the centrifugation is performed at high temperature. Since we wished in these experiments to be able to band $^{13}C^{15}N$ RNA we found it necessary to use temperatures of 65°C to obtain the desired solubility of CsCl.

2.52 g of CsCl (Harshaw Chemical Co., Cleveland, Ohio) were added to 1.4 ml. of the RNA solution to be analysed to give a final density of 1.87 g/cm^{-3} and a volume of 2.05 ml. The solutions were warmed to dissolve the CsCl, and added to Beckman polyallomer tubes. The tubes

were filled with 3 ml. of paraffin oil and placed in a preheated Spinco SW50E rotor. The samples were sedimented at 37000 rev./min for 36 hrs or longer at 65°C in the Spinco Model E ultracentrifuge. On completing the run, fractions were collected using a prewarmed Buchler piercing unit giving 220 to 240 drops per gradient.

It is possible that the preparations of RNase-resistant duplexes are slightly contaminated with single-stranded RNA. To eliminate such a source of error, all CsCl gradient samples are treated with 25 γ/ml. RNase for 30 min. at 37°C, in 0.1 M NaCl, 0.1 M tris pH 7.4, 10^{-2} M $MgCl_2$ and then precipitated with TCA, prior to counting.

It might be expected from the inactivation of RNA by high temperatures (J. H. Strauss, 1966) that a significant number of hits would be introduced into RNA during approximately 40 hr at 65°C. We do indeed observe that MS2 RNA (27S) sediments as a broad peak of about 8S after centrifugation at 65°C for 48 hr; however, there is little, if any, change in the sedimentation of a RNase-resistant duplex, after centrifugation at 65°C for 40 hr.

(f) Density Differences

2 ml. of a CsCl solution of density 1.870 g/cm^3 were centrifuged for 48 hr at 65°C in a Spinco SW50E rotor. Fractions were collected and densities determined at various points along the gradient by weighing a known volume. Assuming constant drop size, the difference in density between the ends of the gradient is 0.130 g/cm^3. The calculated value expected for this difference is 0.164 g/cm^3 (P. Rüst,

personal communication). Use of the former value results in a difference in density between fully heavy and fully light RNA of 0.032 ± 0.001 g cm^3, whereas use of the latter gives 0.041 ± 0.002. Density differences given subsequently refer to the measured value.

3. RESULTS

(a) Parental RNA enters a duplex

The first experiment asked if the ^{32}P of the parental RNA strand enters a RNase-resistant duplex directly or through breakdown and reincorporation. Two parallel cultures of E. coli C3000 were grown, the first in heavy minimal medium, the other in light minimal medium. When the cell concentration was 2×10^8 cells/ml., the "heavy" culture was divided into two equal aliquots, one half being infected with $^{13}C^{15}N^{32}P$ MS2 phage, the other with $^{12}C^{14}N^{32}P$ MS2. The "light" culture was infected with $^{13}C^{15}N^{32}P$ MS2. Ten minutes after infection, the cultures were chilled and the cells centrifuged. RNase-resistant duplexes were isolated from each culture as described above.

The ^{32}P-labeled duplexes were mixed with light ^{3}H-labeled duplexes as density marker and sedimented to equilibrium in a CsCl equilibrium density gradient. Fractions were collected and assayed for ^{32}P and ^{3}H radioactivity. The results presented in Fig. 1 indicate that the density increment of the hybrid density duplexes (relative to the density of the light ^{3}H-labeled duplexes) is exactly half that of the duplexes in which both strands are heavy. This is true whether the heavy label of the hybrid duplexes is in the parental or the complementary RNA strand. This indicates that the RNase-resistant material is indeed a duplex and that the parental RNA enters

FIGURE LEGENDS

Fig. 1. The density of duplexes containing the input parental strand.

(a) Cells growing in heavy medium were infected for 10 min with $^{13}C^{15}N^{32}P$ MS2. The ^{32}P-labeled RNase-resistant duplexes were sedimented to equilibrium in CsCl, using ^{3}H-labeled light duplexes as density marker ($\rho = 1.87$). The difference in density between the peaks is 0.032 ± 0.001 g cm^{-3} using the measured density gradient

(b) $^{13}C^{15}N^{32}P$ MS2 were used to infect cells growing in light medium. The density difference is 0.017 ± 0.001.

(c) $^{12}C^{14}N^{32}P$ MS2 were used to infect cells growing in heavy medium. The density difference is 0.0165 ± 0.001.

(—o——o—) represents ^{32}P cts/min; (--•---•--) ^{3}H cts/min. The density of fully heavy duplex (HH) is indicated.

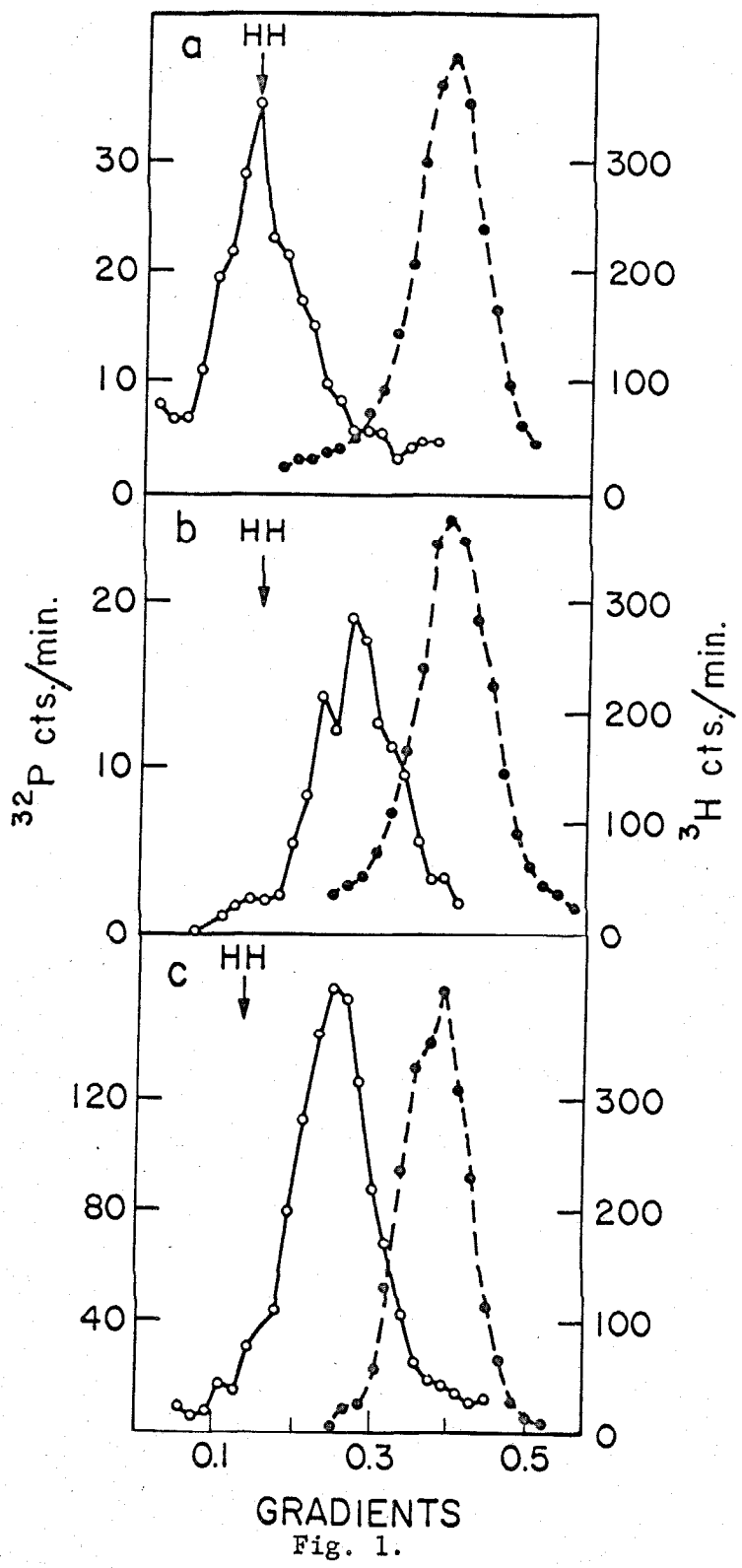
a
HH
30
20
10
0
300
200
100
0
b
HH
20
10
0
300
200
100
0
^{32}P cts./min.
^{3}H cts./min.
c
HH
120
80
40
300
200
100
0
0.1
0.3
0.5
GRADIENTS

Fig. 1.

the duplex directly. Even at 25 min after infection ^{32}P radioactivity is found solely in the hybrid "half-heavy" region of the CsCl gradients.

(b) Production of progeny duplexes

The use of density labeled parental RNA and equilibrium density centrifugation has enabled us to study the formation of the first complementary strand and the kinetics of synthesis of the subsequent RNase-resistant duplexes.

To synchronize the infection process, cells grown to 1 x 10^8 cells/ml. in light minimal medium were infected at a multiplicity of 5, with $^{13}C^{15}N^{32}P$ MS2 at 0^oC for 10 min. The cells were brought rapidly to 37^oC and ^3H-uracil (15 c/mM; 10 μc/ml.) added. This was taken as zero time. Aliquots of the culture were precipitated with TCA at minute intervals to ensure that the ^3H-uracil was not exhausted. At the required times, the samples were chilled with dry ice and acetone to 0^oC, the culture was centrifuged to sediment the cells and the resistant duplexes were then isolated.

Since the first ^3H-labeled material to be synthesized has the same density as the hybrid density parental duplex, it presumably represents synthesis of the strand complementary to the input parental RNA. Before maximum conversion of parental to duplex has occurred, material of light density appears, associated with the synthesis of progeny duplexes. Since the density of the ^{32}P-labeled parental duplex is "half-heavy" it is reasonable to assume that it contains equal amounts of progeny and parental material. Given

this assumption, the ratio of ^{3}H to ^{32}P radioactivity for this "half-heavy" duplex can be used to calculate from their ^{3}H content the numbers of light progeny duplex synthesized per parental duplex.

The results of four such experiments are given in Fig. 2. Whereas it is obvious that too much weight should not be placed on the results obtained at very early times (when the light progeny duplexes appear as a skewing of the ^{3}H peak of the hybrid duplexes, rather than by the presence of a separate peak) it seems clear that there is one progeny duplex per parental duplex at about 8 min after infection, and that the kinetics of synthesis of progeny duplexes can be considered exponential with a doubling time of about 70 seconds.

(c) Replication of the parental duplex

The procedure used to determine whether or not the parental duplex is conserved is closely similar to that of Meselson and Stahl (1958). $^{13}C^{15}N^{32}P$ phage were used at multiplicity 5 to infect a culture of E. coli C3000 grown to 2.5 x 10^{8} cells/ml. in heavy minimal medium at 37°C. Ten minutes after infection the cells were chilled, sedimented and washed thoroughly with cold minimal medium to remove the heavy isotopes. A sample was removed to give a "zero time" sample and the remainder diluted into the light chase medium described above. After 5 and 10 min of incubation at 37°C samples were chilled and cells centrifuged. The RNase-resistant duplexes were isolated and their density determined by equilibrium density centrifugation with an appropriate density marker.

Fig. 2. The increase in the ratio of progeny duplexes to parental duplexes as a function of time after infection.

Cells were infected with $^{13}C^{15}N^{32}P$ phage and labeled with ^{3}H-uracil. After sedimentation in a CsCl density gradient, the ratio of the specific activities of ^{3}H and ^{32}P was determined and used to calculate the number of progeny duplexes per parental duplex. The data from four such experiments are given.

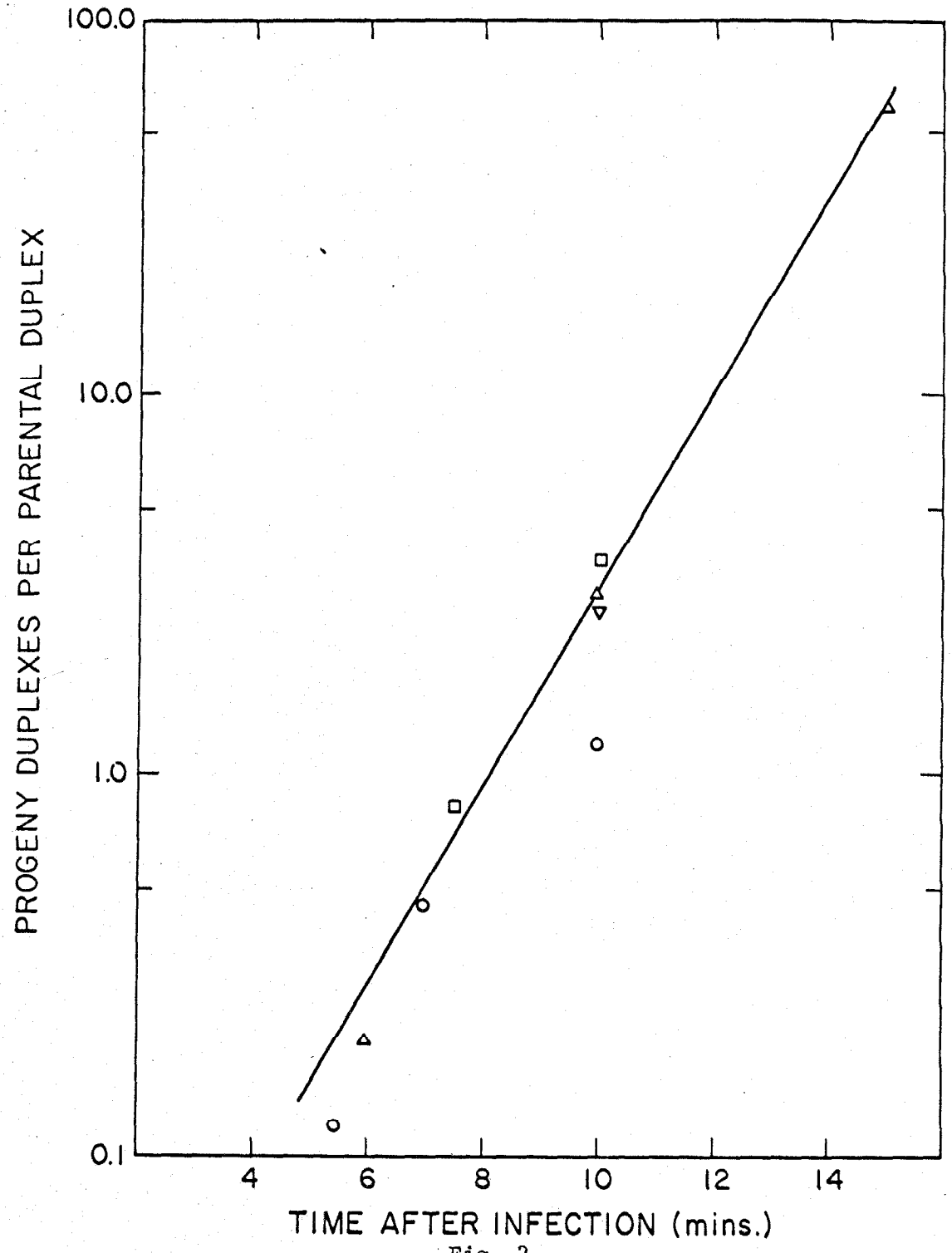
100.0
10.0
1.0
0.1
PROGENY DUPLEXES PER PARENTAL DUPLEX
4
6
8
10
12
14
TIME AFTER INFECTION (mins.)

Fig. 2.

Even if replication of duplexes is conservative most of the ^{32}P-labeled material seen after the chase could be "half-heavy" if the majority of the parental molecules are first converted to duplexes during the chase. The fraction of parental material in RNase-resistant duplex, however, was 9 to 10% for each of the three samples, indicating that maximum conversion of parental to resistant duplex had occurred prior to the chase. In preliminary experiments we had found that parental RNA begins to appear in resistant duplex four minutes after infection and the conversion is essentially complete at eight minutes after which there is little change.

The density of the parental material during the chase relative to a light duplex marker is shown in Fig. 3. The fully heavy and the fully light duplexes are separated by 0.0311 $\pm$ 0.0008 g/cm^{-3}; after a 5 min chase the density of the parental peak is reduced by 0.0064 $\pm$ 0.0008 g/cm^{-3}; and after 10 min of chase the density of the peak has reduced by 0.0153 $\pm$ 0.0008 g/cm^{-3}, although there is considerable skewing towards heavier densities.

Since the peak of parental material goes, very largely (75%), from a fully heavy density to a "half-heavy" density we can conclude that the majority of the parental duplexes can take part in a non-conservative process. The skewing of the ^{32}P-peak observed in Figure 3(c) suggests the presence of some conserved duplexes (25%). This could indicate a definite probability of a conservative replication at each synthesis or it could merely be an indication of the non-participation of some duplexes in further replication, perhaps as a consequence of the high multiplicity

Fig. 3. The density of the parental duplex during a chase from heavy to light medium is shown.

(a) $^{13}C^{15}N^{32}P$ MS2 were used to infect cells growing in heavy minimal medium for 10 minutes. The ^{32}P-labeled duplexes were sedimented in a CsCl equilibrium gradient with a light ^{3}H-labeled double-stranded marker.

(b) After removing the heavy medium and continuing the incubation a further 5 min in light medium, the ^{32}P-labeled duplexes were isolated and their density determined as in 3(a).

(c) As in 3(b) using an 10 min incubation time in light medium.

^{32}P cts/min (—o——o—); ^{3}H cts/min (--•---•--). The position of a fully heavy duplex (HH) is indicated.

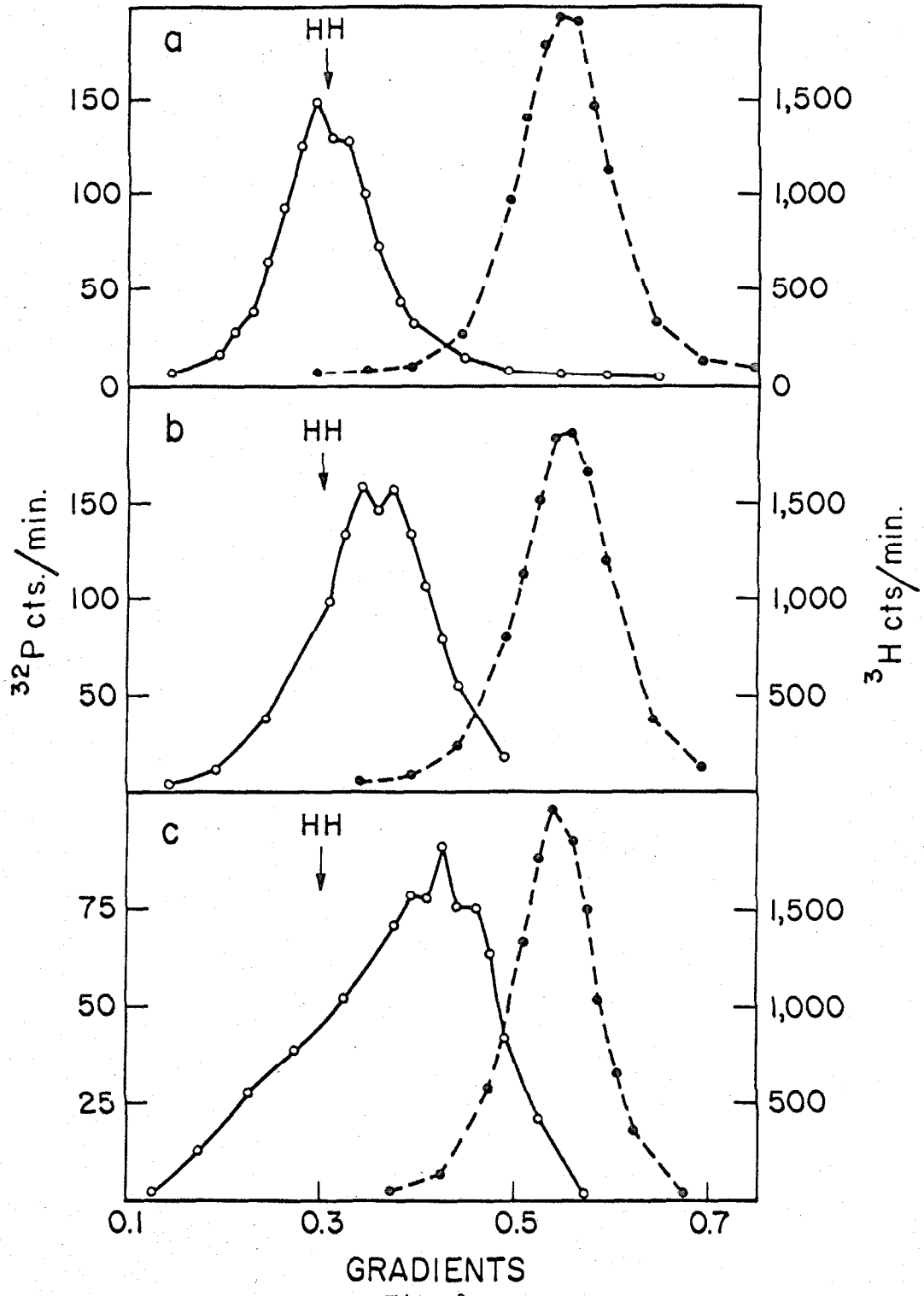
a
HH
150
100
50
0
1,500
1,000
500
0
b
HH
150
100
50
1,500
1,000
500
c
HH
75
50
25
1,500
1,000
500
0.1
0.3
0.5
0.7
GRADIENTS
^{32}P cts./min.
^{3}H cts/min.

Fig. 3.

of infection.

(d) Replication of progeny duplexes

Twenty min after infection there are approximately 100 resistant duplexes per cell, and the rate of synthesis of infective RNA and resistant duplexes is high. It is, therefore, a convenient time to study the replication of progeny duplexes.

Unlabeled light MS2 phage were used at a multiplicity of 3 to infect E. coli C3000 grown to 2 x 10^8 cells/ml. at 37°C in heavy minimal medium. ^{32}P orthophosphate (60 μc/ml., carrier free) was added 5 minutes after infection. Twenty minutes after infection the cells were chilled, centrifuged and washed extensively at 0°C with minimal medium. The cells were resuspended in prewarmed light minimal medium containing excess light glucose, ^{31}P-phosphate and light ammonium chloride. Aliquots of equal volume were removed after 0', 1', 2', 3', 5', and 8' of incubation at 37°C, chilled to 0°C and the cells pelleted.

Conversion of heavy single-stranded ^{32}P-RNA present in the cell before the chase to a duplex during the chase would give rise to a hybrid "half-heavy" duplex. Since the separation of such material from fully heavy material is of the order of two half-widths, a large peak of ^{32}P-labeled "half-heavy" material arising in this way could conceal the existence of conserved duplexes. It is thus necessary to measure the amount of ^{32}P-labeled duplex relative to that at zero time. Therefore an equal number of infected cells,

grown in light medium, and labeled with ^{3}H-uracil from 20 to 25 min after infection were added to the pelleted ^{32}P-labeled cells prior to lysis.

Resistant duplexes were isolated from the combined cultures as described previously and sedimented to equilibrium in a CsCl density gradient. For brevity, we give in Fig. 4 only the results of 0', 5' and 8' chase.

It seems clear that by 8 minutes less than 25% of the original duplexes have been conserved. At 5 minutes the ^{32}P peak is at a lighter density, but not yet at the hybrid "half-heavy" position. The half-width of the ^{32}P-labeled peaks does not significantly alter during the chase. The ratio of ^{32}P to ^{3}H increases by only 25% during the chase which excludes the possibility that the conserved duplexes are concealed by the half-heavy peak.

The experiment was repeated twice. In one of these the supplemented chase medium described in Materials and Methods was used without significantly altering the kinetics of the density shift. The results of these three experiments are summarized in Fig. 5. Despite the scatter of points it appears as if the density of the duplex decreases for about 8 minutes and then remains constant at approximately the "half-heavy" position. The results obtained for the parental duplex (Fig. 3) are included for comparison. Although in all cases the final density difference is slightly higher than the expected value of 0.5, this difference is thought to be within experimental error and thus is probably not significant.

Fig. 4. The density of progeny duplexes during a chase from heavy to light medium is shown.

(a) 20 min after infection cells growing in heavy medium containing ^{32}P orthophosphate were collected and washed. An aliquot was taken, the ^{32}P-labeled duplexes isolated and sedimented to equilibrium in a CsCl density gradient. The density marker in Figs. 4(a),(b) and (c) is light ^{3}H-labeled double-stranded RNA.

(b) An aliquot of the washed cells was incubated at 37°C in a light chase medium for 5 min, and the resistant duplexes analyzed as before.

(c) As in 4(b) with a "chase" time of 8 min.

(—o——o—) is ^{32}P cts/min; (--•---•--) ^{3}H cts/min; the positions of fully heavy (HH) and fully light (LL) duplexes are indicated.

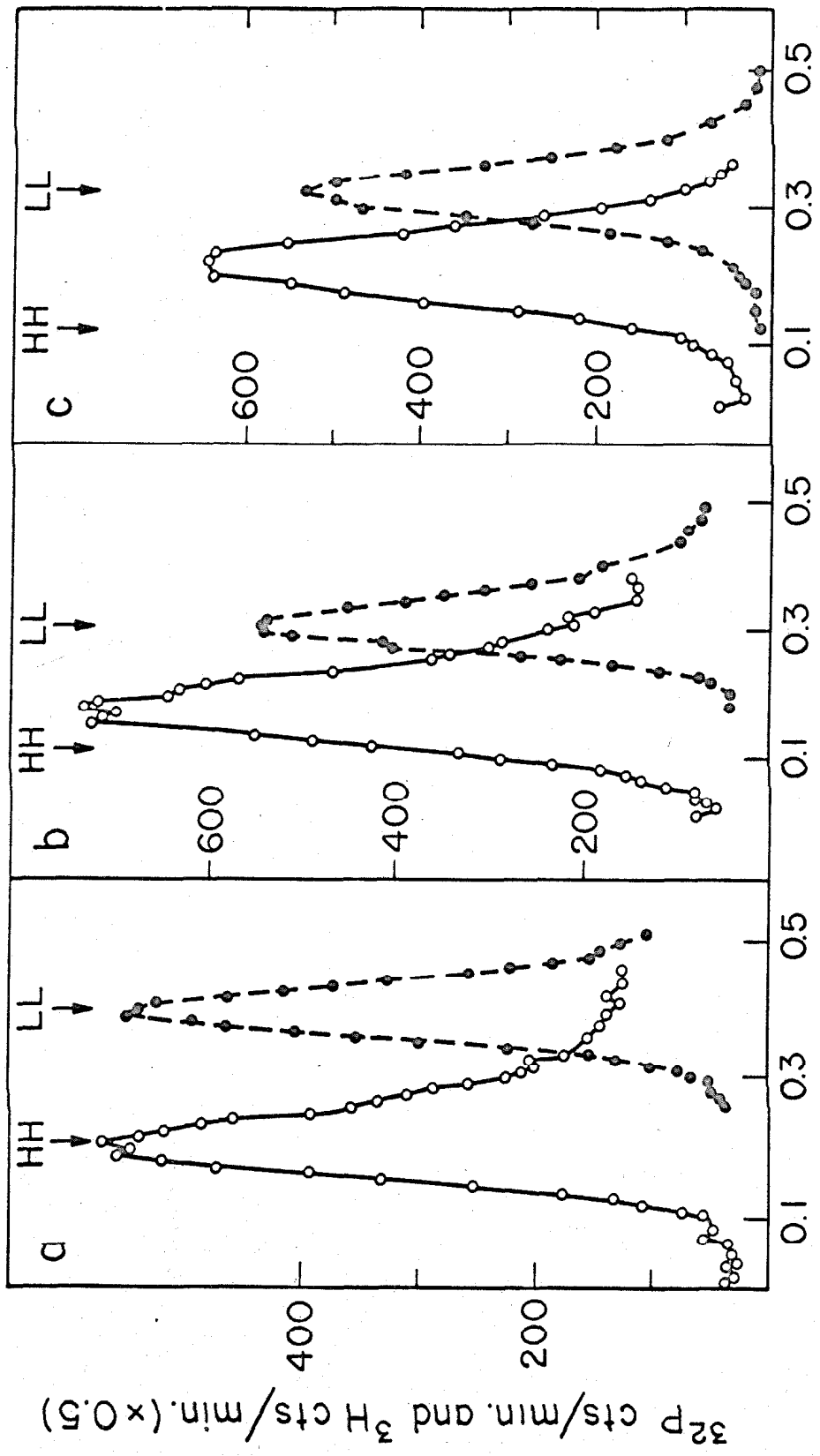
a
b
c
HH
LL
200
400
600
0.1
0.3
0.5
^{32}P cts/min. and ^{3}H cts/min. (x 0.5)
GRADIENTS

Fig. 4.

Fig. 5. The kinetics of the density decrease of progeny duplexes during a chase from heavy to light medium.

The results of three similar experiments are given. The symbol o represents all the data from the experiment in Fig. 4; • is a similar experiment; □ uses the rich chase medium; x refers to the parental duplex chase experiment (Fig. 3) and is included for comparison. ρ is the density of the ^{32}P material, ρ_{LL} is the density of the fully light RNA duplex and ρ_{HH} that of the fully heavy RNA duplex.

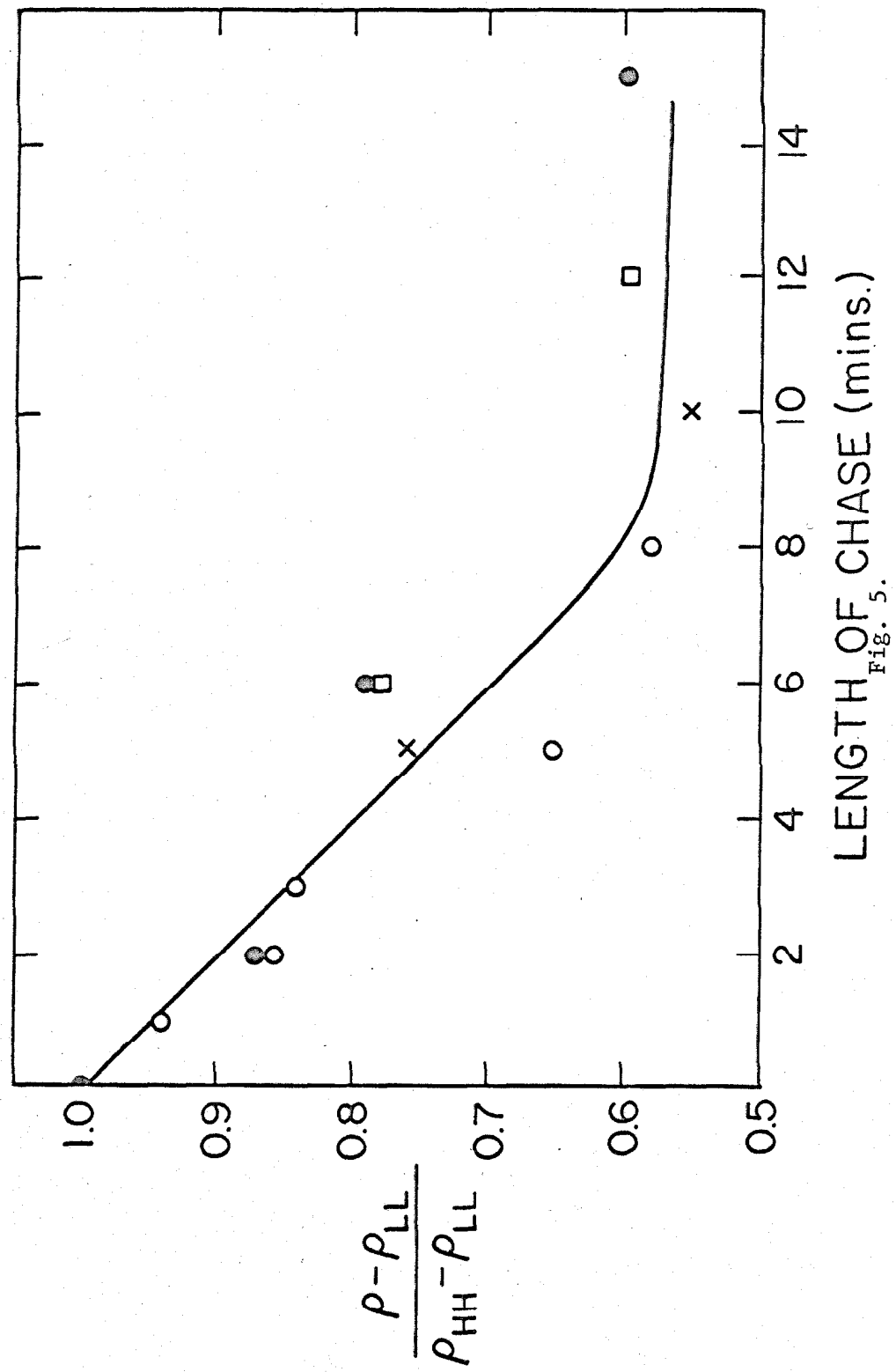
1.0
0.9
0.8
0.7
0.6
0.5
$\frac{\rho-\rho_{LL}}{\rho_{HH}-\rho_{LL}}$
2
4
6
8
10
12
14
LENGTH OF CHASE (mins.)

Fig. 5.

4. DISCUSSION

These experiments indicate that the input parental RNA enters a duplex structure without significant breakdown and reincorporation. The results therefore bear on the manner of progeny RNA synthesis from the duplex. In the proposed model of "semi-conservative" replication (Martin, 1966; Erikson & Franklin, 1966) the parental or viral strand is displaced from the duplex as a new strand is formed. The displaced, single-stranded portion of the viral strand is then subject to cleavage by the treatment with RNase.

In this regard, it is noteworthy that the density of the hybrid duplexes containing parental RNA is the same, after the RNase treatment, whether the density label is in the parental or the non-parental portion of the duplex. From the "semi-conservative" model, one would expect to observe duplexes of all densities from fully light to "half-heavy" when heavy parental virus is used to infect "light" cells, and from fully heavy to "half-heavy" when light virus is used to infect cells in heavy medium.

Since the radioactive label would be in the displaced parental strand the counts will, of course, be weighted toward the half-heavy duplexes in both cases. Nevertheless, we can calculate, assuming the replication points (the points of displacement of the parental strand in each parental duplex) are, at any instant, uniformly distributed over the lengths of all duplex-containing structures, that an average density difference of 0.01 g/cm^3 would be expected between the duplexes from these two modes of infection (heavy virus

in light cells and vice versa). This difference is not observed.

Various explanations of this result may be proposed. At any time a large proportion of the duplexes may not be involved in replication. Completed duplexes have indeed been observed both in the case of polio virus (Baltimore, 1966) and the phage M12 (Hofschneider, Ammann & Francke, 1966). Alternatively, parental RNA caught while synthesizing the complementary strand of a duplex would behave as partially RNase-resistant molecules and would give rise only to "half-heavy" duplexes after RNase treatment. If the kinetics of RNA synthesis during infection is such that a majority of parental RNA is in this stage of the replication cycle at any moment, the observed result would be obtained.

Alternatively, the synthesis of progeny RNA from the duplex may be in part "conservative." As has been previously observed (Sinsheimer & Lawrence, 1964; Chamberlin & Berg, 1964) the choice of the strand to be released from the duplex (parental or progeny) may not be absolute; if the chance that progeny strand is to be released is 0.5 or greater, the present results would be compatible with those of Lodish & Zinder (1966b).

Use of heavy parental phage has allowed us to quantitate the number of progeny resistant duplexes per parental resistant duplex. It is clear that the kinetics of progeny duplex formation can be considered exponential up to 15 minutes after infection. Other experiments have shown that the rate of progeny duplex formation

falls off after 20 minutes of infection. The "doubling time" for duplex formation is 1 to 1.5 min, and there are about 60 duplexes per cell, 15 min after infection. From measurements of the production of infective RNA under identical conditions, the number of infective RNA's produced per cell per minute at 15 minutes after infection is 200. Assuming the RNase-resistant duplexes arise from molecules which are all acting as replicative templates (and regardless of the number of replication points per duplex), then an average rate of synthesis of one infective RNA per template every 20 seconds can be calculated. If some duplexes are inactive, the rate must be greater, or if some duplexes have more than one replication point, the time of synthesis of a strand will be greater.

The results also support the conclusion that most of the parental duplexes and substantially all of the progeny duplexes are not conserved over a several minute period. We cannot definitively conclude that the replicative process is semi-conservative since hybrid density material could arise from the conversion of single-stranded heavy RNA to duplexes during the chase. This also complicates the interpretation of the ratio of ^{32}P to ^{3}H in Fig. 4, for the small increase in this ratio could be due to new duplex formation, or to an incomplete chase.

The kinetics of the density shift shown in Fig. 5 are not easily understood. The change in density of both parental and progeny

duplexes takes about 8 minutes to be completed. If the resistant duplex is the core of a replicative intermediate being transcribed in an asymmetric semi-conservative fashion a replication time per molecule of less than 20 seconds would be expected. On the other hand, even if the duplexes replicated in a symmetrical semi-conservative fashion the change in density should be complete in about 1-1/2 min, 5 times shorter than the observed time. The constant half-width of the peak of ^{32}P-labeled progeny duplexes during the chase argues against lack of synchrony as an explanation. The most probable conclusion is that the observed kinetics represent the slow depletion of the heavy nucleotide pool even in the presence of excess light nucleosides and bases. This is supported by the difficulty we have experienced in attempting to perform chases after labeling with ^{3}H-uracil under similar conditions.

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(b) Discussion of the "chase" procedure.

Evidence is presented later (Part V) that a duplex can be transcribed both conservatively and semi-conservatively. Suppose that use of a conservative or semi-conservative mechanism is a matter of chance, each occurring randomly on a given duplex but with equal probability. Let us consider the kinetics of the density shift if infected cells are transferred from "heavy" medium containing ^{32}P non-radioactive to "light" medium for a time equal to a transcription time. Duplexes which have been transcribed semi-conservatively, will now be "half-heavy" in density but will have only half of the radioactivity of the conserved duplexes which are, of course, still full heavy. Assuming no conversion of single-stranded RNA to duplexes then, after a chase equivalent to two transcription times, 5/8 of the original material will remain, 1/4 full heavy and 3/8 half-heavy. If fully and half-heavy material are not resolved then such a mechanism of replication could result in a slow shift in the density of the maximum, such as was described in Section II(a) (Figure 5.).

This is a very pleasing interpretation but some of the predictions that this model makes are not fulfilled. In the first place, a variation in the half-width of the ^{32}P labeled peak is predicted but is not observed (Section II(a)). Secondly, if ^{3}H-uracil is present in the "light" chase medium, and the chase is longer than a transcription time, ^{3}H-labeled duplexes should have

a "half-heavy" (and eventually a fully light) density. In fact the ^{3}H-uracil peak is only slightly lighter than the ^{32}P peak but denser than "half-heavy" even at times when, by the criterion of the synthesis of ^{3}H-labeled single-stranded MS2 RNA, more than one transcription has occurred. This result implies that the mean density of the nucleotide pool during a chase in "light" medium is not fully light.

This conclusion is supported by the results which were obtained when "pulse-chase" experiments were attempted. If cells were infected with $^{13}C^{15}N^{32}P$ MS2 and labeled with ^{3}H for the first 6 minutes of infection (at 37^{o}C) the majority of the RNase-resistant (14S) ^{3}H material is in the parental duplex i.e. only the strand complementary to the input RNA is labeled. If now the infected cells were washed thoroughly to remove the ^{3}H-uracil and re-suspended in medium containing a thousandfold excess of uracil, it should be possible, in principle, to determine if the ^{3}H radioactivity can be chased out of the complementary strand, as would be expected if replication is semi-conservative. This approach failed. In several experiments, although no more radioactivity was incorporated into cells precipitated with trichloracetic acid, the amount of ^{3}H radioactivity in double-stranded RNA increased several fold during the chase, which made the results uninterpretable. This incorporation is explained if labeled cellular RNA is breaking down to nucleotides during the chase period and these nucleotides are re-incorporated in preference to added nucleosides and bases.

This section has been devoted to discussing the artifacts possible in the chase experiments used in the previous section.

The justification of this discussion of a trivial phenomenon is that chase techniques are widespread and that the limitations of their use should be appreciated.

(c) Pulse-labeling Experiments.

There is another way to determine whether parental duplexes are conserved. By 8 minutes after infection the maximum conversion of parental to duplex has taken place. If replication is semi-conservative the parental must be released from the duplex and direct the synthesis of another complementary strand, i.e. the parental duplex can still incorporate radioactivity. If replication is conservative no more radioactivity will be incorporated into the parental duplex after 8 minutes.

Since the amount of radioactivity involved in these experiments is small, a less equivocal experiment is to compare the incorporation of ^{3}H-uracil into the parental duplex with that into the progeny duplex. If cells growing in TPG are infected with $^{13}C^{15}N^{32}P$ MS2 and labeled with ^{3}H-uracil between 8 and 10 minutes after infection (i.e. after the maximum has been reached) all ^{3}H-uracil incorporation will be into fully light material if the parental duplex is conserved. If the parental duplex is not conserved ^{3}H will be incorporated into both the parental and progeny duplexes. For labeling times less than the doubling time of the duplexes, the Relative Incorporation Rate, (defined as the ratio of radioactivity incorporated into progeny duplexes (fully light) to that incorporated into the parental

duplex ("half-heavy")) will be proportional to the ratio of parental to progeny duplexes at the start of the labeling period. As a control it is convenient to compare the Relative Incorporation Rate for the 2 minute pulse to that for a sample uniformly labeled from 0 to 10 minutes after infection. If replication is conservative the Relative Incorporation Rate will be much higher for the 8 to 10 minute labeling than for the uniformly labeled control, since incorporation into the parental duplex does not occur in the former. If replication is semi-conservative the Relative Incorporation Rate should be smaller for the pulsed than for the uniformly labeled sample.

In the experiment described in Table II - 1, cells infected with $^{13}C^{15}N^{32}P$ MS2 were labeled with ^{3}H-uracil from 0 to 6, 0 to 8 and 0 to 10 minutes after infection, and the distribution of ^{3}H radioactivity between progeny and parental duplexes compared to samples labeled from 4 to 6, 6 to 8 and 8 to 10 minutes respectively. The procedure for distinguishing the radioactivity in parental and progeny duplexes has already been described (Section 11(a)).

The important part of Table 4 is the last row, in which the Relative Incorporation Rate is given for each of the samples. It can be concluded that parental RNA molecules are synthesizing complementary strands quite late in infection. Thus, in the light of the discussion above, the duplexes are not conserved.

TABLE II - 1

Comparison of Pulse and Uniformly Labeled Samples

	0' - 6'	0' - 8'	0' - 10'	4' - 6'	6' - 8'	8' - 10'
$\frac{H_{HL}+H_{LL}}{P_{HL}}$	0.8	2.3	4.5	0.7	0.6	0.9
$\frac{H_{HL}}{P_{HL}}$	0.45	0.55	0.5	0.4	0.3	0.2
$\frac{H_{LL}}{H_{HL}}$	0.7	2.8	7.2	0.6	1.0	4.0

In this experiment the distribution of ^{3}H radioactivity between the parental and progeny duplexes is considered as a function of time and the duraction of the labeling period. H_{HL} represents the ^{3}H radioactivity in the "heavy-light" parental duplex, H_{LL} that in the fully light progeny duplex, and P_{HL} is the ^{32}P radioactivity in the parental duplex.

Part III

PURIFICATION OF THE REPLICATIVE INTERMEDIATE

INTRODUCTION

Since double-stranded RNA is not present in uninfected cells it has been convenient to isolate it from infected cells as an RNase-resistant (14S) duplex. Using this purification procedure the kinetics of synthesis and the nature of the replication of duplexes has been studied and the results given in the first two parts of this thesis. The limitation of such a procedure is that RNase digestion introduces single chain scissions into double-stranded RNA (vide infra). In addition the involvement of RNase-sensitive RNA in replication cannot be investigated. A large part of RNA phage research has been to discover ways of separating the replicative intermediates from the host cell components without use of RNase. Some ways in which this can be done will be discussed in this section of the thesis.

Early experiments which employed actinomycin D to depress host synthesis are first described. Since this technique is prone to artifacts an attempt was made to develop a centrifugal method for separating replicative intermediates from the other nucleic acids in infected cells. Studies on the denaturation of RNA with dimethylsulfoxide suggested appropriate conditions for such a separation, and these were used to purify a partially double-stranded RNA. The sedimentation properties of this RNA in its native and fully denatured states are compared.

to the method of Martin & Ames (1961).) These three components are distinguishable on the basis of their physical properties and seem to have different roles in the replication process.

2. Materials and Methods

(a) *Media*

TPA medium contains, per liter: 0·5 g NaCl, 8·0 g KCl, 1·1 g NH_4Cl, 12·1 g tris, 0·046 g KH_2PO_4, 0·8 g sodium pyruvate, 150 mg tyrosine, 150 mg cystine, 150 mg leucine, 2·25 g of special Natural L-Amino Acid Kit Mixture except L-Cys, L-Tyr, L-Leu (Nutritional Biochemicals Corporation, Cleveland, Ohio), 1·0 ml. of 0·16 M-Na_2SO_4, 10 H_2O, 10 mg adenine. The pH is adjusted to 7·4. After autoclaving, 0·1 mg of $FeCl_3$, 6 H_2O; 2 ml. of 1 M-$CaCl_2$; 1 ml. of thiamin hydrochloride (10 mg/ml.) and 20 ml. of 10% dextrose are added, all of which are sterile.

TPA–sucrose medium is identical with the above except that it is also 10% (w/v) in sucrose.

[^{14}C]MS2 RNA was a kind gift from Mr J. H. Strauss, Jr. Actinomycin D and chloramphenicol were generous gifts from Merck, Sharpe & Dohme, Inc. and Parke-Davis & Co. respectively.

(b) *Preparation of [^{32}P]MS2*

Ten ml. of a log phase culture of *Escherichia coli* C3000 growing in TPA medium containing 4 mc of [^{32}P]orthophosphate (carrier-free, obtained from Nuclear Consultants Glendale, Calif.) are infected at a titer of 1×10^8 cells/ml. with MS2 phage at a multiplicity of one. The lysate is purified exactly as described elsewhere (Davis & Sinsheimer, 1963).

(c) *Preparation of protoplasts*

This procedure is based on that of Guthrie & Sinsheimer (1960). *E. coli* C3000 are grown to about 3×10^8 cells/ml. in TPA medium containing also 0·2 mg/ml. $MgCl_2$. The cells are spun down and washed in cold medium. For every 20 ml. of cells, 0·35 ml. of 1·5 M-sucrose is added, the cells resuspended, and then 0·17 ml. of 30% bovine serum albumin, 20 μl. of lysozyme (2 mg/ml. in 0·25 M-tris, pH 8·1) and 40 μl. of 4% EDTA are added, shaking after each addition.

Three ml. of cold TPA medium are added and the cells examined under a microscope to ensure they have all taken on the spherical shape of protoplasts.

(d) *Uptake of label in actinomycin D*

Following the procedure of Haywood & Sinsheimer (1963), cells to be treated with actinomycin D are first converted to protoplasts as described above and, after 15 min in an ice bath, to each 3 ml. of protoplasts is added 0·12 ml. actinomycin D (250 μg/ml.). All solutions containing actinomycin D are protected from the light.

The protoplasts are then added to 6·0 ml. of pre-warmed (37°C) TPA–sucrose medium which contains 10 μg/ml. of actinomycin D, 2 mg/ml. of $MgSO_4$, and the appropriate amount of [^{3}H]uracil, to give a final volume of 10 ml. of protoplasts at about 6×10^8 cells/ml.

After the required incubation at 37°C is completed, the protoplasts are collected using a modification of the technique described by Guthrie & Sinsheimer (1963). The protoplasts to be collected are poured into a centrifuge tube which has a layer of 75% w/w sucrose filling the bottom of the tube, and above this a very thin layer of 6% bovine serum albumin and 60% sucrose. The tubes are then centrifuged either in a Spinco model L, SW25 rotor for 10 min at 10,000 rev./min, or in a refrigerated International swinging bucket rotor, at 1500 *g* for 20 min. After centrifugation, the protoplasts are in a thin band at the bovine serum albumin layer. After removing most of the clear supernatant solution with a pipette, the thin band of protoplasts can be easily removed using a Pasteur pipette, and the nucleic acid obtained by phenol extraction.

MATERIALS AND METHODS (continued)

RNA Samples

Single-stranded MS2 RNA was a gift of Dr. J.H. Strauss. The preparation of ^{32}P MS2 was described in Section II(a). ^{32}P MS2 RNA was extracted from the phage using the phenol method, immediately prior to use as a centrifugal marker. Double-stranded (14S) "core" material was isolated from ^{3}H labeled infected cells using the procedure outlined in Section II(a). To obtain double-stranded RNA in quantities large enough for optical measurements, the amber coat protein mutant of f2, sus-11, was used. In cells infected with this mutant up to one-quarter of the total RNA is double-stranded and has a sedimentation coefficient of about 6S. The purification of the double-stranded RNA, which has been called su-11-RNA, has already been described (Strauss, 1966).

Optical Rotation Measurements

Optical rotation measurements were made using a Jasco ORD/uv-5 polarimeter. A 5mm. path length cell was used, with a water jacket to allow temperature control. When used to determine melting temperatures the volume correction was less than experimental error.

Since DMSO has a large refractive index, measurements of this index were made for the various DMSO solutions using an Abbe refractometer. The Lorentz correction was applied where appropriate, e.g. Figure III - 5. Although the position of the peak is different for double- and single-stranded RNA, rotations were always measured at 280mμ.

Infectivity Assay

An assay for the infectivity of single-stranded RNA has been described (Strauss, 1964). The use of DMSO to determine the infectivity of denatured double-stranded RNA is described in section IV(a).

Centrifugation

All centrifugation was performed preparatively using 5 to 20% sucrose density gradients. Samples were centrifuged at 65,000 r.p.m. in a Spinco SW65 rotor for a length of time sufficient for the fastest component to travel about 2/3 the length of the tube.

Samples from the gradients were always precipitated with trichloracetic acid and filtered before counting to eliminate quenching artifacts.

As is described in Part V, before a sample is centrifuged in 99% DMSO, it is dissolved in 50% v/v dimethylformamide and 25% v/v DMSO.

Miscellanea.

To determine the RNase-resistance of a sample it is incubated for 30 minutes at 37 °C in 25 γ/ml. RNase, 0.1M NaCl, 0.01M tris pH 7.2, and 0.01M $MgCl_2$. Details on pulse-labeling of infected cells are given in Part V. The preparation of uniformly labeled RI by 50% DMSO sedimentation is described in Part IV.

RESULTS

(a) Phage specific RNA in actinomycin D-treated cells.

The first section of Part III, and part of the Materials & Methods section, are taken from a paper by Kelly, Gould and Sinsheimer published in the Journal of Molecular Biology, volume II, pages 562 to 575. It is included with the permission of the Academic Press Inc. (London) Ltd.

20% w/v sucrose density-gradient (0·1 M-tris, pH 7·0). This ribonuclease concentration is sufficient to digest all single-stranded RNA to a sedimentation rate less than 12 s (average 2 to 4 s), yet minimizes any single-chain scissions in double-stranded RNA. After centrifugation at 37,000 rev./min in the SW39 rotor of a Spinco model L for 12 hr, fractions are collected, the 12 s material isolated, and stored at − 20°C.

3. Results

(a) *Phage-specific RNA in actinomycin D-treated cells*

Using actinomycin D to suppress host RNA synthesis in protoplasts, it has been possible to study the type and amount of phage-specific RNA present in the cells at various times after infection. The host bacterium, *E. coli* C3000, was grown to 1×10^8 cells/ml. in 400 ml. of TPA medium, infected with 3 phage/bacterium, converted to protoplasts and resuspended in 200 ml. of TPA–sucrose medium containing 10 μg/ml. actinomycin D and 1 ml. of [^{3}H]uracil solution (1080 mc/m-mole, 0·5 mg/ml.). From 50-ml. samples of cells, removed at 15, 25 and 40 minutes, the RNA was extracted, dissolved in 1 ml. of 0·1 M-tris buffer, and 100 μl. of each RNA preparation placed on a sucrose density-gradient. After drop collection, the samples were assayed for ultraviolet absorbance and ^{3}H counts. The results are shown in Fig. 1.

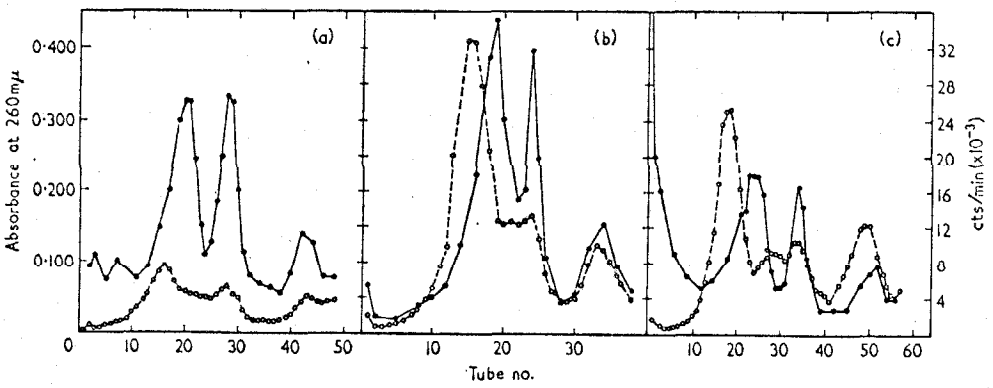

FIG. 1. Distribution of ultraviolet absorbance (—●—●—) and ^{3}H (--○--○--) after sucrose gradient sedimentation of RNA from infected protoplasts, incubated in a medium containing actinomycin D and [^{3}H]uracil for 15 min (a), 25 min (b) and 40 min (c). Unless otherwise stated, all sucrose density-gradients used in this work are 5 to 20% (w/v) linear sucrose gradients (0·1 M-tris, pH 7·0). Samples were centrifuged for 6 hr at 37,000 rev./min in a Spinco model L, SW39 rotor at 5°C.

Viral RNA (27 s) synthesis occurs quite rapidly between 15 and 25 minutes, with a similar but slower synthesis of the 14 to 22 s material tentatively called "replicative form". Continuing the incubation from 25 to 40 minutes produces little more 27 s viral RNA, but suggests that the 14 to 22 s RNA can be resolved into two components (designated as 15 and 20 s) and that material of sedimentation coefficient slightly greater than 4 s is accumulating.

The results obtained with the actinomycin procedure are somewhat variable. In another experiment in which the above procedure was repeated, the cells were

grown to 4×10^8/ml., infected with 5 phage/bacterium, incubated as protoplasts in [^{3}H]uracil (1080 mc/m-mole, 0·5 mg/ml.) and samples removed at 30, 50 and 70 minutes. The RNA's extracted from these samples were sedimented in sucrose density-gradients and drops assayed for ultraviolet absorbance, total ^{3}H counts and ^{3}H counts from material resistant to ribonuclease digestion. The results obtained for the 30-minute sample are shown in Fig. 2.

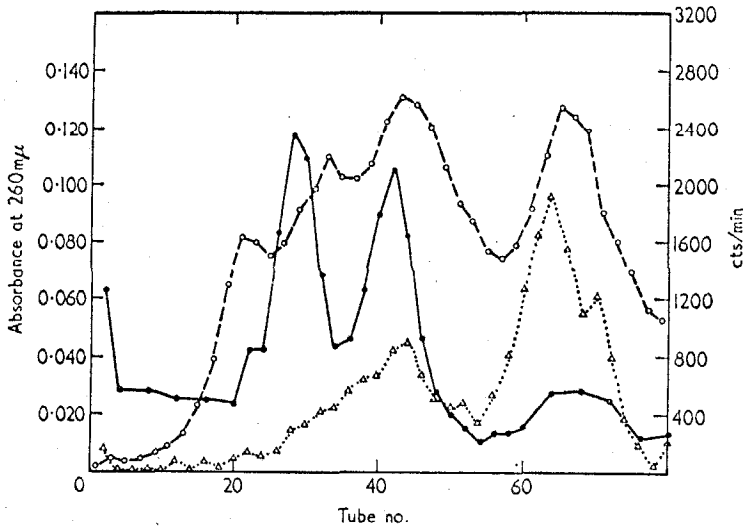

FIG. 2. Distribution of ultraviolet absorbance (—●—●—), total ^{3}H (--○--○--) and ^{3}H resistant to ribonuclease digestion (..△..△..) after sucrose gradient sedimentation of RNA extracted from infected protoplasts incubated for 30 min in a medium containing actinomycin D and [^{3}H]uracil.

It is evident that, in this case, very little 27 s viral RNA has been made relative to the 15 to 20 s "replicative form". This latter can again be resolved into two components, one at about 20 s, the other at about 15 s. Two peaks of ribonuclease-resistant RNA are observed, one at 15 s and one at 6 s.

Analysis of the samples removed after 50 and 70 minutes of incubation showed little additional tritium incorporation.

The RNA sedimenting at 6 s, which is 85% ribonuclease-resistant in this experiment, is also observed in cells treated, prior to infection, with ultraviolet light (see below).

These experiments suggest that there are at least four phage-specific RNA components in an infected cell: a 27 s molecule identical to that which can be extracted from phage; a 6 s molecule which is extensively double-stranded; and two components at least partially separated by sucrose gradient sedimentation into a peak at 15 s and another at 20 s.

(b) Discussion of the results using Actinomycin D.

It has recently been pointed out that Actinomycin D can be introduced into cells if they have been treated with EDTA alone, without converting them to spheroplasts (Lieve, 1965; Haywood & Harris, 1966; Lunt & Sinsheimer, 1966). Indeed it is possible that spheroplasts are permeable to actinomycin D only because EDTA is present during the incubation with lysozyme. In addition it was observed that the extent to which actinomycin D interfered with RNA phage biosynthesis was dependent on its concentration. It is possible, therefore, to explain the variability that was observed in the action of actinomycin D if different preparations of protoplasts vary in their permeability to actinomycin D.

The 20S component described above has also been observed by other authors using the actinomycin D procedure (Haywood & Harris, 1966; Lunt & Sinsheimer, 1966). A similar 20S peak of parental material is often found in the nucleic acids of cells infected with ^{32}P labeled MS2 (Kelly et al., 1965; Godson & Sinsheimer, 1967). Martin (1966) has reviewed the occurence of what he calls "interjacent" RNA in cells infected with animal viruses. On the basis of its sensitivity to RNase, the ionic strength dependence of its sedimentation rate and its buoyant density in CsCl it was concluded (Kelly et al., 1965) that the 20S RNA is a single-stranded RNA. From the results obtained during a synchronized infection it was hypothesized that the 20S molecule was viral RNA with a less compact secondary structure, and that viral RNA adopted this

structure in order to direct protein synthesis.

This hypothesis is attractive but attempts to confirm it have been fruitless. The "synchronization experiment" on which the hypothesis is based has proved difficult to investigate because only very rarely has it been possible to repeat the observations. In addition, at no time after infection is a peak of 20S reproducibly observed. Experiments in which attempts were made to convert 27S to a 20S form by infecting in the absence of RNA synthesis (uracil starvation) and protein synthesis (histidine starvation) were unsuccessful. Finally, 20S RNA can arise by degradation of viral RNA (Argetsinger & Gussin, 1966; Godson & Kelly, unpublished observations). Because the 20S RNA component is only occasionally observed, and since it can arise through degradation of intact RNA it seems probable that the presence of a 20S component in an RNA preparation is due to limited scission of the RNA strands. Perhaps a theory in fact needs more than elegance to be correct (Gamow, 1967).

The most striking feature of infected cells treated with actinomycin D is the accumulation of the double-stranded 6S molecule. Late in infection when high concentrations of actinomycin D are used the majority of the phage specific RNA is in this form. A molecule with identical properties also accumulates in very large amounts in cells infected with amber mutants of f2 that are defective in coat protein synthesis (Lodish & Zinder, 1966). If infected

cells are incubated at temperatures greater than 40°C, the majority of the double-stranded RNA is also of this 6S form (Lodish, 1966). By annealing studies it has been shown that this RNA is phage specific and that the small pieces of viral RNA which it contains are not derived from a unique region of the viral genome, but are representative of the entire genome (C. Weissmann, personal communication to Lodish (1966)). This suggests that 6S RNA arises by degradation of single or double-stranded viral RNA.

The 15S peak is partially resistant to RNase and is heterogeneous in sedimentation. Since this component is the first to be labeled in short pulse experiments it could be the precursor to viral RNA (Fenwick, Erikson & Franklin, 1964). These authors also showed that gentle digestion of the heterogeneous 15S peak with RNase, gave as one of the products a homogeneous 14S peak of double-stranded RNA. It was postulated that the heterogeneity in the 15S peak was due to single-stranded "tails" attached to a 14S double-stranded "core". From the RNase sensitivity of this component they calculated that there were several tails per molecule.

The assumption inherent in this model is that the RNase sensitive and insensitive structures are both parts of the same molecule since they sediment together. To test this assumption it is only necessary to sediment the 15S component under different ionic strength conditions since variation of the ionic strength greatly alters the sedimentation rate of single-stranded but not

double-stranded RNA. In Figure III-3, a partially RNase-sensitive component which sedimented at 15S in 0.1M tris, split into two components when sedimented in 10^{-3} M EDTA, a highly resistant peak and a fully sensitive peak. Clearly double-stranded RNA can be contaminated by fragments of single-stranded RNA and thus caution must be used in interpreting the ribonuclease sensitivity of a component from a sucrose density gradient.

We can conclude that phage specific RNA molecules are observed if host cell synthesis is depressed with actinomycin D, but that the synthesis of the RNA is not normal. It is not known why actinomycin D has such a striking effect on phage biosynthesis, for animal virus replication is not affected (Reich et al., 1961) nor is the in vitro RNA synthesizing system derived from phage-infected cells (Weissmann et al., 1963). RNA synthesis is normal in the presence of the drug until 20 minutes after infection (Lunt & Sinsheimer, 1966); addition of the drug after 20 minutes has no effect (Haywood & Harris, 1966). Since it is known that the synthesis of polymerase stops at about this time (Lodish, Cooper & Zinder, 1964), and that the amber mutant which produces a great deal of 6S RNA also produces excess amounts of polymerase (Lodish & Zinder, 1966) it is tempting to suppose that the action of actinomycin D is somehow to prevent the repression of polymerase synthesis.

Legend to Figure III -3.

Sedimentation of the 15S component at low ionic strength. Cells were infected with ^{32}P MS2 phage. Eight minutes later, the cells were chilled, lysed and the nucleic acids extracted. These were sedimented in a sucrose density gradient in 0.1M tris, pH 7.2. The 15S component was collected and sedimented again in a sucrose density gradient containing 10^{-3} M EDTA pH 7.0. Total (—•———•—) and RNase-resistant (-o---o) radioactivity.

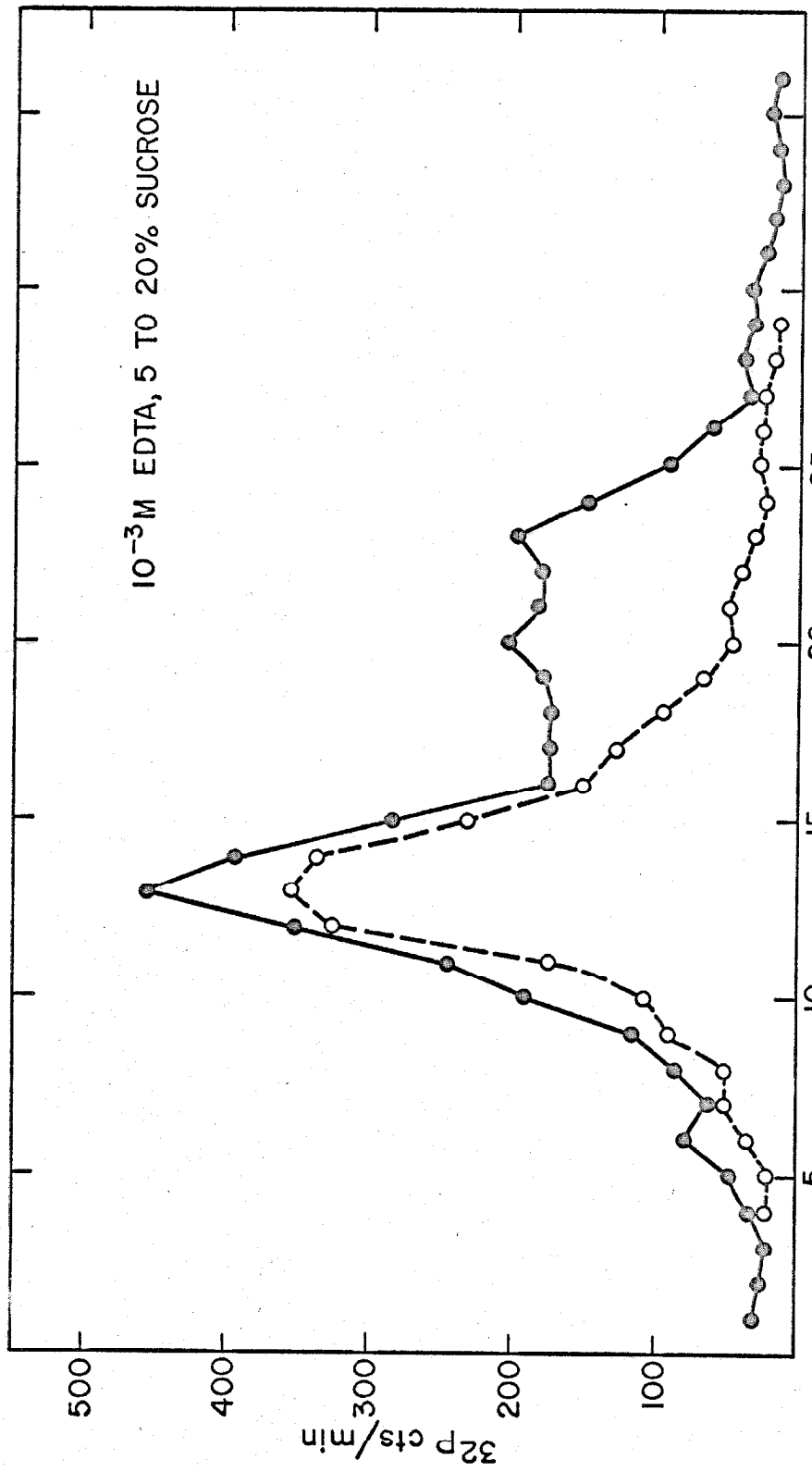
10^{-3}M EDTA, 5 TO 20% SUCROSE
^{32}P cts/min
100
200
300
400
500
5
10
15
20
25
30
35
FRACTION

Fig. III - 3.

(c) The Denaturation of double and single-stranded RNA

In the isolation of double-stranded RNA, a problem arises through contamination with single-stranded RNA fragments (Figure III-3). Although these fragments are small they have a low frictional coefficient, and thus have the same sedimentation coefficient as the much larger double-stranded RNA. It was hoped that conditions could be found to denature (unstack) single-stranded RNA without destroying the secondary structure of double-stranded RNA, and that the sedimentation rate under such conditions would be a more accurate reflection of molecular weight. If the replicative intermediate could be purified by centrifugation under these conditions, it would be informative to denature them and look for the existence of covalently closed double-stranded rings, nascent strands and infective strands. For these applications of denaturation to be successful it was essential that RNA could be studied while denatured, and that it is not degraded during the denaturation. This led to the choice of the organic solvent dimethylsulfoxide (DMSO) as the denaturing agent (Strauss, Kelly & Sinsheimer, in preparation).

The commonest method of studying the denaturation of nucleic acids is the measurement of hyperchromism at 260mμ. In this case, however, since DMSO absorbs strongly at 260mμ and since there is a complex dependence of hyperchromism on DMSO concentration, (Strauss 1966) the optical rotation of RNA at 280mμ is a more convenient measure. Both double and single-stranded RNA have a positive Cotton

effect with a peak at about 280mμ and a trough at about 250mμ (Figure III-4). Comparison of the position of the peak for the three samples indicate that the wave length at which the maximum occurs depends on the double-stranded character of the RNA, but no striking change is observed in the height of the peak. Since optical rotation depends on the stacking and not the hydrogen bonds in a nucleic acid this result is to be expected.

A detailed description of the denaturation of RNA by DMSO is in preparation (Strauss et al.) and consequently only a brief account is given here. The effect of increasing DMSO concentration on the rotation of double and single-stranded RNA is shown in Figure III-5. Single-stranded RNA is, by this criterion, "half-melted" at 48% DMSO, and double-stranded RNA at 67%. The apparent biphasic nature of the denaturation of double-stranded RNA probably reflects a contaminant. The temperature of the sample was varied at each DMSO concentration and the melting temperature determined as the mid-point of the sharp transition (Figure III-6). At low DMSO concentrations the dependence of the melting temperature of double-stranded RNA on the concentration of the denaturant parallels that of single-stranded RNA, but at concentrations greater than 50%, the melting temperature is much more heavily dependent on DMSO concentration. Data from hyperchromicity measurements (Strauss, 1966) are included to demonstrate the equivalence of the two methods.

Legend to Figure III - 4

The molar residue rotation at 25°C of MS2 RNA in 0.1 M NaCl, 0.001 M EDTA (—•———•—) and in 0.001 M EDTA, (.□.....□.), and of su-11-RNA in 0.001 M EDTA, 0.005 M tris, pH 7.1 (-o---o-) is given as a function of wavelength. The points are the average of two scans. Extinction coefficients of 7.7 and 6.8 x 10^3 were used for the single- and double-stranded RNA respectively. A 5mm. path length, water-jacketed cell was used, and the RNA concentration was approximately 80 γ/ml.

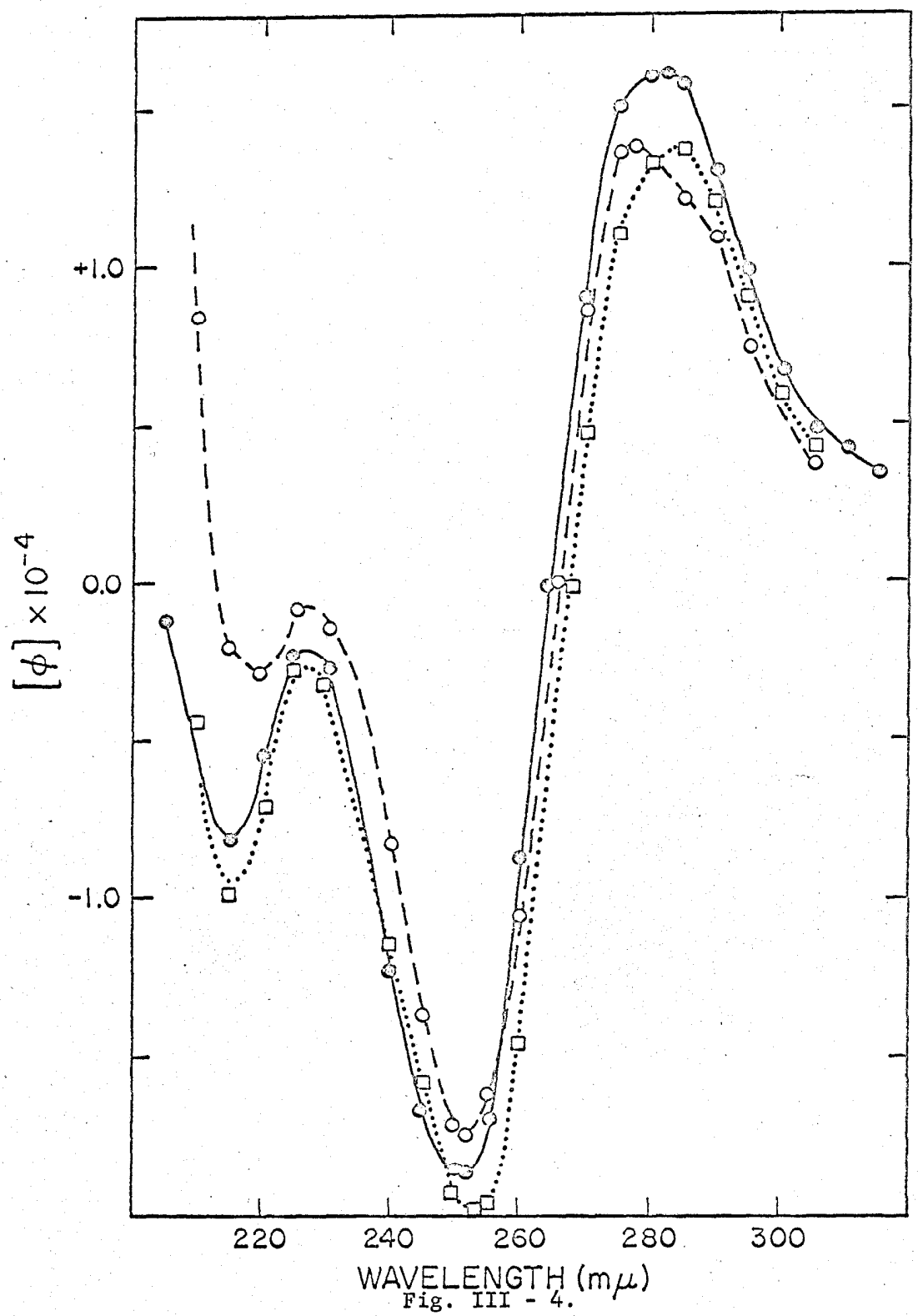

Fig. III - 4.

Legend to Figure III - 5.

The reduced molar residue rotation at 280 mμ of double-stranded, su-11-RNA (-o-----o-), and single-stranded MS2 RNA (-●———●-) is given as a function of DMSO concentration. Samples of RNA were diluted quantitatively into DMSO at the appropriate concentration. The buffer in all cases was 0.001 M EDTA, and 0.005 M tris, pH 7.1. The remainder of the experimental details are the same as in Figure III - 4.

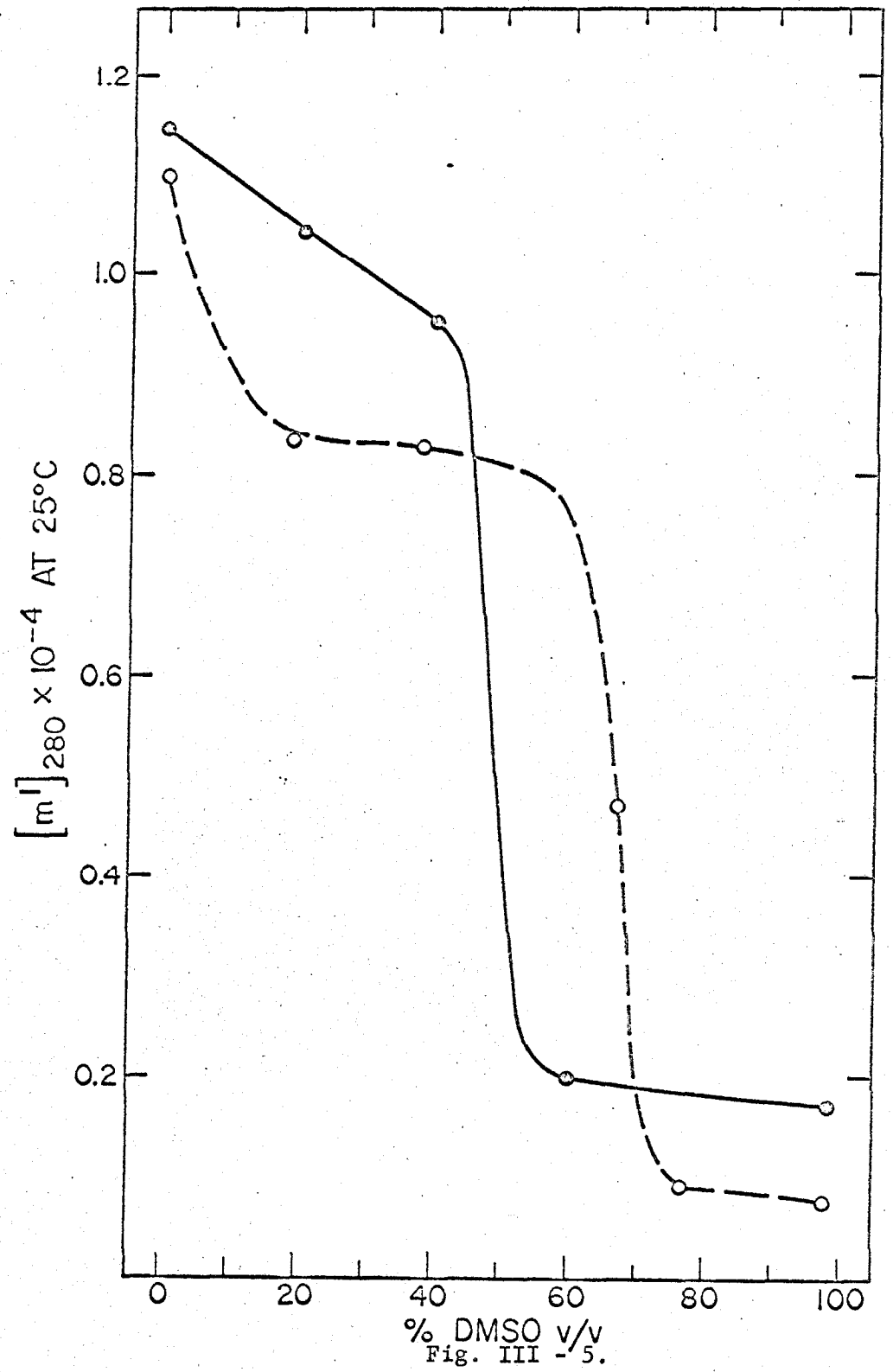

Fig. III -5.

Legend to Figure III - 6

The melting temperature of single and double-stranded RNA as a function of DMSO concentration. For each DMSO concentration, the optical rotation at 280mμ was observed at various temperatures and the mid-point of the melting transition noted. The melting temperature of double- (□) and single-stranded RNA (x) is indicated. The corresponding values from measurements of hyperchromicity (o,Δ) are included for comparison.

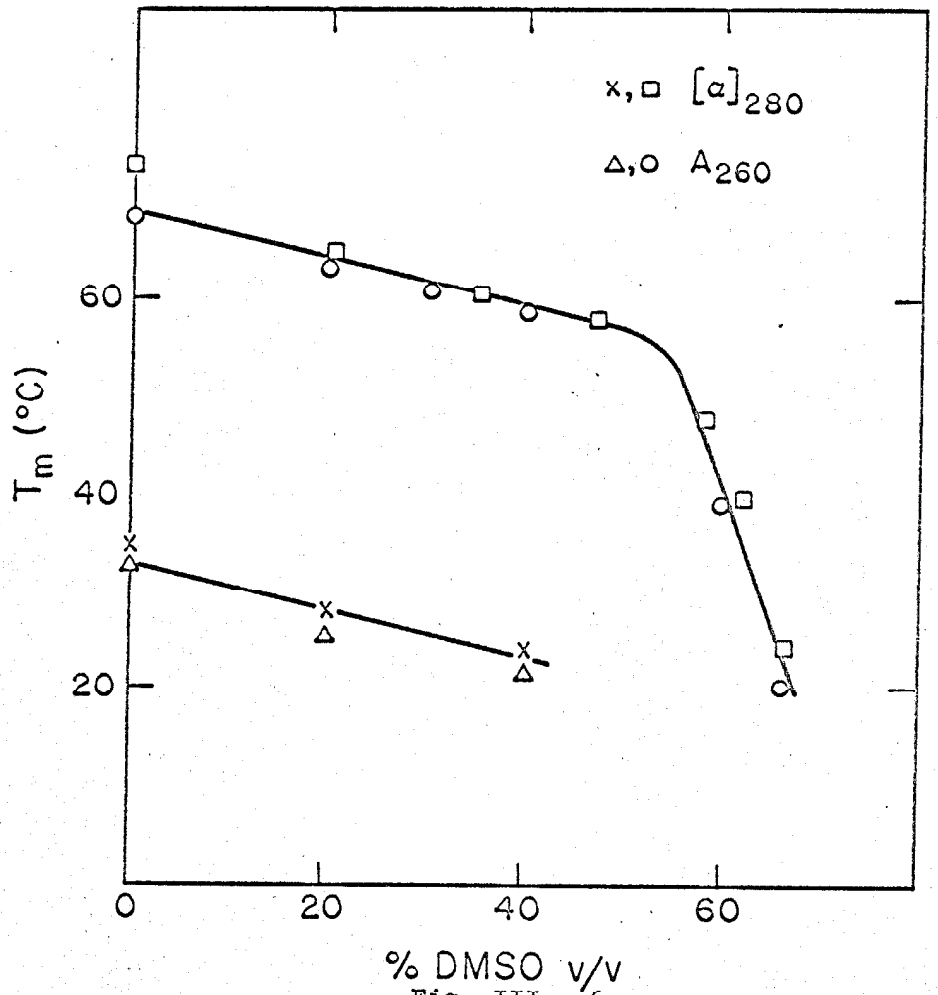

Fig. III - 6.

It is known that carefully purified double-stranded RNA from infected cells is not infective in its native state but is infective after denaturation (Ammann et al., 1964). This also can be used as a method for measuring denaturation, or more specifically, irreversible denaturation, since the RNA molecules are removed from the denaturing condition before assaying for infectivity. MS2 replicative intermediate, purified by the method described in Part IV, was denatured with DMSO at 37°C, diluted and assayed using the protoplast system (Strauss, 1964). A 10^5 fold increase in infectivity was observed (Figure III-7) with "half-melting" occuring at about 68%, at 37°C in contrast to the value of 62% determined optically. The melting temperature seemed independent of salt concentration in the range measured.

Since the above assay presumably detects only irreversible denaturation it implies that strand separation has taken place. More direct proof that small regions rich in guanine, cytosine hydrogen bonds will be denatured by these conditions was obtained by studying the hyperchromicity of poly rG: rC (Strauss, 1966). Data on the optical rotary dispersion of rG:rC in water and in DMSO supported this conclusion.

The conclusions relevant to the remainder of this thesis are that, at 25°C DMSO denatures all RNA at concentrations greater than 90%, and that in the 50 to 60% DMSO concentration range, single-stranded RNA is denatured but double-stranded is not.

Legend to Figure III - 7

A purified preparation of partially double-stranded RNA was diluted into different concentrations of DMSO at 37°C. Samples were diluted ten-fold and assayed immediately using the protoplast infectivity system. Three ionic strengths and RNA concentrations were used. These were:

(●) 0.06M NaCl, 0.001M tris pH 7.4; 0.06 ɣ/ml. of RNA.

(×) 0.024M NaCl,0.001M tris pH 7.4; 0.024 ɣ/ml. of RNA.

(o) 0.006M NaCl,0.001M tris pH 7.4; 0.006 ɣ/ml. of RNA.

10^{-7} phage per single strand equivalent is the limit of sensitivity of the assay, and is not a plaque count.

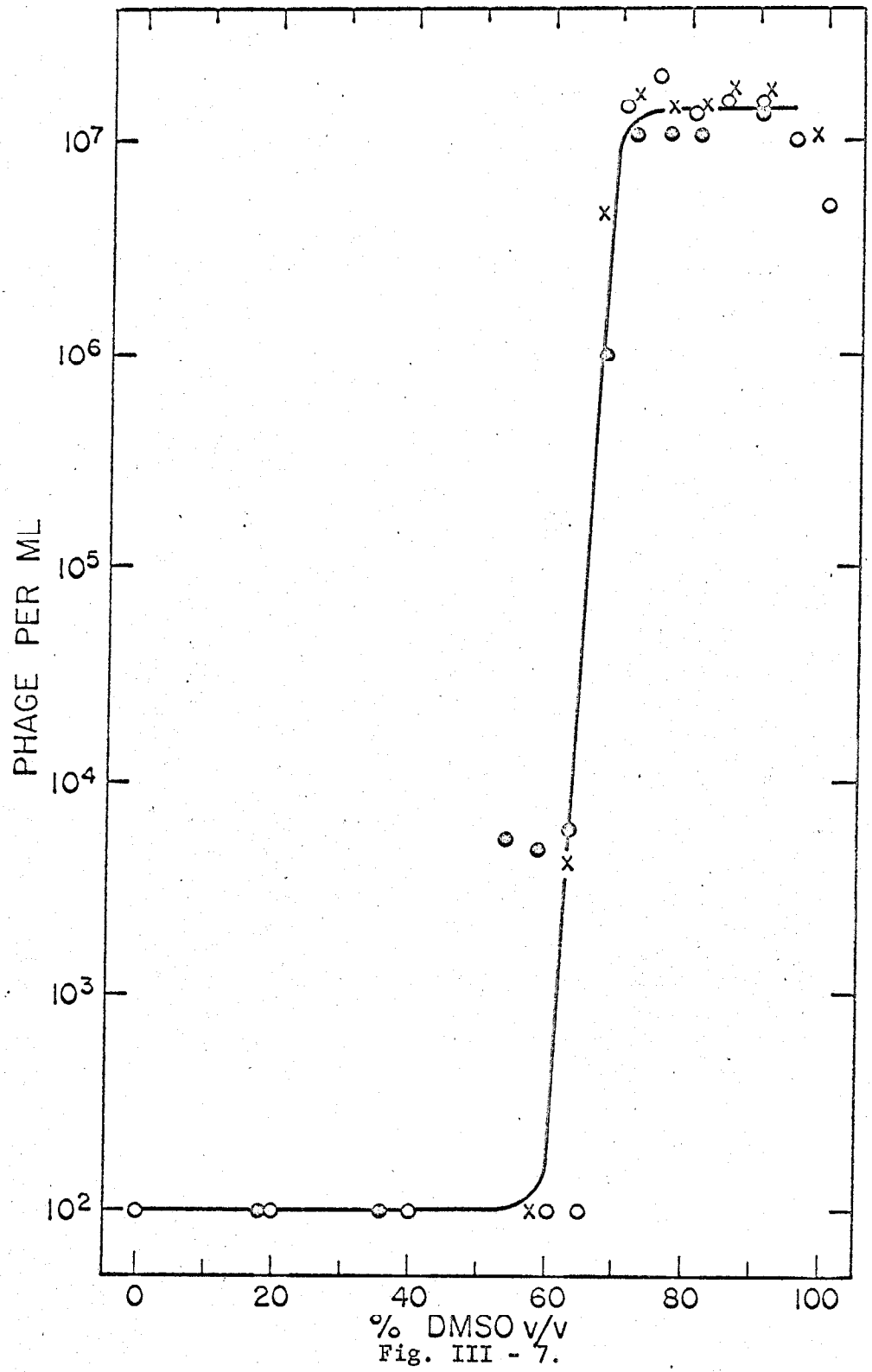

Fig. III - 7.

(d) Centrifugation of RNA under denaturing conditions.

It is clear from the previous discussion that DMSO can be used as a selective denaturing agent for single-stranded RNA. Thus we are in a position to look for conditions under which double-stranded RNA will sediment faster than single-stranded. Since only isotopically labeled material was available this work was done using sucrose density gradients containing the required volume fraction of DMSO. Since the viscosity and density of solutions of DMSO and sucrose are not known, it was not possible to calculate an $S_{20,W}$. Instead of making viscosity and density corrections in the following data, it was assumed that the distance travelled by an RNA under a set of denaturing conditions was proportional only to the time of centrifugation and the square of the angular velocity. The distance travelled by an RNA was then normalized to the distance a single-stranded RNA molecule would have sedimented in a sucrose gradient containing 0.1 M tris, at that speed and for that time (see Legend to Fig.III-8).

It was pointed out earlier that the heterogeneity of the 15S component may be due to single-stranded tails attached to a double-stranded core. This is obviously not suitable material for investigating the difference in the sedimentation characteristics of double and single-stranded RNA and thus "core" material was used, i.e. 15S material from which "tails" have been trimmed with RNase.

Data on the sedimentation behaviour of material which has not been treated in this way will be presented later.

In Table III-1, the sedimentation rates of double- and single-stranded RNA are compared in different ionic strengths and DMSO concentrations. To simplify the discussion some of the data are also plotted in Figure III-8. Decreasing the ionic strength clearly has a marked effect on single-stranded RNA but not double-stranded. Increasing the DMSO concentration up to 50% likewise slows down the single-stranded RNA but has a smaller effect on double-stranded. At 40 to 50% DMSO the double-stranded RNA travels 25 to 50% faster than the single strand, which is about the ratio expected on the basis of mass (S is usually proportional to M to the power 0.3 to 0.5). At concentrations of DMSO greater than 50% the sedimentation rate of double-stranded RNA begins to rise, while that of single-stranded RNA continues to decrease, producing a marked increase in the ratio of the sedimentation rates. As Strauss observed (1966), the sedimentation of single strand begins to increase again at the very high concentrations of DMSO, perhaps due to a viscosity effect.

The explanation of the dependence of sedimentation rate on DMSO is not apparent, largely because of the lack of information on the possible viscosity effects, dependence of sedimentation rate on the degree of stacking and the partial specific volumes

Legend to Table III - 1 and Figure III - 8

Comparison of the sedimentation properties of double and single-stranded RNA under denaturing conditions. ^{32}P-labeled MS2 RNA and ^{3}H-labeled core were centrifuged under the conditions described. ^{3}H (0); ^{32}P (0).

The Relative Velocity is the ratio of the distance travelled under the denaturing conditions to that in 0.1M tris, corrected for angular velocity and time of centrifugation.

TABLE III - 1

The Sedimentation Rate of RNA under Denaturing Conditions

%DMSO	Salt used	Relative Velocity		Ratio of double- to single-strand.
		MS2 RNA	"core"	
0	0.1M tris	1.00	0.51	0.51
0	0.003M EDTA	0.68	0.50	0.73
0	0.001M EDTA	0.56	0.46	0.81
20	"	0.42	0.43	1.03
40	"	0.20	0.25	1.24
50	"	0.18	0.27	1.50
55	"	0.14	0.36	2.55
60	"	0.12	0.39	3.26
65	"	0.10	0.42	4.20
75	"	0.09	-	-
100	"	0.18	-	-

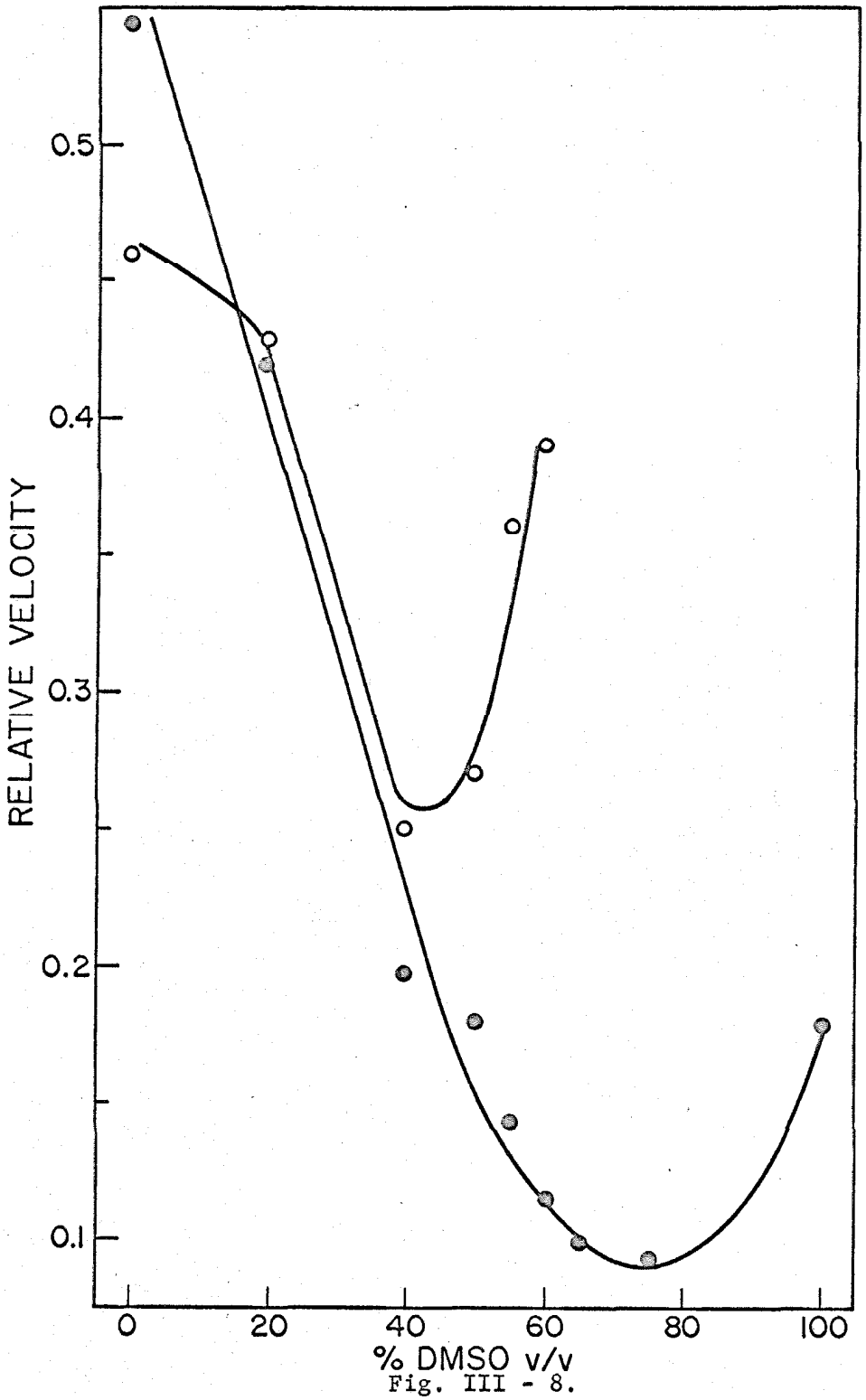

Fig. III - 8.

of RNA in various concentrations of DMSO. It is noteworthy that the sharp change in the ratio of the sedimentation rates corresponds approximately with the onset of denaturation. This might suggest that the increased sedimentation rate of the double strand reflects a partial denaturation. Indeed, if fractions from a sucrose gradient in 60% DMSO are immediately digested with RNase it is found that the double-stranded RNA has become significantly RNase-sensitive.

The data on centrifugation under partial denaturing conditions have been of great practical value in purifying the replicative intermediates and examining their structure. Unfortunately the data are too meagre to allow any satisfactory theory of the basis of this separation.

(e) Purification of Replicative Intermediate on DMSO Sucrose Gradients.

In the previous sections the sedimentation properties of single- and double-stranded RNA have been studied when subjected to various degrees of denaturation. It has been pointed out that in 50 to 65% DMSO double-stranded RNA sediments 1.5 to 4 times faster than a single strand of half its molecular weight, and that this could be useful in purifying replicative intermediate. In this section such a purification is described and the sedimentation properties of the intermediate characterized.

If infected cells are labeled for short periods of time before lysing, a large fraction of the radioactivity is associated with the partially RNase sensitive replicative intermediate. In Fig. III-9, the nucleic acids from cells labeled for 30 seconds with ^{3}H-uracil were sedimented through a 5 to 20% sucrose density gradient containing 60% DMSO and 10^{-3} M EDTA. The partially RNase-resistant peak moving ahead of the MS2 RNA marker presumably represents replicative intermediate. Since this peak is the fastest sedimenting component it should not be contaminated with fragments of single-stranded RNA.

Using this procedure, uniformly labeled intermediate was isolated and sedimented once again through a sucrose gradient, in this case with 0.1 M tris as the solvent. Compared to a single-stranded MS2 RNA marker, the replicative intermediate sediments more slowly in a broad peak, the faster components of which are more RNase-sensitive than the slower (Figure III-10).

At concentrations greater than 90% DMSO the double-stranded RNA is fully denatured. If replicative intermediate, purified on a 50% DMSO sucrose gradient, is sedimented on a 99% DMSO sucrose gradient, it gives the distribution shown in Figure III-11. The ^{3}H radioactivity sediments slightly more slowly than the MS2 RNA marker. Using the dependence of S on molecular weight derived by Strauss (1966), the peak of ^{3}H radioactivity corresponds to a molecular

Legend to Figure III - 9

Separation of RI by centrifugation in 50% DMSO. Cells were grown at 37°C and infected with MS2 phage. After 20 minutes, they were cooled to 25°C and labeled with ^{3}H-uracil for 30 seconds. The cells were chilled, lysed and the nucleic acid extracted with phenol. After alcohol precipitation the RNA was re-suspended in 0.001M EDTA and centrifuged in the presence of a ^{32}P MS2 RNA marker (.x.....x.). The ^{3}H-labeled material was assayed for total (-●———●-) and RNase-resistant (-o-----o-) radioactivity.

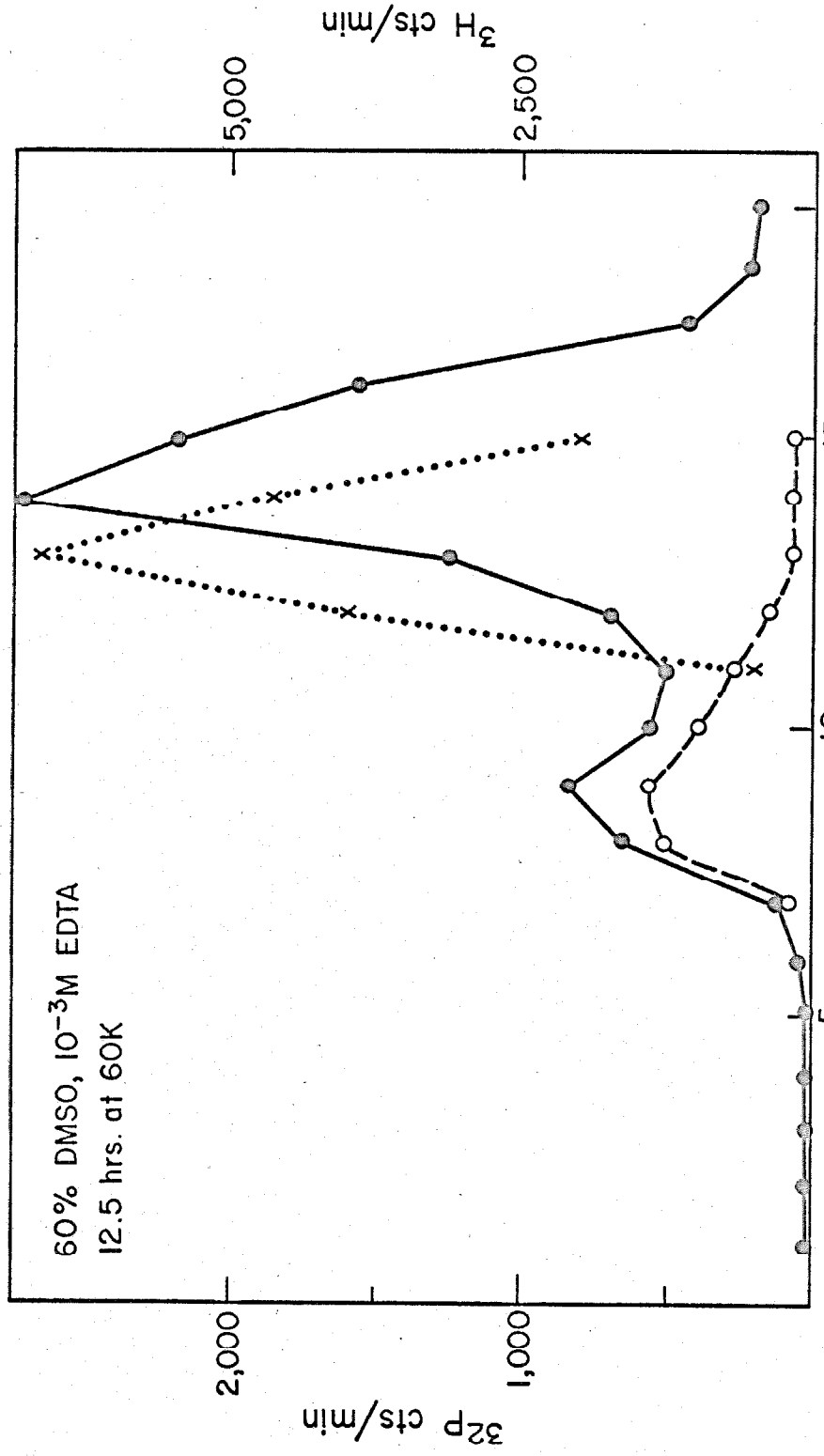
60% DMSO, 10^{-3}M EDTA
12.5 hrs. at 60K
^{32}P cts/min
^{3}H cts/min
2,000
1,000
5,000
2,500
5
10
15
20
FRACTION

Fig. III - 9.

Legend to Figure III - 10

The sedimentation behaviour of purified RI on a non-denaturing sucrose density gradient is given. Uniformly labeled RI was purified using a procedure identical to that in Figure III - 9 . The RI peak was precipitated with alcohol, re-suspended, and centrifuged for 2 hours at 65,000 r.p.m. (5°C), in a sucrose density gradient containing 0.1M tris pH 7.2. A ^{32}P MS2 RNA marker (.x.....x.) was included. The ^{3}H-labeled material was assayed for total (—●———●—) and RNase-resistant (-o----o-) radioactivity.

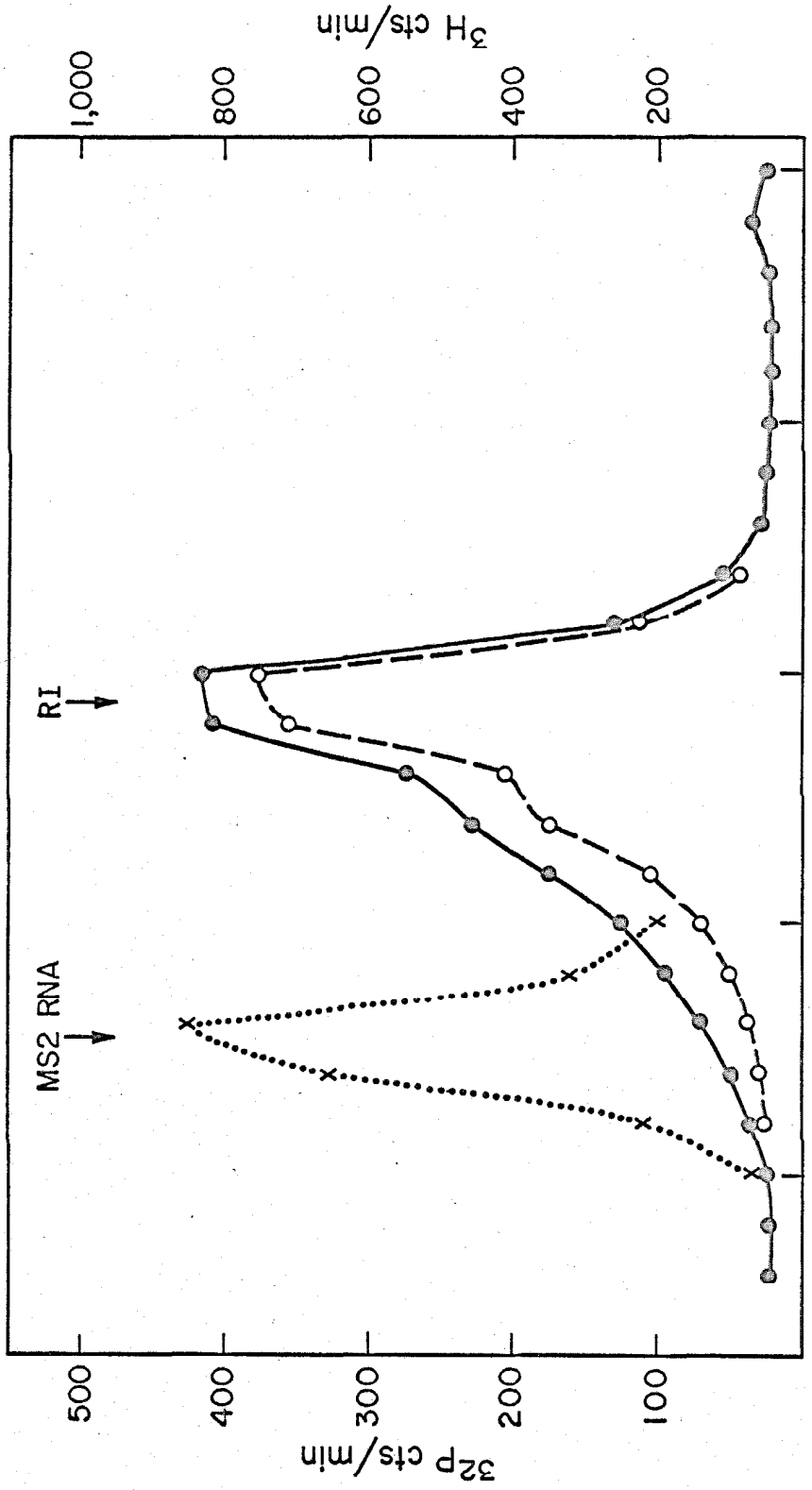

Fig. III - 10.

Legend to Figure III - 11

The sedimentation behaviour of purified RI on a fully-denaturing (99% DMSO) sucrose density gradient. 50 l. of the uniformly labeled RI sample described in Figure III-10 were mixed with 50 ul. of DMSO and 100 ul. of dimethylformamide. The mixture was layered on a sucrose gradient containing 99% DMSO, 0.001M EDTA and centrifuged 15 hours at 65,000 r.p.m. at 25°C. A ^{32}P MS2 RNA marker was included and fractions assayed for ^{32}P (-o----o-) and ^{3}H (-●——●-) radioactivity.

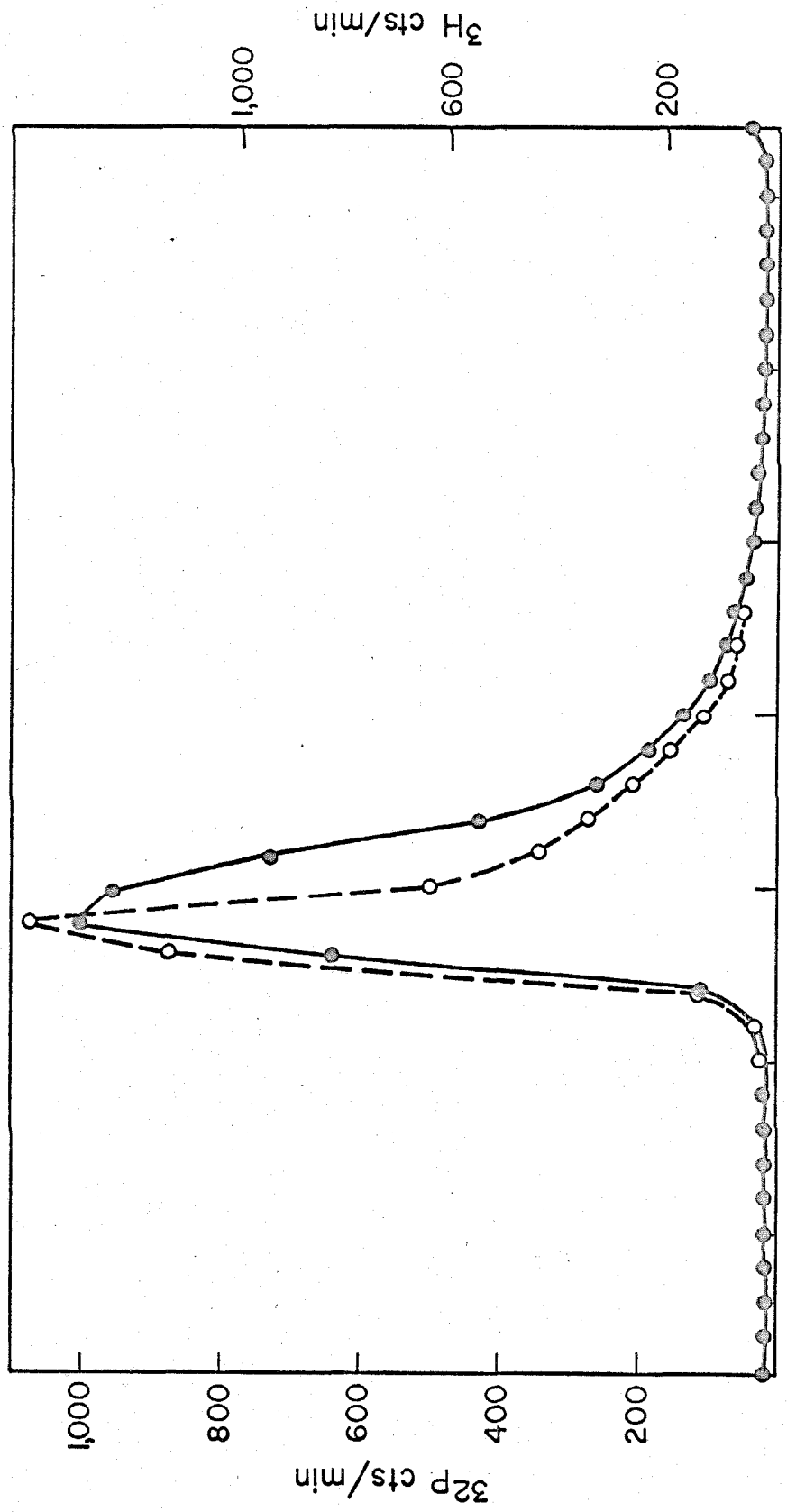
^{32}P cts/min
1,000
800
600
400
200
^{3}H cts/min
1,000
600
200
5
10
15
20
25
30
35
FRACTION

Fig. III - 11.

weight of 0.91 that of the MS2 RNA marker. The calculated weight average molecular weight for a distribution of intermediates in which all tail lengths (including no tail at all) are equally probable is 0.93 times that of MS2 RNA. It must be remarked, however, that the agreement with the measured value might be quite fortuitous if degradation in the preparation of intermediates is compensated for by the presence of a pool of duplexes with no tails. In addition large changes in molecular weight cause only small changes in the sedimentation rate.

Several conclusions can be drawn, however. There is no evidence for covalently-closed double-stranded rings such as are observed in the DNA of Polyoma virus (Dulbecco & Vogt, 1963; Weil & Vinograd, 1963) or ØX RF (Burton & Sinsheimer, 1965). Secondly, no ^{3}H radioactivity sediments faster than the MS2 RNA marker, which is a linear molecule (Strauss, 1966). Since circular ØX DNA sediments faster than the linear form under these conditions, it is unlikely that MS2 replicative intermediate contains any covalently-linked circular RNA strand. This argues against the model of viral replication proposed by Brown & Martin (1965), which required a circular complementary strand. Finally it has been proposed that the partially RNase sensitive structure which is observed corresponds to a complete viral strand with small pieces of complementary strand attached. From the data in this experiment the average molecular weight for the "small pieces" is at least 0.7 times that of a complete strand.

If the "core" of the replicative intermediate is isolated by gentle RNase digestion (0.1 γ/ml. RNase, 10 min. at 37°C) and sedimented under fully denaturing conditions, fragments of RNA are obtained much smaller in size than an MS2 RNA marker (Figure III-12). The conclusion that RNase makes single chain scissions in double-stranded RNA has also been made by Ammann et al., (1964).

We can conclude that the heterogeneous 15S component can be purified by centrifuging under partially denaturing conditions. The sedimentation behaviour of the purified material, in either a native or denatured state, is consistent with a model in which the heterogeneity is due to tails of different length attached to a double-stranded core. The tails can be "trimmed" from the core by use of RNase. RNase is not specific for single-stranded RNA but can cause single-chain scissions in a duplex.

Legend to Figure III - 12

The sedimentation behaviour of purified core material on a fully denaturing (99% DMSO) sucrose density gradient. The nucleic acids from MS2-infected cells, uniformly labeled with ^{3}H-uracil, were treated with 0.1 /ml. RNase, and the 14S RNase-resistant peak isolated by centrifugation. The remainder of the procedure was identical to that given in Figure III - 11. (—o——o—) ^{3}H radioactivity; (-o---o-) ^{32}P MS2 RNA marker.

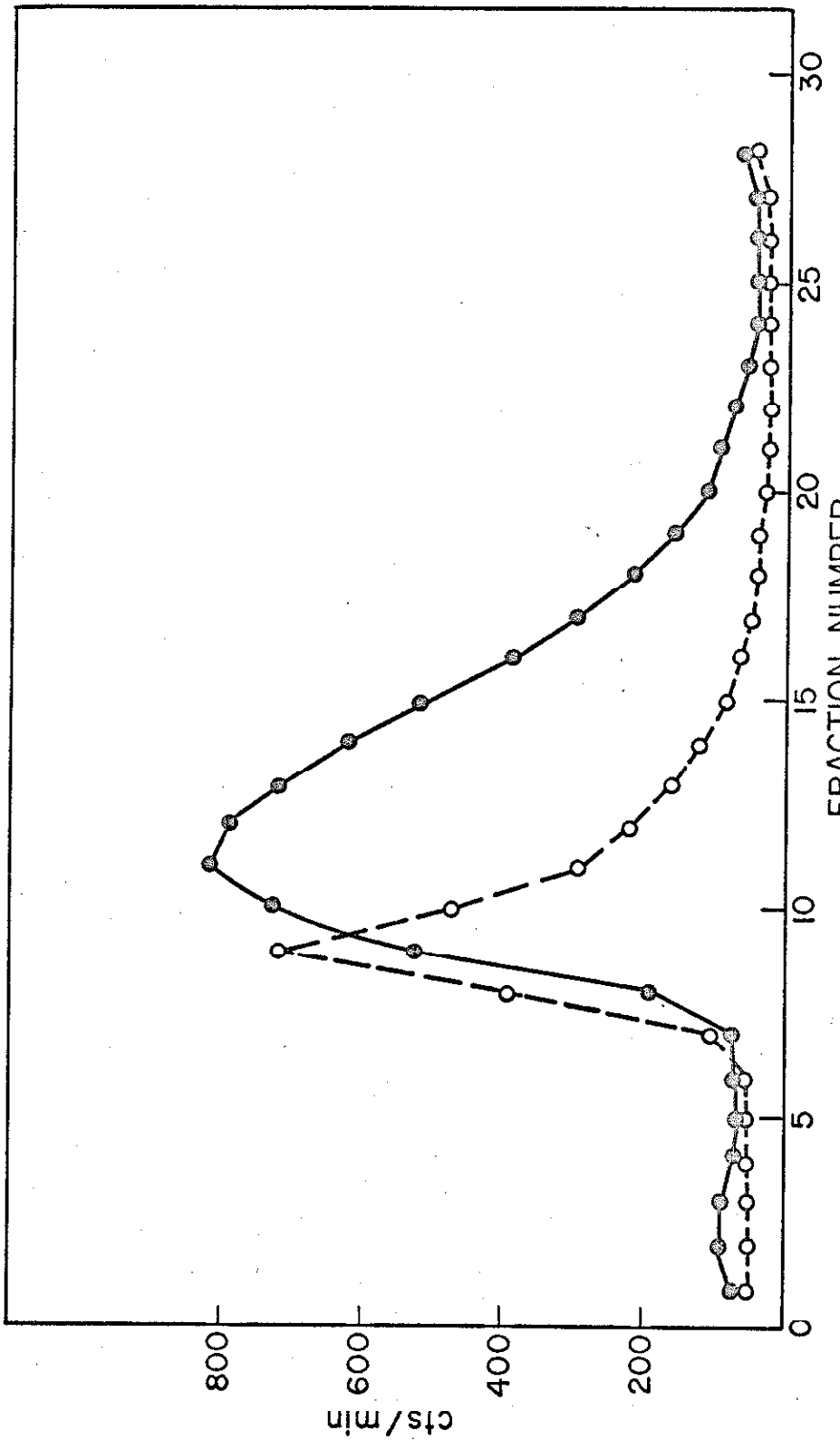

Fig. III - 12.

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Part IV

FRACTIONATION OF THE "REPLICATIVE INTERMEDIATE" USING A BNC COLUMN.

INTRODUCTION

The purification and partial characterization of RI has been described in Part III. In this section a column method is described which is useful for large-scale preparation and fractionation of RI. Further evidence is given in support of the RI structure discussed in the previous section, namely, a duplex of constant size to which a single-stranded tail is attached. The data are best explained if there is one tail per RI. By the centrifugation of pulse-labeled RI under denaturing conditions, the nascent strand is identified.

In addition, since purified RI was available it was possible to compare the melting of the RI as determined by the appearance of infectivity, with that determined by the disappearance of RNase-resistance. The former assay requires stronger denaturing conditions than the latter.

RESULTS

(a) Fractionation of RI on a BNC column

The results in this section have been submitted for publication in the Journal of Molecular Biology. The paper is included in the form in which it was submitted.

SUMMARY

The template on which single strands of MS2 RNA are synthesized, often called the replicative intermediate, is a partially double-stranded molecule. This unusual secondary structure permits the purification of the replicative intermediate on columns of benzoylated, napthoylated DEAE cellulose, which have been shown to fractionate nucleic acids with respect to the extent of their double-stranded character. The replicative intermediate elutes from such a column after DNA and before single-stranded MS2 RNA, but shows heterogeneity. The fractions of replicative intermediate which elute at higher sodium chloride concentrations are more sensitive to RNase and have a buoyant density closer to that of single-stranded RNA. The results suggest that the replicative intermediate molecule consists of a double-stranded "core" to which is attached one single-strand tail of variable length and that these molecules are fractionated on the BNC column with respect to their tail length. All the fractions of replicative intermediate are infective upon denaturation.

1. INTRODUCTION

It has been shown that a double-stranded ribonucleic acid (RNA) appears in cells infected with the single-stranded RNA bacteriophage, MS2 (Kelly & Sinsheimer, 1964; Weissmann, Borst, Burdon, Billeter & Ochoa, 1964). It has also been established that this double-stranded RNA is in large part, only the "core" or a more complicated RNA molecule, which has been termed the "replicative intermediate" or RI (Erikson & Franklin, 1966). The RI is heterogeneous in sedimentation rate and partially sensitive to ribonuclease (RNase) digestion (Erikson, Fenwick & Franklin, 1964). The structure first proposed for the RI (Fenwick, Erikson & Franklin, 1964) was of a "core" of RNA duplex of constant size to which are attached one or more single-stranded "tails" of variable length.

The partial single-stranded character of the RI can be used to obtain it in purified form and to separate the RI into fractions with different lengths of tail. This has been accomplished with a column procedure recently described (Sedat, Kelly & Sinsheimer, 1967), which fractionates nucleic acids on the basis of their secondary structure.

2. MATERIALS AND METHODS

(a) Media

MS2 broth and TPA have already been described (Kelly, Gould & Sinsheimer, 1965).

(b) Preparation of carrier

Unlabeled, infected cell RNA for use as carrier in column fractionations was prepared by growing E. coli in broth to 2 x 10^8 cells/ml., and then infecting with MS2 at a multiplicity of 5. After 30 minutes of infection, the cells were chilled and centrifuged, and the nucleic acids extracted and precipitated as previously described (Kelly et al., 1965).

(c) Preparation of ^{3}H-labeled RI

To prepare ^{3}H labeled RI, bacteria were grown in TPA medium to 2 x 10^8 cells/ml, and infected with MS2 at a multiplicity of 5. Twenty minutes later, ^{3}H-uracil was added (15 c/mM, 12.5 μc/ml.). After 5 minutes, the cells were chilled, lysed and the nucleic acids extracted with phenol. After precipitation with ethanol, the nucleic acids were resuspended in 10^{-3} M EDTA, pH 7.0.

By optical rotary analysis, the appropriate concentrations of dimethylsulfoxide (DMSO) to remove all secondary structure from single-stranded MS2 RNA without denaturation of double-stranded RNA have been determined (Strauss, Kelly & Sinsheimer, 1967). When

centrifuged under such conditions, the RI sediments faster than does either single-stranded MS2 RNA or ribosomal RNA and thus can be purified from single-stranded RNA.

Not more than 200 μg of the RNA were layered on a 5 to 20% sucrose gradient, containing 50% v/v DMSO and 10^{-3} M EDTA. Samples were centrifuged at 25°C for 10 hrs. at 65,000 rev./min in a Spinco SW65 rotor. The RI, as detected by resistance to RNase, sedimented approximately 2/3 the length of the gradient, twice as far as MS2 RNA.

Since it is known that the doubling time of progeny duplexes, and thus of complementary strands is between 1.5 to 2 minutes (Kelly & Sinsheimer, 1967a), over 2.5 replications have occurred in the 5 minute labeling period. Consequently, greater than 80% of the complementary strands will be labeled, as well as all of the nascent strands, and either all or greater than 80% of the viral strands of the RI, depending on the mechanism of replication (Erikson & Franklin, 1966). To this extent the ^{3}H-labeling of the RI may not be uniform.

(d) Preparation of ^{32}P parental-labeled RI

Cells were grown in broth to 2 x 10^{8} cells/ml., and infected with ^{32}P- labeled MS2, at a multiplicity of one. After 8 minutes the cells were chilled, blended in a Waring Blendor for 2 minutes to remove phage which had adsorbed but not penetrated, and then centrifuged. The nucleic acids were then extracted.

(e) Fractionation on BNC columns

Benzoylated-naphthoylated DEAE cellulose (BNC) was prepared according to the procedure of Tener, Gilliam, von Tigerstromm, Milward & Wimmer (1966, also personal communication). BNC columns of 1 cm x 20 cm were prepared and washed with 0.4 M NaCl, 0.01 M tris, pH 7.4, to remove any ultra-violet absorbing material. The nucleic acid sample, in 0.4 M NaCl, was applied to the column, using concentrations of less than 0.5 mg/ml. to reduce the viscosity of the DNA. As much as 4 mg of nucleic acid has been fractionated on a column of these dimensions.

Elution was performed with a linear gradient of 0.4 to 1.0 M NaCl, 0.01 M tris pH 7.4, using a total volume of 100 ml. and flow rates of less than 0.5 ml./min. Ultraviolet absorbance of the eluate was determined either using a Gilford absorbance recorder or, after collecting fractions, with a Bausch & Lomb spectrophotometer. All operations were performed at room temperature.

The RI eluted from the column in a total volume of 20 to 30 ml. It could be concentrated by dilution to lower the salt concentration to 0.4 M NaCl and reabsorption of the RNA to BNC in a small column (6 cm x 0.5 cm). This column was then eluted with 5 ml. of 0.65 M NaCl. The bulk of the RI eluted in 1.0 ml. Washing with higher molarity NaCl released no more RI.

(f) Ethidium bromide - cesium chloride density gradients

On addition of the dye, ethidium bromide, RNA can be centrifuged to equilibrium in cesium chloride solutions of densities attainable at 25°C. To 1.4 ml. of the RNA sample to be characterized, in 0.1 M tris pH 7.4, was added 0.1 ml. of a solution of ethidium bromide (2 mg/ml.) and 2.2 g CsCl. This provided a final density of 1.80 $g\text{-}cm^{-3}$. The solution was placed in a polyallomer centrifuge tube, and, after adding 3ml. of paraffin oil, was centrifuged in a Spinco SW50 rotor for more than 24 hrs. at 37,000 rev./min and at 25°C. Fractions were collected and their radioactivity determined.

The density values given below are calculated values using $\beta = 1.22 \times 10^9$ (Vinograd & Hearst, 1962) to determine the density gradient.

TCA precipitation, filtration and counting procedures have all been described previously (Kelly et al., 1965).

To determine RNase-resistance, samples were treated with 25 γ/ml. RNase, 0.1 M NaCl, 0.1 M tris pH 7.4, and 10^{-2} M $MgCl_2$ for 30 minutes at 37°C, prior to precipitation.

(g) Infectivity assay

The infectivity of single-stranded MS2 RNA was determined using the procedure of Strauss (1964).

Double-stranded RNA or RI is not infectious in this assay (Kelly & Sinshcimer, 1964) unless it has been previously denatured to release the infectious viral strand (Ammann, Delius & Hofschneider, 1964).

To determine the infectivity of RI, the RNA sample was diluted 10 fold into 99% DMSO at 37°C for longer than 1 minute. 50µl. of this solution was then diluted into 0.4 ml. of 0.05 M tris pH 7.4, at 0°C. The infectivity assay was either performed immediately or the samples frozen until required. Control dilutions were made without denaturation, through 0.05 M tris, instead of DMSO, to determine the background of single-strand RNA infectivity.

RESULTS

(a) Fractionation of ^{3}H-labeled RI on a BNC column

The BNC column (Sedat, Kelly & Sinsheimer, 1967) appears to fractionate nucleic acids almost entirely on the basis of secondary structure. Double-stranded DNA and double-stranded RNA are both eluted from such a column with approximately 0.6 M NaCl, and single-stranded MS2 RNA with 0.8 M. It seemed reasonable to predict that the partially single-stranded RI molecules would be eluted at an intermediate ionic strength.

Since the RI is only about 1% of the total cell nucleic acid, a preliminary purification was necessary. Therefore, ^{3}H-labeled RI was prepared using the 50% DMSO sucrose gradient described above. 1.2 mg of infected-cell RNA was added as carrier, and the material fractionated on a BNC column.

The elution pattern is shown in Fig. 1. Four peaks of optical density can be seen corresponding to DNA, MS2 RNA, 16S and 23S ribosomal RNA respectively. (The first peak represents "run-off" material.) A RNase-resistant peak of ^{3}H-counts is found in the place expected for double-stranded RNA (fraction 21), and another, RNase-sensitive peak at the position of MS2 RNA (fraction 54). The majority of the radioactivity elutes between the double and the single-stranded RNA, and represents the expected replicative intermediate.

FIGURE LEGENDS

Fig. 1 The fractionation of ^{3}H-labeled RI on BNC. ^{3}H-labeled-RI, partially purified on a 50% DMSO sucrose gradient, was fractionated on a BNC column with MS2-infected cell RNA as carrier. Elution was performed with a linear gradient of NaCl, 0.4 to 1.2 M. There were 84 fractions. Samples were assayed for absorbance, and for TCA-precipitable radioactivity, before and after treatment with RNase. (-•--•-) ^{3}H-cts/min; (.o....o.) ^{3}H-cts/min after RNase digestion. Absorbance at 260 mμ is the continuous line.

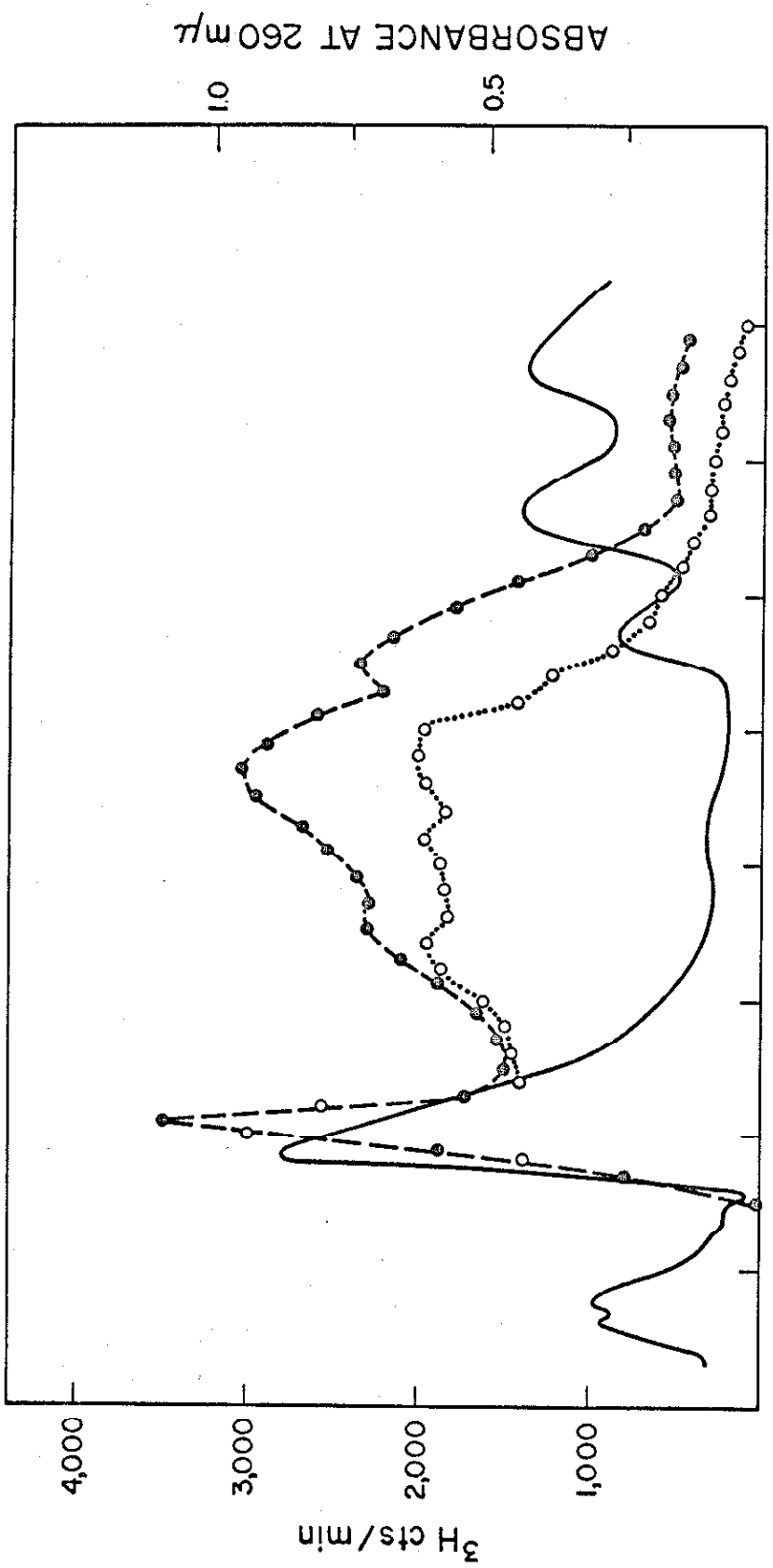
ABSORBANCE AT 260 mμ
1.0
0.5
^{3}H cts/min
4,000
3,000
2,000
1,000
10
20
30
40
50
60
70
80
FRACTION NUMBER

Fig. 1.

The RNase-sensitivity of the RI increases from about 0% at fraction 25 to about 30% at fraction 50. If the RNase-sensitivity of the purified material is a valid criterion for the proportion of single-stranded RNA in the RI, then the column must be fractionating on the basis of the amount of tail. Assuming that the RNA has been uniformly labelled, 30% RNase-sensitivity is equivalent to one full-length tail per duplex.

The fully RNase-resistant peak of ^{3}H-radioactivity (i.e., fractions 17 to 23) is alkali-sensitive and has a sedimentation coefficient of 14 to 15S. It could correspond to the completed duplexes observed by Hofschneider, Ammann & Francke (1966). Alternatively, it may be "core" material derived from RI, from which the tail has been removed prior to fractionation either by the 50% DMSO sucrose gradient or by single-strand scission.

From another chromatography of a portion of the same RNA preparation the equivalents of fractions 24 to 50 were collected, pooled and concentrated. Approximately 2% of the starting material (A_{260}) was recovered as RI. While this material may be partially contaminated with DNA it contains, by infectivity assay, less than 1 part in 10^{5} single-stranded MS2 RNA.

The specific infectivity (infectivity/A_{260}) after denaturation, of several such RI preparations has been found to be in the range of 0.1 to 1.0 times the specific infectivity of viral MS2 RNA. A specific infectivity of about 0.4 might be expected if only the viral strand from the RI is infective. The range of observed values

probably reflects the inaccuracy of the infectivity assay. Variable inactivation of the RI or of the single-stranded RNA standard may be the cause.

(b) Fractionation of RI infectivity

In the above, partial RNase-resistance has been used as the criterion for the presence of RI. An alternative characteristic of the RI is that it is not infective in the spheroplast assay, unless it is denatured. The elution of such RI infectivity from BNC columns has been studied.

Four mg of unlabeled RNA from infected cells were fractionated on a BNC column (using 200 ml total eluting volume) (Fig. 2). Four peaks of ultra-violet absorbance corresponding to sRNA, DNA, 16S and 23S ribosomal RNA were resolved. Fractions were assayed for immediate, single-stranded-RNA infectivity, and for infectivity after denaturation. As before, single-stranded MS2 RNA (infective prior to denaturation) is released slightly before 16S ribosomal RNA. RI infectivity is eluted at a fairly constant level from the DNA peak to that of MS2 RNA. Since a yield of 10^2 phage per ml. is the lower limit of the assay it is clear that the infectivity of the RI fractions increases more than 1,000 fold with denaturation.

We can again conclude that RI is eluted from the column as predicted for a partially single-stranded molecule, and that it is not released as a homogeneous component. Assuming that most of the RI is eluted before MS2 RNA, and that each denatured RI molecule is just as infective as a single-stranded MS2 RNA, there are about 50 MS2 RNA molecules for each RI molecule in the extract prepared 30 minutes after infection.

Fig. 2 The fractionation of RI, as determined by infectivity, on a BNC column. An unpurified nucleic acid preparation from MS2-infected cells was applied to the column and eluted using a 0.4 to 1.0 M NaCl gradient. There were 73 fractions. Infectivity before denaturation (.△....△.); after denaturation (-▲---▲-); absorbance at 260 mμ is the continuous line. The nature of each absorbance peak is indicated. Small differences in the elution profile occur between batches of BNC, as is shown by a comparison of figures 1 and 2.

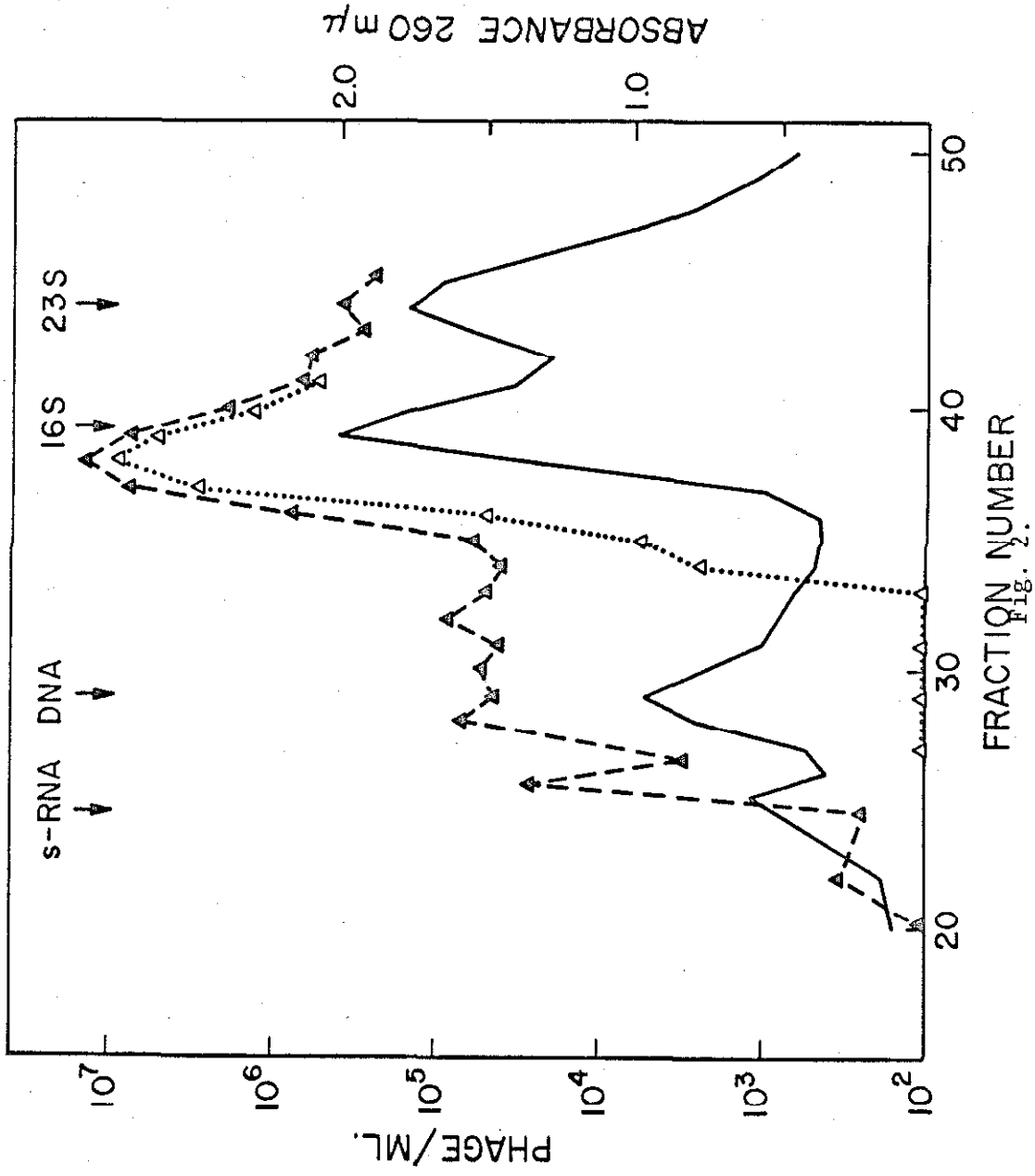
ABSORBANCE 260 mμ
2.0
1.0
50
40
30
20
FRACTION NUMBER
PHAGE/ML.
10^7
10^6
10^5
10^4
10^3
10^2
s-RNA
DNA
16S
23S

Fig. 2.

(c) Fractionation of ^{32}P parental-labeled material

When ^{32}P MS2 phage is used to infect cells and uninjected phage are removed by blending, a large fraction of the residual radioactivity is in the RI. This procedure is a useful way to label the RI in order to study its properties.

1.5 mg of unlabeled, infected-cell RNA was added to the nucleic acids extracted from cells infected for 8 minutes with ^{32}P MS2. The elution profile obtained upon fractionation on a BNC column is given in Fig. 3. A large fraction of the parental-labeled material is eluted between DNA and MS2 RNA. This material is partially RNase-sensitive. The sensitivity increases progressively in the fractions eluted with higher ionic strength, rising from 0% sensitive to about 50% of the label. Fractions 30, 36 and 42, when precipitated, redissolved, and sedimented on a sucrose density gradient, have average S values of 15, 17 and 19S respectively, but are each heterogeneous.

All the RNase-resistant material can be shown to be in a 14S "core" by isolation and analysis of the RNase-resistant duplex (Kelly & Sinsheimer, 1967a). This is true even for the RNase-resistant material which is eluted in the same fractions as the MS2 RNA. This material might either represent a chromatographic trailing of the RI because of insufficient resolution, or duplexes with an unusually large amount of tail (vide infra).

Fig. 3 Fractionation of nucleic acids from cells infected for 8 minutes with ^{32}P labeled MS2 RNA. The positions of the DNA and 16S ribosomal RNA, as determined by absorbance at 260 mμ, are indicated. Total ^{32}P cts/min (●——●); ^{32}P cts/min resistant to RNase (-o----o-).

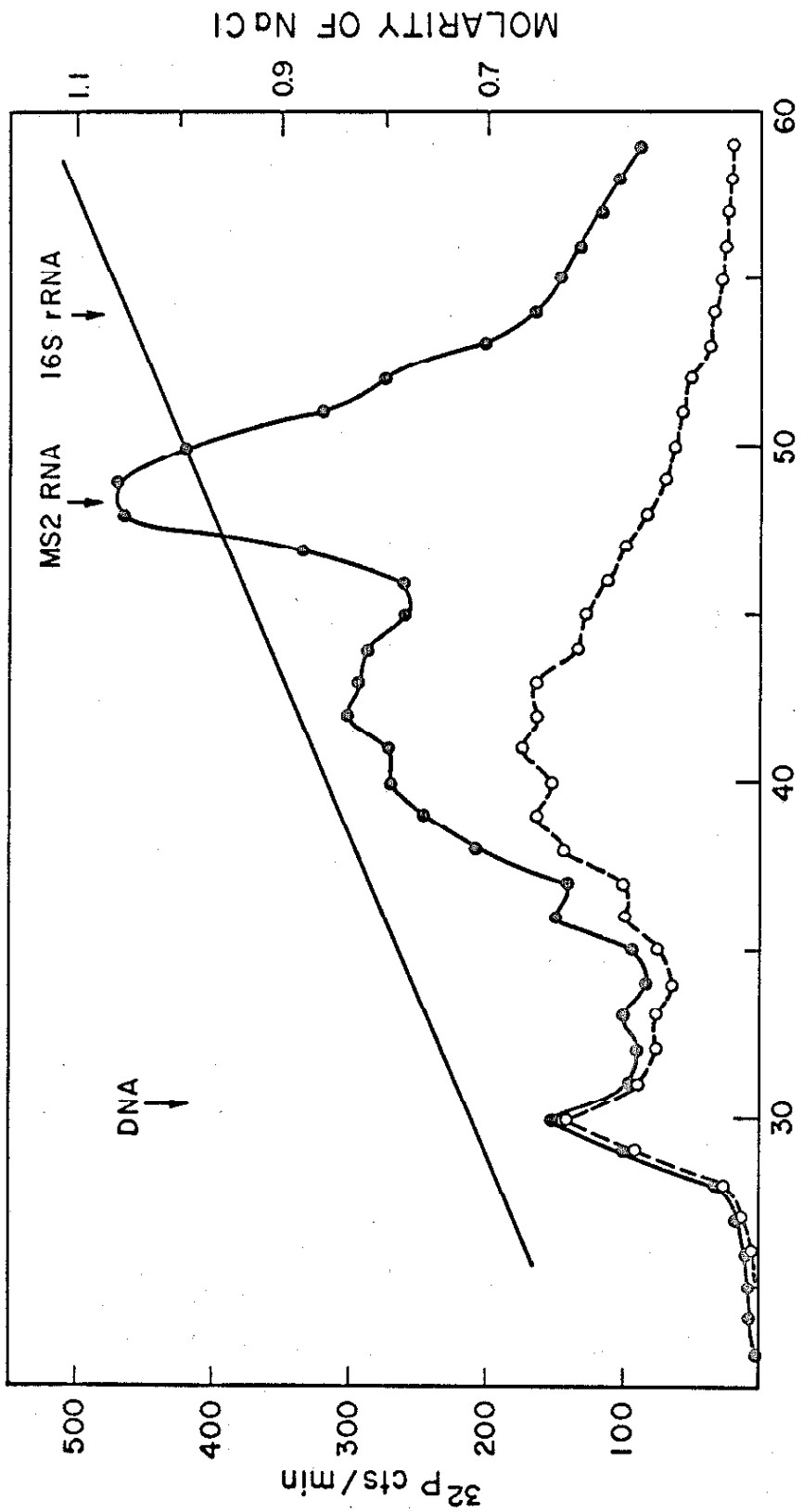
MOLARITY OF NaCl
1.1
0.9
0.7
16S rRNA
MS2 RNA
DNA
^{32}P cts/min
500
400
300
200
100
30
40
50
60
FRACTION NUMBER

Fig. 3.

That these successive fractions from the column are, indeed characterized by increasing length of tail could be verified by a determination of their buoyant densities. As we have pointed out (Kelly & Sinsheimer, 1967a), the use of CsCl gradients at 65°C for this purpose is precluded by the scissions which occur in single-stranded RNA under such conditions. It has recently been shown, however, that RNA can be analyzed in CsCl gradients at 25°C in the presence of the dye, ethidium bromide (Dr. J. B. LePecq, personal communication).

Fractions from a column equivalent to that of Fig. 3 were therefore, centrifuged in a cesium chloride density gradient containing ethidium bromide for 40 hrs. at 25°C, using ^{3}H-labeled RNase-resistant duplex as a density marker. After collecting fractions, samples were TCA-precipitated and counted. Assuming a constant density gradient, the densities of the various column fractions were determined and are shown in Fig. 4. It is clear that fractions which are eluted at increasing ionic strength (and thus have increasing RNase-sensitivity) have densities closer to that of single-stranded RNA.

This result is in accord with the concept that the BNC column fractionates RI on the basis of its proportion of single-stranded RNA. The density difference between the most dense RI (presumably duplexes with little or no tail) and single-stranded RNA is 0.046 g-cm^{-3};

Fig. 4 The buoyant density of RI fractions in ethidium bromide-cesium chloride density gradients. Nucleic acids from cells infected with ^{32}P-MS2 RNA were fractionated on a BNC column. Selected fractions were mixed with ^{3}H-labeled, RNase-resistant duplex (RRD) and sedimented to equilibrium for 50 hours at 37,000 rev./min in a cesium chloride density gradient. Fractions were collected, precipitated and counted. The densities were calculated assuming a uniform density gradient. Fractions 36 and 52 correspond to fractions 30 and 44, respectively, in Fig. 3.

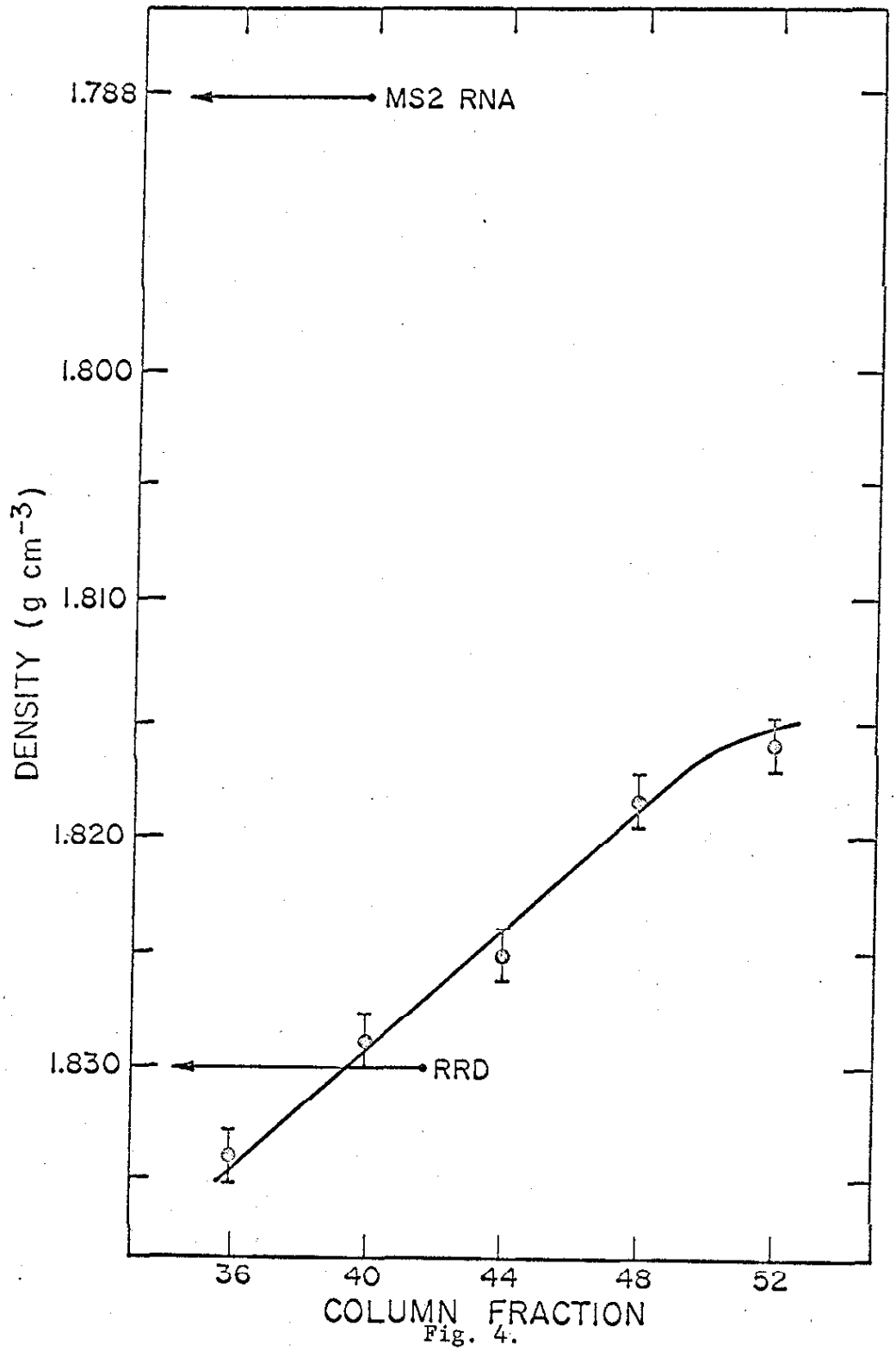
1.788
MS2 RNA
1.800
1.810
1.820
1.830
RRD
DENSITY (g cm^{-3})
36
40
44
48
52
COLUMN FRACTION

Fig. 4.

whereas, the density difference between the most dense and the least dense RI (presumably maximum tail) is 0.018 g-cm^{-3}. The maximum density difference observed, between RI molecules is, therefore, 39 $\pm$ 6% of that observed between duplexes and single strand. Assuming simple additivity and that there is one tail per molecule, a value of 33% would be expected.

As was mentioned it seemed possible that the double-stranded material which was eluted at the same salt concentration as the single-stranded MS2 RNA might represent a fraction of RI with an unusually large amount of tail. When a column fraction equivalent to the single-stranded MS2 peak in Fig. 3 was centrifuged to equilibrium in a ethidium bromide - cesium chloride density gradient two labelled peaks were resolved. The majority of the radioactivity had the density of single-stranded RNA; however, a minor component appeared with a density of 1.818 $\pm$ 0.001 g-cm^{-3}. This density corresponds to a duplex with approximately one full tail. This result indicates that some RI is eluted with single-stranded MS2 RNA because of the imperfect resolution of the column, and that RI molecules with more than one tail, if they exist at all, are present in small amounts.

DISCUSSION

Since the BNC column appears to fractionate nucleic acids on the basis of secondary structure, it was predicted that the greater the proportion of single-stranded RNA in an RI molecule, the higher would be the salt concentration required to elute it. The increasing RNase-sensitivity of the successive fractions obtained by elution of uniformly labeled RI supports this prediction. It is also apparent from the elution pattern that a one-step purification of RI by sedimentation on 50% DMSO gradients does not completely remove all single-stranded MS2 RNA.

In Figure 1, only 20% of the counts in RI are in complete duplexes (fractions 17 to 23). Similarly, in Figure 2, there is no large peak of RI infectivity corresponding to complete duplexes. The absence of a large pool of such complete duplexes implies that most of the duplexes can be used as templates, and that the lag time between the completion of a cycle of transcription, and the initiation of a new cycle cannot be longer than the time required for transcription.

Fractions from the column with higher sensitivity to RNase have a greater sedimentation rate and have buoyant densities approaching that of single-stranded RNA. A partially single-stranded structure for RI seems, therefore, a logical deduction. Measurements of the

RNase-sensitivity and density of the successive column fractions lead to the conclusion that the heterogeneity in the RI represents variation in the length of one single-stranded tail, from zero to a length almost equal to that of the viral RNA.

It is surprising that the density of single-stranded MS2 RNA is less than that of double-stranded RNA in ethidium bromide - cesium chloride gradients. This result implies that the dye is bound preferentially to the single-stranded molecule. (This conclusion is partially ambiguous because of the large amount of secondary structure in MS2 RNA (Strauss & Sinsheimer, 1963)). The density of the RNase-resistant duplex (RRD), which is the "core" material obtained from RI by RNase digestion, is less than that of untreated duplexes with no tail (Fig. 4). This suggests that the RNase treatment may produce some single-stranded regions in the RRD molecule.

The BNC column is clearly useful for fractionation of the RI. Where it is used to purify the intermediate, it has two disadvantages: the preparation is contaminated with DNA, and the RI is eluted in a large volume. The latter problem has been met by re-absorption of the sample to a small column. DNA can be removed by treatment with DNase, followed by gel filtration. The column also appears to be a very effective method to eliminate single-stranded RNA from the RI.

The significance of the partial RNase-sensitivity of the ^{32}P parental-labeled RI will be discussed in the following publication (Kelly & Sinsheimer 1967b).

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(b) Denaturation of Column fractions by DMSO

The effect of a denaturant on the secondary structure of a nucleic acid can be determined either at the ambient conditions or after removal of the denaturant. Since the former method is a measure of the denaturation at the ambient conditions whereas the latter determines the irreversible denaturation at these conditions, Geiduschek (1962) has named these procedures the "d-assay" and the "i-assay". Irreversible or "Type II" denaturation requires the dissociation of G:C rich "nuclei". In Part III, the difference between the "half-melting" concentration (C_m) of DMSO determined either by optical or by infectivity measurements was interpreted in these terms.

This explanation was only one of several since the sources of double-stranded RNA for the two measurements were different. Sufficient purified RI was not available for optical measurements. As an alternative approach it is possible to compare the concentration of DMSO required to sensitize labeled RI to RNase digestion, with that required to release infectivity. We assume that complete strand separation is required for infectivity and that the denatured regions will not significantly renature when the samples are rapidly diluted out of DMSO into a solution containing RNase, i.e. the denaturation is "frozen-in" (Dove & Davidson, 1962). The advantage of this procedure is that

comparison can be made under identical ionic and temperature conditions on the same batch of DMSO.

A dependence of melting temperature on the length of the duplex has been observed on several occasions (e.g. Crothers, 1964). Because of this effect and the complex structure of the RI, the C_m might be a function of the tail length. To check this point fractions of pulse-labeled RI of different tail length were used. Infected cells were pulse-labeled for 30 seconds at 25°C, lysed and the nucleic acids purified (see Procedure in Part V(a)). Using the BNC column described above the nucleic acids were chromatographed and three RI fractions obtained of different tail lengths.

The fractions were diluted into various concentrations of DMSO, all containing 10^{-3} MEDTA. After 10 minutes at 37°C a sample was diluted 10 fold into cold buffer and immediately assayed for infectivity. The remainder was diluted 5 fold with a solution of 100 μg/ml. RNase, incubated at 37°C for 30 minutes, precipitated and counted. The results are shown in Fig. IV-5.

Clearly there is no significant difference between the melting properties of the three fractions. There is a striking difference in the C_m's measured by the two procedures, the C_m for the infectivity assay being higher. Similar results were obtained when the inactivation of transformation markers by heat was compared to optical melting data (Ginoza & Zimm, 1961). It would appear from these results that the release of infectivity does indeed

Legend to Figure IV-5.

Comparison of the denaturation of RI as measured by RNase-sensitivity and infectivity. 100 µl. of three fractions of RI (29, 31 and 33 in Fig. IV-2) were diluted into 2.4 ml. of DMSO at different concentrations, containing 10^{-3} MEDTA, pH 7.0. After 10 minutes at 37°C, 40 ul. were diluted into 0.4ml. of cold 0.05M tris, pH 7.4, and assayed for infectivity. To the remainder were added 10 ml. of a solution of 100 µg/ml. RNase, 0.1 M NaCl, 0.1 M tris, pH 7.4, and 0.01 M $MgCl_2$. After digesting for 30 minutes at 37°C the sample was precipitated with TCA, filtered and counted. RNase-resistant counts (—●———●—); phage/ml. after infectivity assay (-0----0-). The DMSO concentrations given are those of the solutions before the RNA was added.

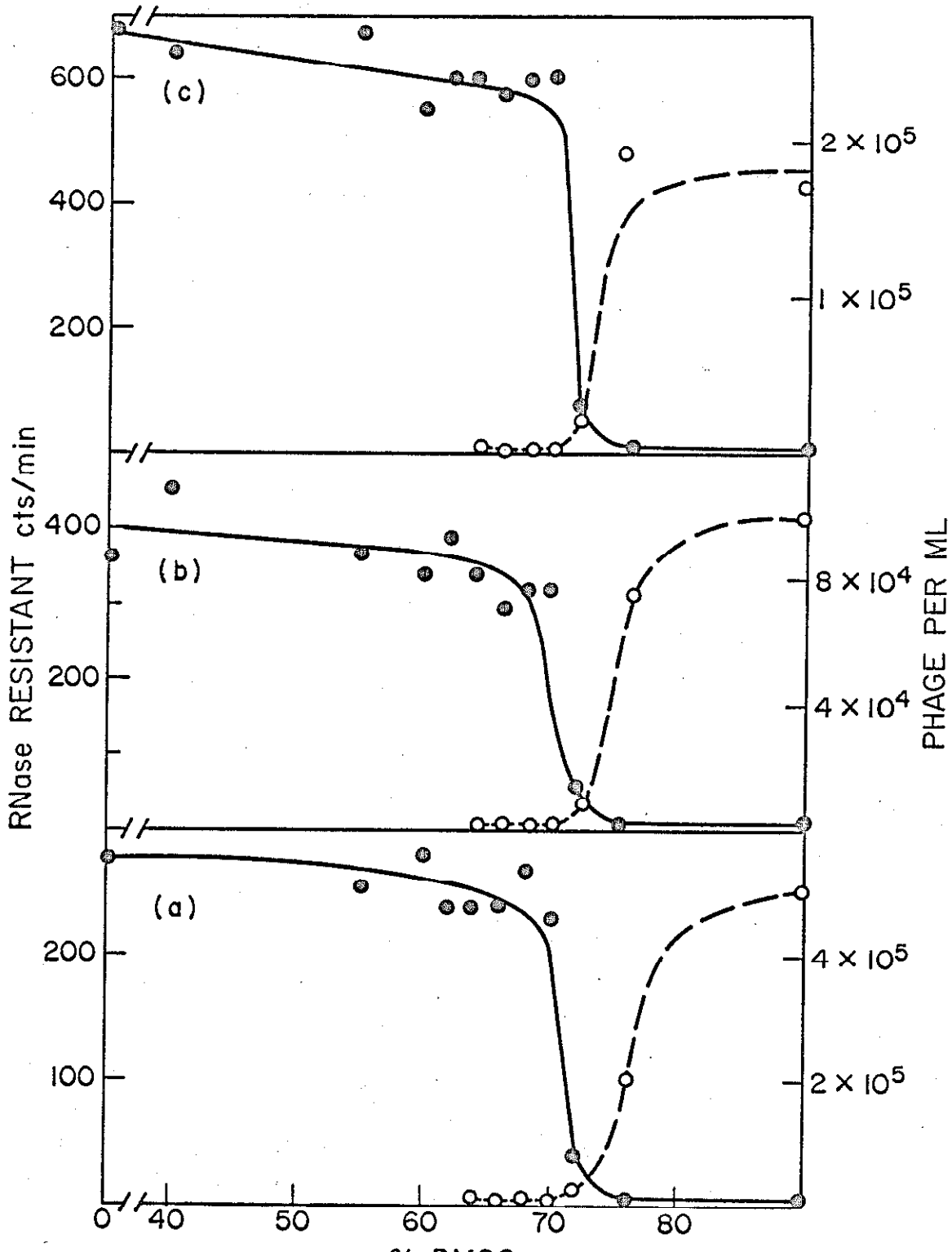
(c)
(b)
(a)
600
400
200
400
200
200
100
2 × 10^5
1 × 10^5
8 × 10^4
4 × 10^4
4 × 10^5
2 × 10^5
RNase RESISTANT cts/min
PHAGE PER ML
0
40
50
60
70
80
% DMSO

Fig. IV - 5.

require the melting of G:C rich nuclei but in the absence of good data comparing optical melting to either of the above methods such a conclusion can only be tentative. The presence of partially denatured states, however, might simplify the explanation of the strange sedimentation properties of double-stranded RNA as the DMSO concentration approached the C_m. (Part III).

(c) The denaturation products of Fractionated RI

If uniformly labeled RI is centrifuged under denaturing conditions, its average sedimentation rate is almost identical to an MS2 RNA marker (Figure III - 11). The presence of a nascent strand could only be deduced from a trailing of the peak. To confirm that there is indeed a nascent strand attached to the duplex in purified RI it was proposed to selectively label the nascent strand by using short pulses of radioactivity. The nascent strand could then be identified by centrifuging pulse-labeled RI under denaturing conditions. Because of the complexities of arguing from a sedimentation distribution to a distribution of tail lengths the RI molecules were first fractionated on the basis of their putative tails, before analysis by centrifugation. If the model of the RI and the theory of BNC fractionation are both correct then column fractions should have nascent strands of increasing length.

The procedures used in this experiment have already been described. RI pulse-labeled for 30 seconds was prepared as described in IV(b), fractionated on BNC (IV(a)), precipitated with alcohol and centrifuged in a sucrose gradient containing 100% DMSO (III(e)). The results are shown in Figure IV - 6.

In (c) the fraction with the longest tail, the bulk of the radioactivity corresponds to material sedimenting 0.85 times as

Legend to Figure IV - 6

Centrifugation of the denaturation products of RI fractions of different tail length. RI, labeled for 30 seconds with ^{3}H-uracil, was fractionated on a BNC column. Three column fractions were precipitated and resuspended in 10^{-3}M EDTA. 50ul. of each fraction were mixed with 50ul DMSO, and 100ul of dimethyl formamide, and centrifuged in a 100% DMSO, 5 to 20% sucrose gradient (10^{-3}M EDTA) for 15 hours at 63K (25°C.) Figures (a), (b) and (c) correspond to increasing tail length. (—●———●—) ^{3}H radioactivity.

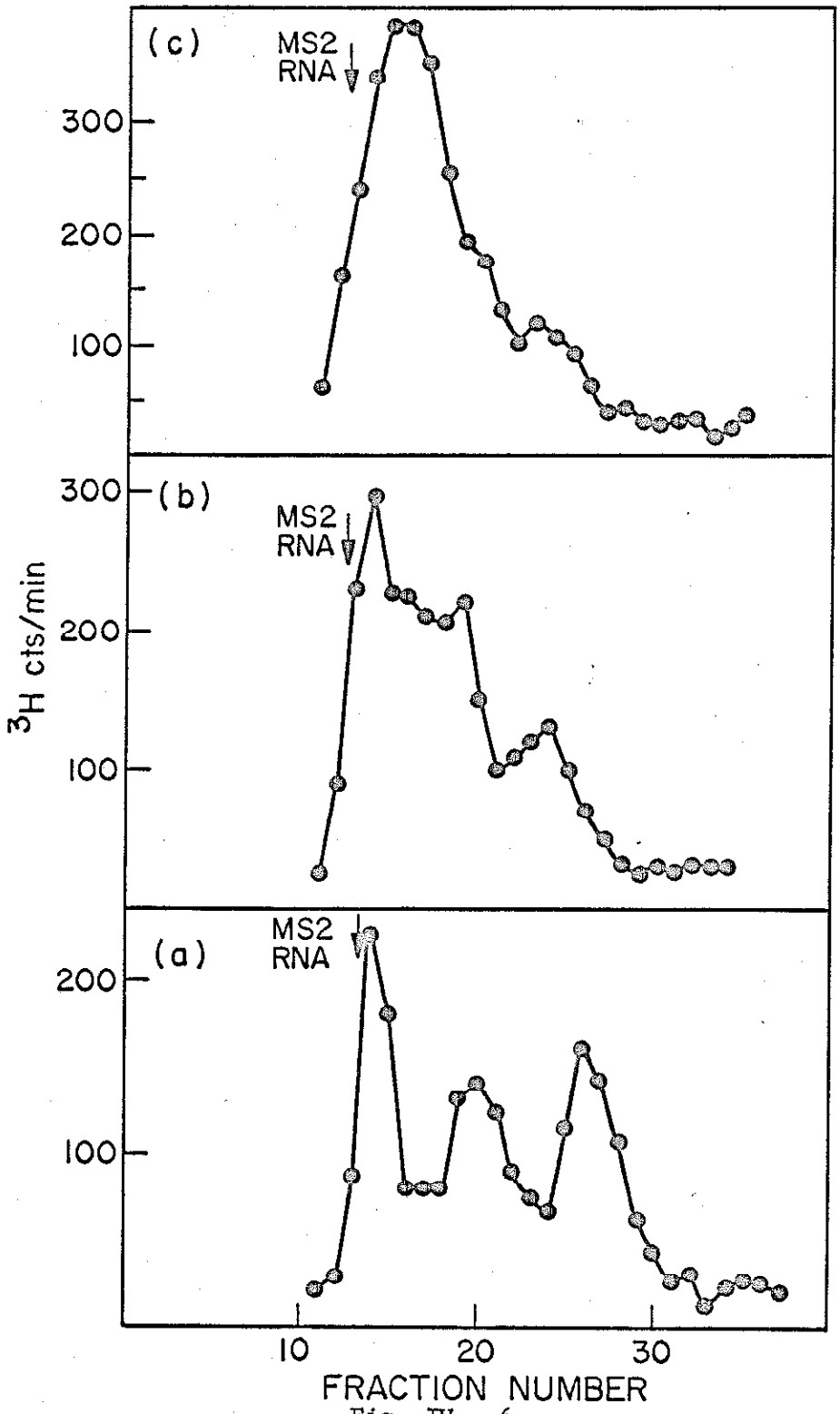
(c)
MS2 RNA
300
200
100
(b)
MS2 RNA
300
200
100
^{3}H cts/min
(a)
MS2 RNA
200
100
10
20
30
FRACTION NUMBER

Fig. IV - 6.

fast as the single strand marker, and presumably corresponding to the nascent strand. In Figures (b) and (a), in which the tail is presumably decreasing in length, the distribution of radioactivity becomes more complex, but reproducible. The fastest peak almost co-incides with the single-stranded marker. Comparison of the results of several experiments of this type show that the fastest peak is especially prevalent in RI fractions with short tails, and that the amount in this form is greater, the longer the labeling time. It is postulated to arise from RI molecules which have completed a transcription during the labeling period (see Part V(a)).

The second fastest peak increases in sedimentation rate in these experiments as the length of the tail increases. It is assumed to be the nascent strand.

The slowest peak is present in all RI preparations. It sediments about 0.4 times as fast as MS2 RNA, corresponding approximately to a molecular weight of 10^5 daltons. It is clearly of interest to determine if this is a phage specific RNA, perhaps even a part of the RI structure, or merely a contaminating host cell RNA fragment. A possible function of an RNA molecule of this size could be to direct the synthesis of a coat protein. The appropriate experiments have, unfortunately, not been done.

The conclusions of these experiments is that pulse-labeling does preferentially label molecules of size smaller than the viral

or complementary strands. As the pulse length increases more radioactivity is seen in completed RNA strands. Finally, another small RNA fragment is found in all RNA preparations but its nature and origin are not known.

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Part V

THE STRUCTURE AND REPLICATION OF THE REPLICATIVE INTERMEDIATE.

INTRODUCTION

Using the techniques for the purification and fractionation of RI, described in previous sections, it is possible to investigate the structure of RI more closely, in the hope of determining the mechanism of transcription. The results of such investigations are given in the present section.

The model which is presented to describe phage specific RNA transcription suggests some conjectures about transcription in general. Some of these are discussed as a conclusion to this section, and the thesis.

RESULTS

(a) The structure and replication of the RI

The results in this section have been submitted for publication in the Journal of Molecular Biology. The paper is included in the form in which it was submitted.

SUMMARY

Experiments are described to determine whether the ribonuclease-sensitive component of the replicative intermediate (RI) formed during RNA bacteriophage infection is the nascent strand (conservative replication) or is a displaced portion of the viral strand of the RNA duplex (semi-conservative replication). Appropriate labeling experiments indicate that this component arises in each RI molecule, with equal probability, from either origin. Those RI molecules in which the nascent strand is ribonuclease-sensitive are more readily denatured than those in which the viral strand is partially displaced. The two types of replicative intermediate can be distinguished on this basis. It is proposed that each RI molecule replicates both conservatively and semi-conservatively, equally often.

1. INTRODUCTION

In order to clarify some of the unsolved problems of nucleic acid replication, extensive study has been made in recent years of the mechanism of replication of the single-stranded ribonucleic acid (RNA) bacteriophages. Evidence has accumulated that a double-stranded RNA molecule appears during the replication cycle (Kelly & Sinsheimer, 1964; Weissmann, Borst, Burdon, Billetter & Ochoa, 1964) and plays a direct role in the production of progeny single-stranded molecules (Fenwick, Erikson & Franklin, 1964; Ammann, Delius & Hofschneider, 1964; Mills, Pace & Spiegelman, 1966). Thus the principle that nucleic acid replication requires the synthesis of a complementary strand seems to be valid also for the replication of single-stranded RNA.

It is not yet known how single-stranded RNA molecules are produced from the double-stranded intermediate. Two mechanisms have been described for the in vitro production of single-stranded RNA from double-stranded templates using the DNA-dependent RNA polymerase. If the template is a synthetic DNA-RNA hybrid, the pre-existing RNA is displaced, at least part of the time, by the newly synthesized RNA strand (Sinsheimer & Lawrence, 164; Chamberlin & Berg, 1964). This mode of transcription has been called semi-conservative replication. If, however, the template is double-stranded DNA (ØX replicative form) the newly synthesized RNA is displaced from the duplex in a conservative mode of replication (Hayashi, 1965). There is no basis for predicting which, if any, of these mechanisms will be used when

the template is a double-stranded RNA.

From a detailed knowledge of the structure of the replicative intermediate (RI), as the in vivo precursor to single-stranded RNA is often called, it should be possible to distinguish between a semi-conservative and a conservative mode of replication. The present evidence strongly supports a structure for the RI consisting of an RNA duplex of constant size (Erikson, Fenwick & Franklin, 1964) to which is attached one single-stranded RNA chain of length less than that of the viral RNA (Kelly & Sinsheimer, 1967b). A structure of this type would be expected if the growing strand does not dissociate from the duplex during the extraction procedure, and can be explained either by a conservative or a semi-conservative replication mechanism (Figure 1). If the duplex is transcribed semi-conservatively it is obvious from Figure 1 that the nascent strand displaces the viral strand. Conversely, if the transcription is conservative the nascent strand, except for a short region at the growing point, is displaced from the duplex. The experiments described below have been designed to determine whether the nascent or the viral strand is so displaced and on this basis to decide whether replication is conservative or semi-conservative. The results suggest that both types of replication occur with equal probability.

FIGURE LEGENDS

Fig. 1 A schematic diagram of possible structures for the MS2 replicative intermediate. v denotes the viral strand, identical to that in the phage. c is the strand complementary to the viral strand. n is the nascent strand, complementary to c, with the growing point indicated by an arrowhead. The single-stranded portion of the RI is referred to as the tail, and the double-stranded portion as the core or RNase-resistant duplex. The tail can be removed from the core by digestion with ribonuclease.

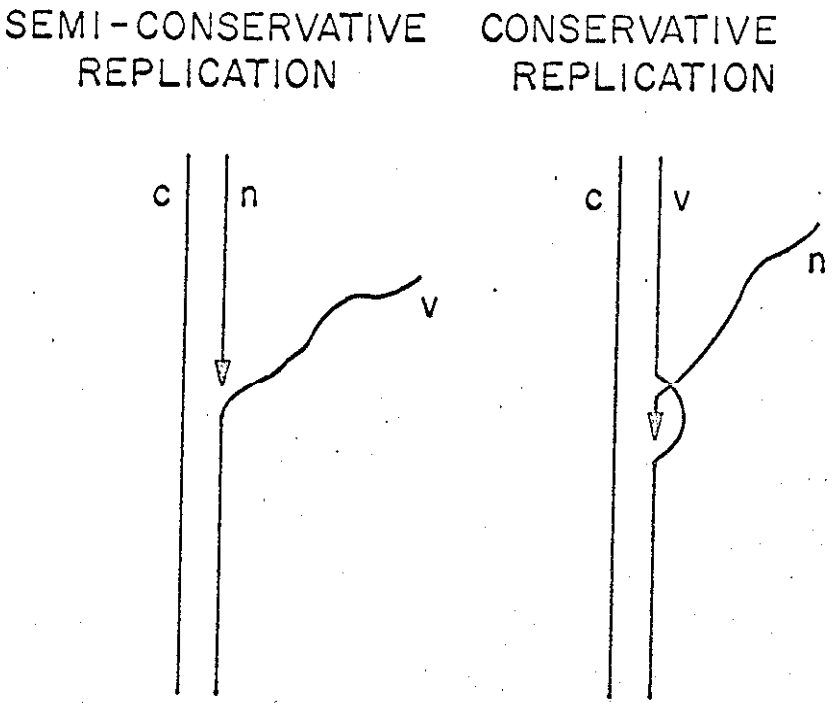
SEMI-CONSERVATIVE REPLICATION
CONSERVATIVE REPLICATION
c
n
v
c
v
n

Fig. 1.

2. MATERIALS AND METHODS

Many of the techniques employed in this paper have been previously described. Information on growth media, and on the methods of lysing the cells, and extracting and precipitating the nucleic acids were described by Kelly, Gould & Sinsheimer (1965). The preparation of $^{12}C^{14}N^{32}P$ MS2 and of $^{13}C^{15}N^{32}P$ MS2 and the procedures for determining the buoyant density of RNA in cesium chloride density gradients at 65°C were outlined by Kelly & Sinsheimer (1967a). Finally, ethidium bromide-cesium chloride density gradient centrifugation, fractionation of nucleic acids on BNC columns, and ribonuclease (RNase) digestion have been discussed by Kelly & Sinsheimer (1967b).

(a) Pulse-labeling

E. coli grown at 37°C can be infected at 25°C by MS2 phage and will produce infective RNA. In order to label infected cells for periods which were short compared to the time of transcription of a viral strand, label was added for a few seconds at 25°C. Cells were grown in TPA medium at 37°C to 2 x 10^8 cells/ml. and infected with MS2 phage at a multiplicity of 5. After 20 minutes incubation at 37°C the cells were chilled to 25°C for 15 minutes. ^{3}H-uracil (2 μc/ml., 15 c/mM) was added to the culture, with vigorous stirring. After several seconds the incubation was stopped by addition of excess crushed ice and sodium azide to a final concentration of

0.01 M. The chilled culture was immediately filtered (Millipore HA, 142 mm) and washed with cold 0.1 M tris, pH 7.4 and 0.01 M sodium azide before resuspending in the same buffer. The cells were concentrated by sedimentation, lysed with SDS and extracted with phenol.

(b) Sucrose gradient sedimentation

Samples to be analyzed by preparative sedimentation were centrifuged in 5 ml. polyallomer tubes containing a 5 to 20% sucrose density gradient. For non-denaturing gradients, 0.1 M tris, pH 7.4 was used as the solvent, and the samples were sedimented in a Spinco SW65 for 2 hrs. at 65,000 rev./min (5°C). For partial denaturing gradients, 60% dimethyl sulfoxide (DMSO) and 10^{-3} M EDTA were used as solvent. Samples were centrifuged 10 hrs. at 65,000 rev./min and at 25°C. For fully denaturing gradients the sucrose was dissolved in 99% DMSO, 10^{-3} M EDTA and the samples centrifuged 12 hrs. at 65,000 rev./min and at 25°C. In the latter case, 50μl. of the sample to be analyzed were mixed with 100μl. dimethyl formamide and 50μl. dimethyl sulfoxide before layering on the gradient. Under these conditions a very stable layer is formed above the 99% DMSO sucrose density gradient, and no convection occurs. Further details of this procedure have been described by Strauss (1966).

3. RESULTS

(a) The RNase-sensitivity of the nascent strand

From Figure 1 it is apparent that - by definition - if the nascent strand is displaced from the duplex the replication is conservative, and if it is not the replication is semi-conservative. If infected cells are labeled for short periods the majority of the phage-specific radioactivity appears in the RI. On denaturing the RI, this radioactivity can be shown to be associated with the nascent strand (vide infra). If, in the RI, the nascent strand is displaced from the duplex it will be sensitive to RNase digestion; if not, it will be resistant. *Consequently, by labeling the nascent* strand with a brief pulse of radioactivity and determining the sensitivity of the radioactivity to RNase, a simple test of the mechanism of replication should be possible.

Cells infected with MS2 for 30 minutes at 37°C were cooled to 25°C and labeled for 10 seconds with ^{3}H-uracil (2 μc/ml., 15 c/mM). The cells were lysed and their nucleic acids extracted with phenol and precipitated with alcohol. After resuspending the nucleic acids in 0.3 M NaCl, ^{32}P MS2 RNA was added as marker and the material chromatographed on a BNC column. Each fraction was assayed for total and RNase-resistant radioactivity (Fig. 2).

The RI radioactivity (fractions 27 to 31) is neither fully resistant to RNase, as a semi-conservative mode of replication predicts, nor fully sensitive, as is predicted by a conservative mode,

Fig. 2 Fractionation of pulse-labeled replicative intermediate on a BNC column. The nucleic acids from MS2 infected cells labeled at 25°C with ^{3}H-uracil for 10 sec. just prior to lysis were applied to a BNC column and eluted with a 0.3 M to 1.0 M linear gradient of NaCl (buffered with 0.01 M tris, pH 7.4). 40 fractions of 2.5 ml. were obtained. Samples were assayed for absorbance (peaks indicated by arrows) and for TCA-precipitable ^{3}H radioactivity both before (—o——o—) and after (-o----o-) RNase digestion. (..x.....x..) indicates the position of a single-stranded ^{32}P MS2 RNA marker.

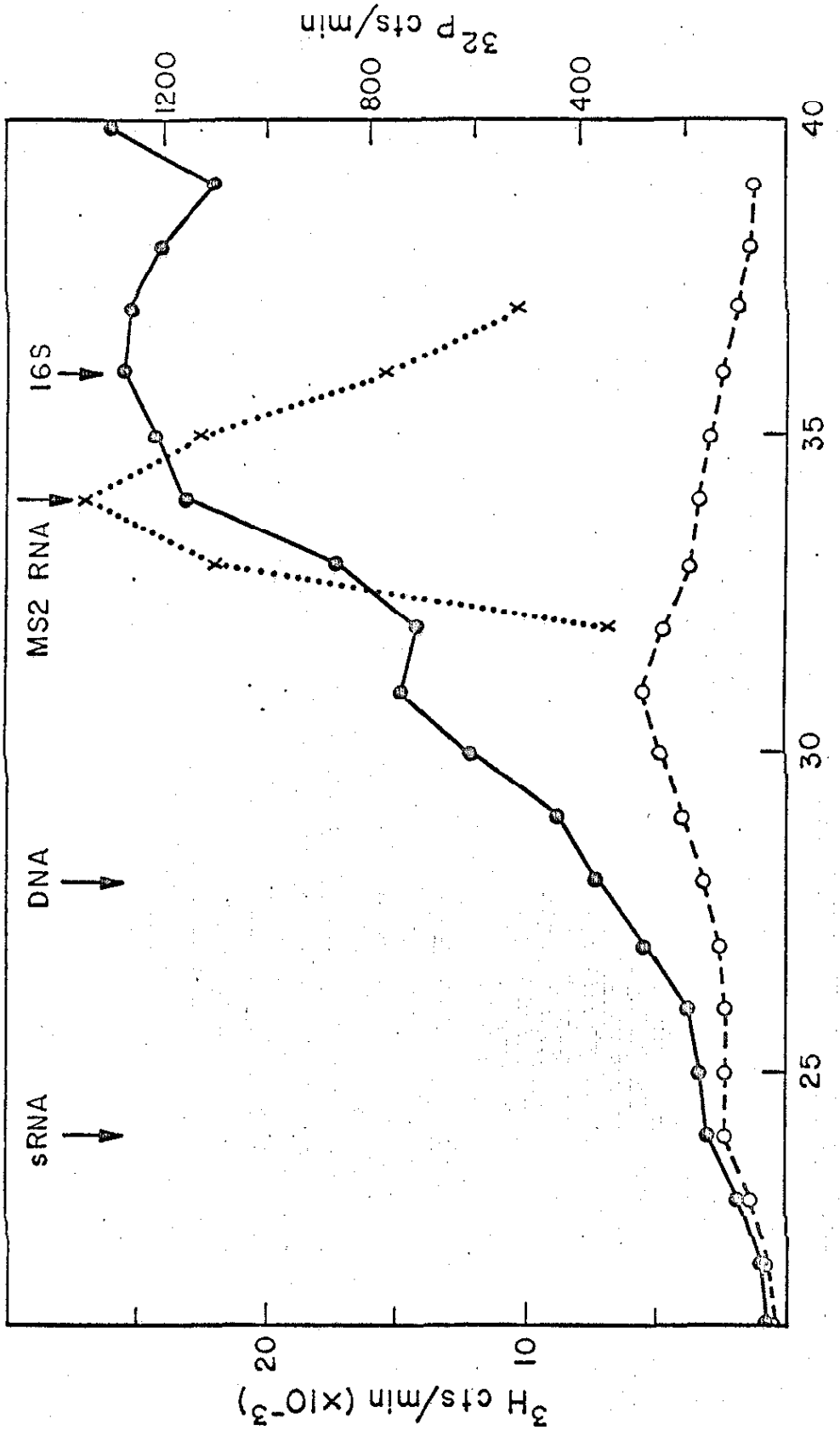
^{32}P cts/min
1200
800
400
^{3}H cts/min (x10^{-3})
20
10
16S
MS2 RNA
DNA
sRNA
25
30
35
40
FRACTION NUMBER

Fig. 2.

but is 40 to 50% resistant. The small shoulder (fraction 34) on the peak of 16S ribosomal RNA, coincident with the MS2 RNA marker perhaps represents some synthesis of completed MS2 RNA. Fractions 23 to 26 consist largely of completed duplexes (vide infra) and thus are highly resistant to RNase.

There are several possible interpretations of a pulse label which is 40 to 50% RNase-resistant. Even if the replication is conservative, the segment of the nascent strand at the growing point (Fig. 1) might be insusceptible to RNase. Hayashi (1965) has shown that during the in vitro synthesis of RNA on a double-stranded DNA template about 50 nucleotides at the growing point of the nascent strand are hydrogen bonded to the template and thus resistant to RNase. The remainder of the nascent strand is sensitive to RNase. If during a conservative replication a similar number of nucleotides were required to bind the nascent strand to the duplex in the MS2 RI the pulse label would only become sensitive to RNase when the labeling time exceeded about 2% of the transcription time. (The transcription time is the time for a replication point to travel from one end of the template to the other). A 50% resistance to RNase could arise if the labeling time (10 seconds at 25°C) was about 4% of the transcription time. This is unlikely, however, since the pulse-label in the various RI fractions remained 50% resistant to RNase when the labeling time was varied from 5 to 30 seconds. It is concluded that significantly more than 100 nucleotides are added to the nascent strand during 10 seconds at 25°C and that another explanation of the resistant

fraction must be sought.

If replication is semi-conservative and the viral strand is displaced by the nascent strand, it can be concluded from Figure 1 that the pulse label in RI will only begin to be RNase-sensitive when the labeling time exceeds the transcription time. Hence to account for the observed 50% or more of RNase-sensitive label in RI one must postulate that the 10 second pulse at 25°C is equivalent to two or more transcription times (and then this result could be obtained only for the RI with long-tails - Table 1). However, in this unlikely event, 50% or more of the label in the RI should be present in full length strands. In Figure 3b it is apparent that, after denaturation, the large majority of the radioactivity, initially present in an RI fraction with relatively long tails, sediments more slowly than does MS2 RNA and is thus in incomplete nascent strands. Thus it is not possible to explain the RNase-sensitivity by any wholly semi-conservative replication process.

A third and trivial explanation would be that the 50 to 60% of the label in the column fractions which is sensitive to RNase is not a part of the RI but corresponds to single-stranded RNA fragments. Indeed when one of the column fractions is sedimented under fully denaturing conditions (Fig. 3b), a small peak (Fraction 19) of labeled RNA is observed which sediments much slower than the nascent strand but this corresponds to only 15% of the total radioactivity of the fraction. The remaining 45% of the label which was RNase-sensitive and the 40% of the label which was RNase-resistant must, after denaturation, have approximately the same sedimentation rate and

TABLE 1

Semi-Conservative Replication

Length of tail	0.1		0.5		0.9	
Number of Replication Cycles	1	⩾2	1	⩾2	1	⩾2
% of Label in Full Length Strands	90	91	50	66 2/3	10	53
% of Label in Nascent Strands	10	9	50	33 1/3	90	47
% of Label which is RNase-sensitive	0	9	0	33 1/3	0	47

Proportion of label expected to be in various components of replicative intermediates of various tail lengths, assuming exclusively semi-conservative replication and labeling for the indicated number of replication cycles.

Fig. 3 Centrifugation of pulse-labeled RI under partial and complete denaturing conditions.

a) A sample of fraction 29, Figure 2, was centrifuged in a 5 to 20% sucrose density gradient containing 60% DMSO, 10^{-3} M EDTA pH 7.1 for 12 hrs. at 60,000 rev./min in a Spinco SW65 at 25°C.

b) A sample of fraction 30, Figure 2, was centrifuged in a 5 to 20% sucrose density gradient containing 99% DMSO, 10^{-3} M EDTA for 12 hrs. at 63,000 rev./min in a Spinco SW65 at 25°C.

^{3}H radioactivity before (—o——o—) and after (-x----x-) RNase digestion. A single-stranded ^{32}P MS2 RNA marker (.o....o.) is included in 3a. The arrow in 3b indicates the distance travelled by an external marker under identical conditions.

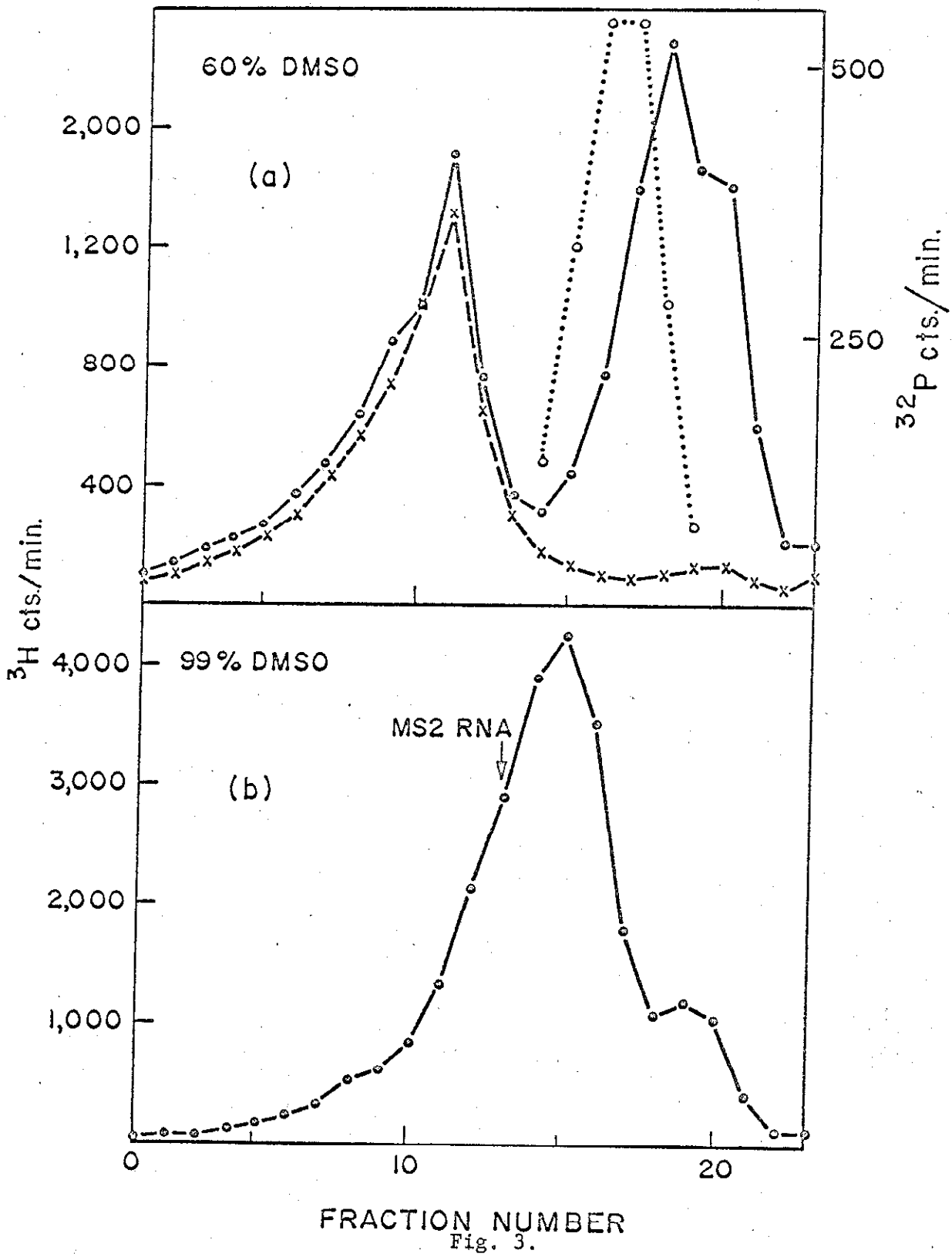
60% DMSO
(a)
2,000
1,200
800
400
500
250
^{32}P cts./min.
^{3}H cts./min.
99% DMSO
MS2 RNA
(b)
4,000
3,000
2,000
1,000
0
10
20
FRACTION NUMBER

Fig. 3.

therefore molecular weight. Also, on centrifuging the pulse-labeled RI to equilibrium in an ethidium bromide-cesium chloride density gradient at 25°C, only one peak of RNA (partially RNase-sensitive) is observed, i.e., the RNase-resistant and RNase-sensitive material are formed at the same density. (The small 15% component is not resolved from the RI in such experiments).

Thus the RNase-sensitive and the RNase-resistant radioactivity elute together from a BNC column, have the same buoyant density and, after denaturation, approximately the same molecular weight. It is evident that these results cannot be explained by either of the postulated replication mechanisms. They are understandable if, in fact, both modes of replication are used. More direct evidence indicative of the presence of both types of RI will now be presented.

(b) Partial denaturation of RI

The melting temperature of double-stranded molecules is a function of the length of the polynucleotide chain (Steiner & Beers, 1961). If the nascent strand is held to the duplex by only a small number of nucleotides (conservative replication) it should be possible by appropriate choice of denaturing conditions to remove the nascent strand from the duplex without denaturing the duplex. Alternatively, if the viral strand is displaced during replication, it should not be possible to remove the nascent strand in this way. The results presented below suggest that in fact the nascent strand can be dissociated from a portion of the RI molecules by centrifugation in

sucrose density gradients containing 60% DMSO.

It has been observed (Kelly & Sinsheimer, 1967b) that a double-stranded RNA sediments faster than its single-strand components in concentrations of dimethylsufloxide which denature single- but not double-stranded RNA. For example, when ^{3}H labeled RNase-resistant core material was sedimented with ^{32}P labeled MS2 RNA in a partially denaturing sucrose gradient (Figure 4) the core sedimented 3.5 times faster than the single strand. When fraction 29 (Figure 2) was sedimented under identical conditions two peaks of radioactivity were observed (Figure 3a); one component is completely resistant to RNase and is faster than MS2 RNA, the other is completely sensitive and slower.

The explanation proposed for this result is that the fast peak of radioactivity corresponds to a fraction of RI molecules in which the unlabeled viral strand has been partially displaced and, in consequence, the labeled nascent strand is fully resistant to RNase. The other peak of label arises from a RI fraction in which the nascent strands have been displaced. This fraction of RI molecules has dissociated, under the conditions chosen, to an unlabeled duplex and a RNase-sensitive nascent strand, which sediments more slowly than MS2 RNA. From the areas of the peaks, 46% of the radioactivity is in the former type of RI molecule.

If this interpretation is correct, the sedimentation rates of the "semi-conservative" RI molecules (the fast peak) and the dissociated

Fig. 4 Comparison of the sedimentation rates of double and single-stranded RNA under partial denaturing conditions. ^{3}H labeled double-stranded RNA was isolated from the nucleic acids of infected cells as a 14S peak after RNase digestion. It was mixed with ^{32}P MS2 RNA and centrifuged, as in Figure 3a, for 4 hrs. at 65,000 rev./min.

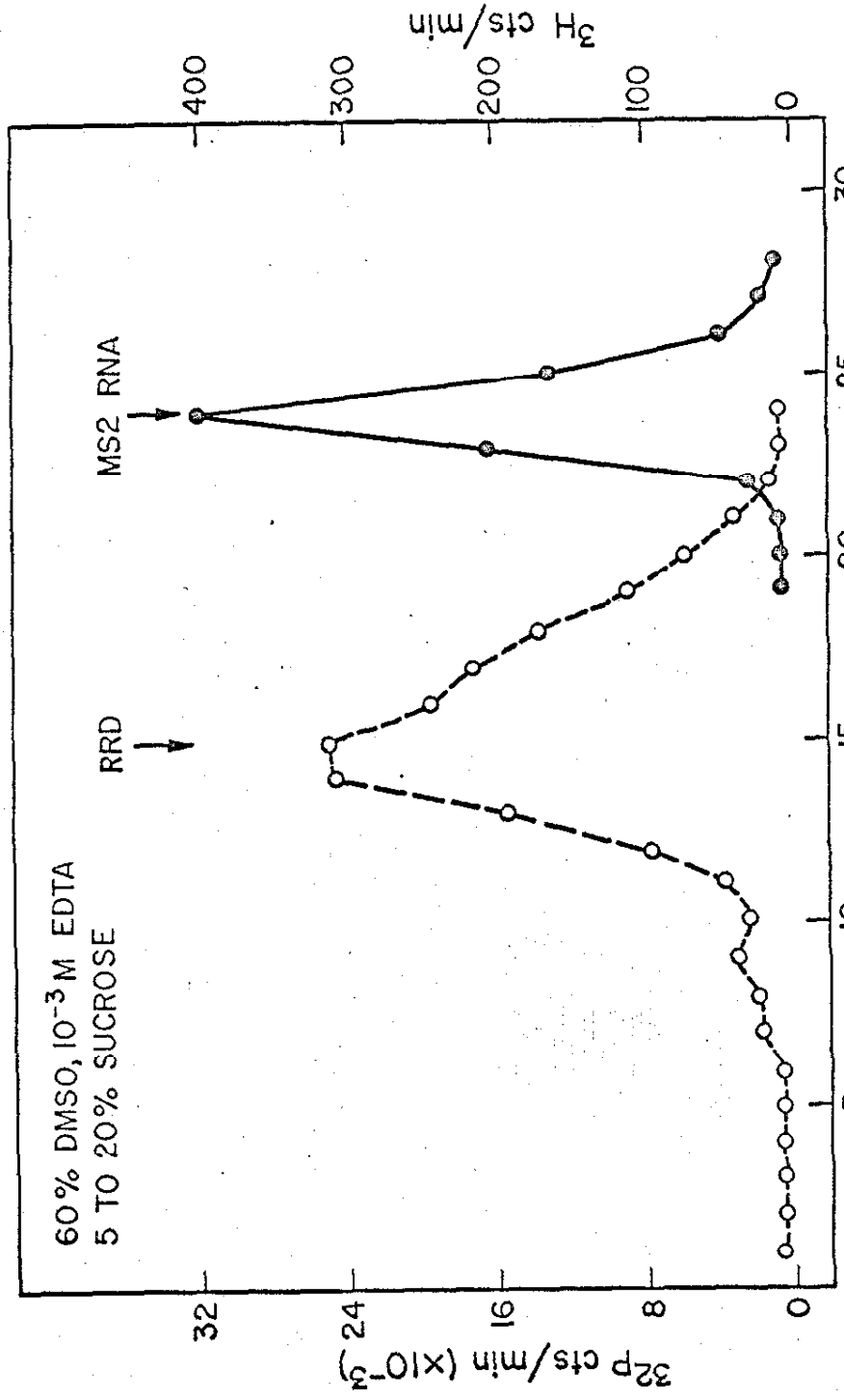
60% DMSO, 10^{-3} M EDTA
5 TO 20% SUCROSE
RRD
MS2 RNA
^{32}P cts/min (X10^{-3})
^{3}H cts/min
FRACTION
0
8
16
24
32
0
100
200
300
400
5
10
15
20
25
30

Fig. 4.

nascent strands should reflect the length of tail present in the original RI molecules. Fractions 23, 27 and 31 (Figure 2) were precipitated and centrifuged under partially denaturing conditions. Each fraction resolved into 2 peaks (Figure 5) a fast peak, presumably RI, and a slow peak, presumably nascent strands. The smaller the tail on the original "semi-conservative" RI molecule, the faster it sediments in 60% DMSO, implying that the tail exerts some drag on the duplex. Conversely, the smaller the tail on the original "conservative" RI, the smaller is the sedimentation rate of the dissociated nascent strand. For fractions 23, 27 and 31 the fraction of the radioactivity in the fast peak was 68%, 49% and 51% respectively.

These experiments strongly support the concept that either the viral strand or the nascent strand can be displaced from the RI during replication with equal probability. Both types of RI elute together from the BNC column, which explains the 50% resistance to RNase of the pulse label, but can be distinguished by centrifugation under partially denaturing conditions.

(c) The RNase sensitivity of the viral strand

If cells are infected with ^{32}P MS2 phage and the RI isolated, only the viral strand of the RI will be labeled. By experiments analogous to those described above it is possible to determine whether or not the viral strand is displaced during replications. If the viral strand is displaced the radioactivity in the viral strand should change from being fully resistant to being fully sensitive to RNase as the length of the nascent strand increases. Conversely,

Figure 5 The sedimentation properties of RI fractions with increasing tail length under partially denaturing conditions. Fractions 23 (short "tails"), 27, and 31 (long "tails") from a BNC column (Figure 2) were centrifuged in 60% DMSO using the conditions described in Figure 3a. The position of ^{32}P MS2 RNA is indicated.

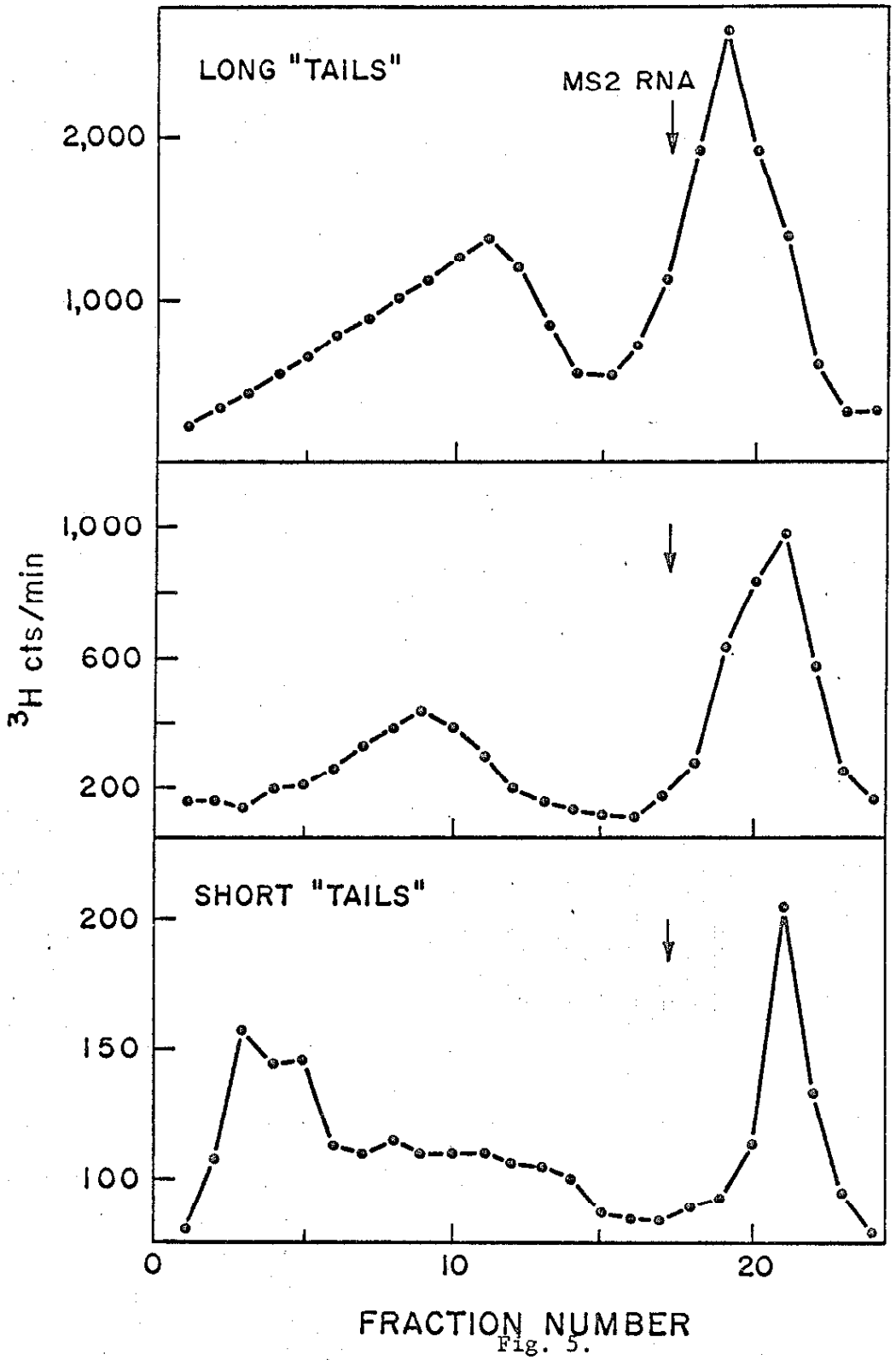
LONG "TAILS"
MS2 RNA
2,000
1,000
1,000
600
200
^{3}H cts/min
SHORT "TAILS"
200
150
100
0
10
20
FRACTION NUMBER

Fig. 5.

if the nascent strand is displaced the radioactivity in the viral strand should remain very largely or fully resistant to RNase, independent of the length of the nascent strand.

An experiment has been described (Figure 3, Kelly & Sinsheimer, 1967b) in which ^{32}P-parental-labeled RI is prepared, fractionated on a BNC column and the density of individual RI fractions measured in an ethidium bromide-cesium chloride density gradient. If the density of the RI fractions was used as a criterion for tail length, then as the tail length increased, the viral strand of the RI became more sensitive to RNase.

This experiment then implies that the viral strand of the parental RI can be displaced by the nascent strand, a result consistent with the observations of Lodish & Zinder (1966). However, in these experiments as with the pulse label, no RI fraction has been isolated in which the sensitivity of the parental strand to RNase is greater than 50%. Thus this experiment is also consistent with the hypothesis that the replication of the parental RI can be semi-conservative or conservative with approximately equal probability.

(d) Density measurements on RNase-treated $^{13}C^{15}N^{32}P$ RI

The experiments just described used RI labeled with ^{32}P in the viral strand, and conclusions were drawn concerning the structure of the RI based upon the fraction of the ^{32}P label which remains in the core after RNase digestion. As another approach, the viral strand of the RI can be labeled with both radioactive and dense isotopes (^{32}P, ^{13}C and ^{15}N). After the tail has been removed with RNase, the

density of the core will be independent of tail length if the nascent strand is displaced during replication; whereas, if the viral strand is displaced, the density of the core must decrease as more of the viral strand becomes accessible to RNase. (Since only RNase-resistant material is studied in such an experiment, the possible artifact of small pieces of contaminating single-stranded RNA is avoided.)

To prepare RI density labeled in the viral strand, cells were grown in broth to a concentration of 2×10^8 per ml. and infected at a multiplicity of one with $^{13}C^{15}N^{32}P$ MS2. After 8 minutes the RNA was extracted from the cells and sedimented in a sucrose density gradient (2 hrs. at 65,000 rev./min, 5°C) to obtain RI fractions with different lengths of tail. The nucleic acids in those fractions which sedimented at 14 to 16S, 17 to 20S and 21 to 28S were precipitated with alcohol. After resuspending each fraction and digesting with RNase to remove the tails, the resultant RNase-resistant duplexes (all 14-15S) were isolated by a second sucrose gradient sedimentation. These fractions have been designated for convenience "15S", "20S" and "25S" respectively to indicate the S of the initial RI. ^3H-labeled fully light RNA duplexes were added as density marker to these ^{32}P labeled cores from each of the fractions, and the samples were then centrifuged to equilibrium in a cesium chloride density gradient at 65°C.

The distribution of ^{32}P and ^3H radioactivity in the fractions from these gradients are shown in Figure 6. The density differences

Fig. 6 The density of core material from RI labeled in the viral strand with ^{32}P, ^{13}C and ^{15}N. Cells were infected with $^{13}C^{15}N^{32}P$ MS2 phage and after 8 mins., chilled and the nucleic acids extracted. The RI was fractionated on a sucrose density gradient into 3 fractions of approximately 15, 20 and 25S. These were digested with RNase (0.1 γ/ml. 10 min. at 37°C) and centrifuged on a sucrose gradient to obtain the 14S core material. The latter was mixed with ^{3}H-labeled, fully light double-stranded RNA and then sedimented to equilibrium in a CsCl density gradient (40 hrs. at 37,000 rev./min, 65°C). Fractions from these gradients were again digested with RNase (25 μg/ml., 30 min. at 37°C) and TCA precipitable radioactivity determined (—●———●—) ^{32}P cts/min; (-O----O-) ^{3}H cts/min.

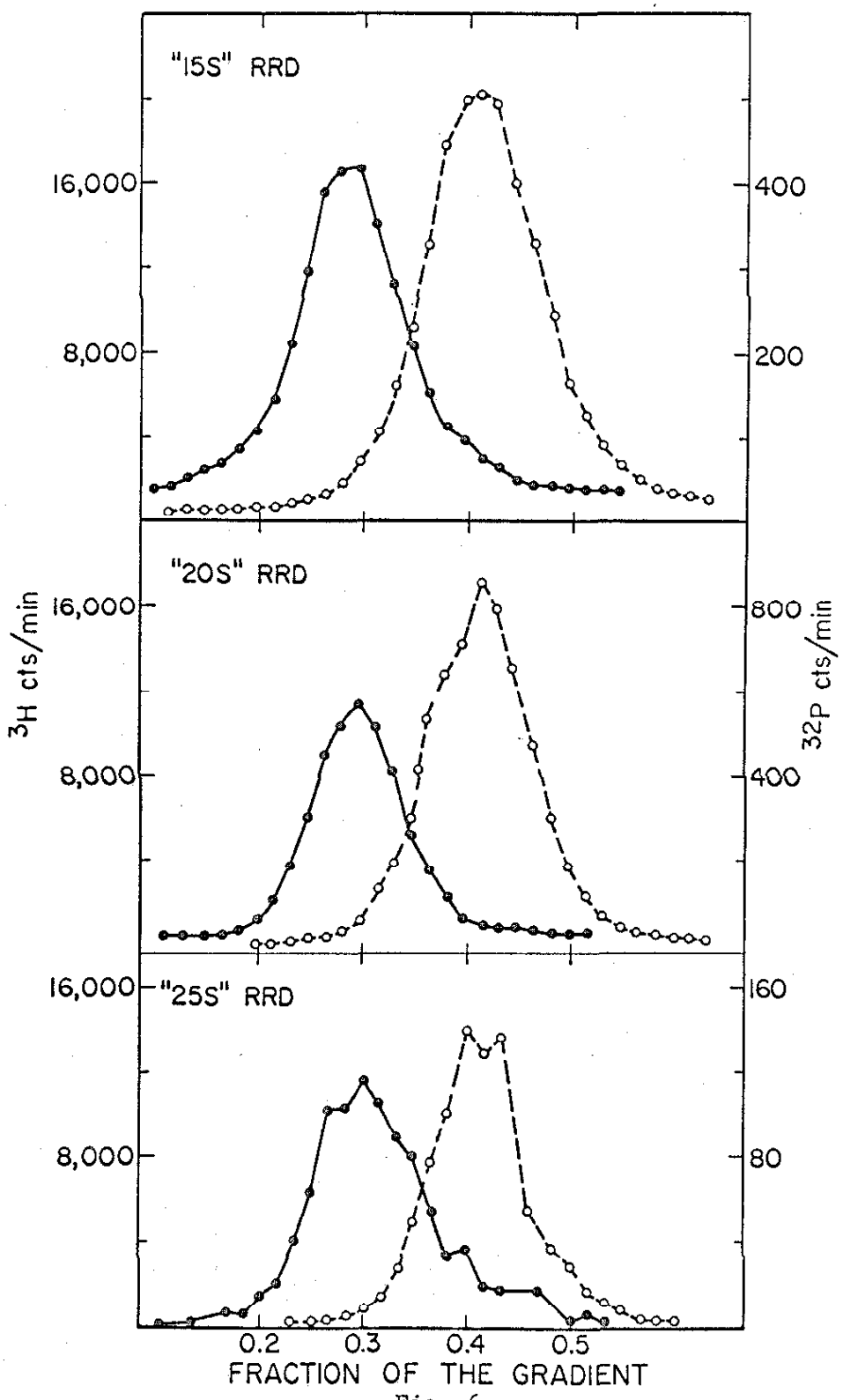
"15S" RRD
"20S" RRD
"25S" RRD
16,000
8,000
400
200
800
160
80
^{3}H cts/min
^{32}P cts/min
0.2
0.3
0.4
0.5
FRACTION OF THE GRADIENT

Fig. 6.

between the ^{32}P labeled material and the marker were found to be 0.016 ± 0.001, 0.015 ± 0.001 and 0.014 ± 0.001 g-cm^{-3} for the "15S", "20S" and "25S" fractions, respectively. This may be compared to the density difference of 0.031 g-cm^{-3} observed between fully heavy and fully light duplexes (Kelly & Sinsheimer, 1967b). There is a skewing of the ^{32}P labeled peaks toward the lighter density especially in the "25S" fraction.

To determine if this result simply reflected a poor fractionation of the RI by sucrose gradient sedimentation, the same nucleic acid preparation was chromatographed on a BNC column and RI molecules of different tail lengths isolated. The cores derived from these RI molecules gave, on equilibrium density gradient sedimentation, a result identical to that in Figure 6, i.e., the radioactivity peak of the cores of all RI fractions is at the same density, which corresponds to that of a duplex in which one strand is "heavy" the other "light". Since we have shown that BNC columns fractionate RI according to tail length (Kelly & Sinsheimer, 1967b) we can conclude that the density of the major labeled component of the core is independent of the length of the tail.[1]

1. A possible objection to this conclusion derives from the observation that scissions occur in single-stranded RNA centrifuged in a cesium chloride density gradient for 48 hrs at 65°C (Kelly & Sinsheimer, 1967a). If during centrifugation, a break occurred in a hybrid density duplex in the vicinity of the replication point, 2 double-stranded fragments would be produced, one fully light (unlabeled) and one "half-heavy" (^{32}P labeled). Thus a duplex of constant density would be observed even if replication was semi-conservative. However, since it has been determined that 48 hrs. at 65°C in a cesium chloride density gradient does not appreciably alter the sedimentation coefficient of core material, it is reasonable to conclude that such cleavage does not, in fact, occur.

The results of this experiment are consistent with the observations described earlier (Kelly & Sinsheimer, 1967a) on unfractionated, RNase-treated RI in which no density difference was observed between duplexes in which the heavy isotopes were in either the viral or the non-viral part of the RI. However, the present experiment could not be explained by the possible presence of a large pool of completed duplexes. Therefore it is again concluded that a fraction of parental RI molecules are involved in conservative replication. It should be emphasized, however, that these experiments are weighted in favor of finding "conservative" RI since only the dense portion of the core is labeled with ^{32}P. Consequently, a duplex of lesser density than a half-heavy duplex will also have less radioactivity. If 50% of the RI molecules containing parental RNA were replicating semi-conservatively, only 33% of the total ^{32}P-labeled core material would be in duplexes of lesser density and would only be detectable as a skewing of the ^{32}P peak toward the fully light density. While there is evidence for such skewing the accuracy of the data is not sufficient to estimate to what fraction of "semi-conservative" RI molecules this asymmetry might correspond.

4. DISCUSSION

Before discussion of the interpretation of these experiments the assumptions on which they are based must be clarified. It has been assumed that the complex RNA structure under examination is, in fact, a replicative intermediate. This is justified by the experiments which indicate that the RI is a precursor to single stranded RNA in vivo (Fenwick, Erikson & Franklin, 1964) and in vitro (Weissmann & Feix, 1966; Mills, Pace & Spiegelman, 1966); and the duplex is not conserved during replication (Kelly & Sinsheimer, 1967b); and that the RI molecule contains an infective viral strand (Ammann, Delius & Hofschneider, 1964). Implicit also in these experiments is the assumption of a model for the RI consisting of a double-stranded core to which only one single-stranded tail is attached (Kelly & Sinsheimer, 1967a). Although this model is supported by the observation that, on denaturing the RI, the nascent strand sediments as a single peak (Figure 3b), evidence has been presented that a small number of RI molecules have indeed more than one tail (Granboulan & Franklin, 1966). The presence of a small number of molecules with more than one tail would complicate the discussion of these experiments but would, however, leave the conclusions unaltered.

Essential also to the experiments described here is the use of RNase digestion as a tool to distinguish single-stranded RNA from double-stranded RNA. If, in fact, the specificity of RNase action

is more subtle than this, interpretation of these data is impossible. Finally it has been supposed that it is a valid experimental approach to the study of the mechanism of replication to investigate the structure of the replicative intermediate. While this is not an unreasonable assumption it ignores changes in structure which might take place upon purification.

When short pulses of radioactivity were used to label the nascent strand, and the RI was fractionated on a BNC column it was apparent that the nascent strand had been displaced from the duplex (and thus became RNase-sensitive), but only in a fraction of the molecules. The converse experiment, in which the viral strand was labeled, gave the same result which leads to the conclusion that either the viral or the nascent strand can be displaced during replication. When the viral strand was labeled with dense and radioactive isotopes, the density of the core material after the tail had been removed was in qualitative agreement with a model in which the nascent strand can be displaced from the duplex in a large fraction of the RI molecules. Because of the nature of the experiment, the presence of "conservative" RI molecules would effectively conceal the presence of "semi-conservative" RI molecules if both were present in approximately equal amounts. The results of these experiments then fit a model in which either the viral or the nascent strand is displaced with approximately equal frequency.

If pulse-labeled RI is sedimented under partially denaturing conditions the radioactivity peak with the sedimentation rate of RI is fully resistant to RNase and the RNase-sensitive portion of the

label now sediments as a single-strand RNA fragment. The best interpretation of this observation is that two classes of RI are fractionated by this procedure; the "semi-conservative" RI molecules, with a displaced viral strand, are not denatured; the "conservative" RI molecules, with a displaced nascent strand, partially denatured, releasing the nascent strand from the duplex. If only a small number of hydrogen bonds hold a nascent strand to the duplex in a "conservative" RI (Figure 1), the basis for this fractionation is easily explained.

From the areas of the peaks in Figures 3a and 4 it is obvious that both classes of RI are present in approximately equal amounts, except in fractions with short tails. In these the fast (semi-conservative) component contains the majority of the pulse label. When such a fraction was sedimented under fully denaturing conditions a peak corresponding to viral RNA is observed as well as a slower peak corresponding to the nascent strand. The preponderance of the fast component in these fractions therefore probably reflects the presence of duplexes which have completed a transcription and initiated another during the labeling period.[2]

2. It is interesting to note that a purely conservative mechanism requires that the radioactivity is released from the duplex when transcription is complete and so labeled complete duplexes would not appear (except, of course, through replication of the duplex). However, using a uniquely semi-conservative mode of replication it would be impossible to explain the appearance of labeled single-strands of MS2 RNA (Figure 2) after labeling times shorter than the transcription time.

This analysis of the structure of the RI clearly leads to the conclusion that the duplex is transcribed both conservatively and semi-conservatively with approximately equal frequency. Since no duplexes are conserved during replication (Lodish & Zinder, 1966; Kelly & Sinsheimer, 1967a) it cannot be argued that one class of molecule always replicates conservatively, and the other semi-conservatively; all molecules must use both mechanisms. This could occur either if there is some mechanism for alternating between the two modes of replication, or simply if the two modes are used randomly with equal probability. It might be possible to distinguish between two possibilities on kinetic evidence but the data of Lodish & Zinder (1966) and Kelly & Sinsheimer (1967a) have insufficient accuracy.

Finally there is some evidence in vitro that removing the MS2 synthetase enzyme from the RI makes the nascent strand less vulnerable to RNase digestion (Borst & Weissmann, 1965). It is therefore possible that the structure of the purified RI molecules is only indirectly related to the structure in vivo. This question can only be settled through examination of the structure of the replicative intermediate with the enzyme still attached.

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DISCUSSION

In the previous section we have shown that in MS2 infected cells there appear to be two classes of RI in approximately equal amounts: in one class, the tail consists of the nascent strand; in the other, it is the displaced portion of the viral strand. This result suggests that replication can occur either conservatively or semi-conservatively and that the two modes are equally frequent.

In the closely analogous transcription process in which DNA-dependent RNA polymerase catalyses the incorporation of ribonucleotides, the mechanism of transcription has been extensively analysed. When the RNA polymerase is primed by its natural template, double-stranded DNA, transcription is conservative. Using homopolymer pairs as primers, Chamberlin (1965) has obtained evidence that the mode of transcription is not rigorously determined by the enzyme but can be influenced by the thermodynamic stabilities of the two alternative duplexes. Thus, when the original duplex has a stability different from the duplex formed if the nascent strand displaces one of the original strands, then that mode of transcription is employed which will produce the more stable duplex.

Suppose, however, that replacing one of the original strands with a newly synthesized strand produces a duplex of identical stability to the original duplex (this is presumably the situation in RNA phage replication). A priori, it would be predicted that

semi-conservative transcription is preferred, since, in conservative transcription, the nascent strand must relinquish its hydrogen bonds to the complementary strand (except for a small number at the growing point) in order to be replaced by the viral strand. Since such an interchange would presumably involve transitional configurations of higher energy, it would be discriminated against in comparison to semi-conservative transcription.

In practice, the situation does not seem so simple. When the RNA polymerase uses a synthetic DNA-RNA hybrid as template for the production of a nascent RNA strand, both alternative duplexes have the same energy. In line with our prediction, it was found that semi-conservative transcription does occur (Sinsheimer & Lawrence, 1964; Chamberlin & Berg, 1964). Unfortunately, the data are insufficient to decide if replication is only partly semi-conservative, and partly conservative. In the phage RNA transcription system described in this thesis once again the two alternative duplexes have the same energy, but the evidence suggested that both modes of transcription occurred. Why is transcription not exclusively semi-conservative? The answer is not known but some plausible conjectures can be made.

1. Mechanisms can be devised in which a conservative transcription must be followed by a semi-conservative one, and vice versa. Such an alternation of modes could produce equal proportions of the two types of RI.

2. If triple-stranded structures are formed, the viral and the nascent strand become, from the energetic standpoint, indistinguishable. If such a structure gave rise to the 2 types of RI observed, they would be expected in equal amounts. As an example of how this could happen, one could postulate that a ribosome attaches at random to either the nascent or the viral strand of the triple-stranded structure. This attachment renders the triple-stranded structure unstable and it dissociates into a duplex, and a tail to which the ribosome is attached.

Evidence has been given (Bishop, et al., 1965) for the presence of triple-stranded RNA in polio-infected He La cells. This structure is destroyed by treatment with phenol.

3. The final possible explanation of the 2 types of RI is that they might only arise when purified and bear little resemblance to the structure originally present inside the infected cell. Suppose that, in vivo, both the viral and the nascent strands are displaced, that is, do not form hydrogen bonds with the complementary strand. When such a structure is removed from its natural environment it will adopt an energetically favorable hydrogen-bonded structure. Since the stability of both types of RI are the same, they should occur in equal quantities.

This type of explanation seems unlikely because the non-hydrogen-bonded RI is so very unstable, but data have been reported supporting such a model (Borst & Weissmann, 1965).

These three possible explanations can all be investigated experimentally. Especially relevant for a further understanding of RNA replication will be a study of the mechanism of phage RNA transcription *in vitro*, and physical characterization of the polymerase-template complex isolated from infected cells as gently as possible, and certainly without the use of detergents or phenol.

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