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Symposium on Replication of Viral Nucleic Acids

III. Replication of Mengovirus Ribonucleic Acid[1]

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INTRODUCTION

Progress in understanding the biochemistry of the replication of ribonucleic acid (RNA) viruses was initially hampered by the difficulties encountered in distinguishing between reactions of the host cell and those related specifically to the replicative process. Recent discoveries which demonstrated that certain antibiotics, notably actinomycin, inhibit cellular synthesis of RNA without interfering appreciably with the viral directed synthesis of RNA have stimulated considerable research in this area. Certain members of the picornaviruses such as poliovirus, Mengo encephalomyelitis virus (mengovirus), encephalomyocarditis (EMC) virus, and mouse encephalomyelitis (Me) virus are the most extensively investigated among the animal viruses. These polyhedral viruses have a diameter of about 30 mμ and contain a single molecule of RNA of approximately 10^6 to 2×10^6 daltons (38, 74). The picornaviruses share many features in common with the RNA-containing bacteriophages (26, 34, 40, 55, 64, 77) and with the polyhedral viruses of plants (17, 57). The use of specific inhibitors has led to the conclusion (76) that the replication of many RNA viruses does not require the synthesis of deoxyribonucleic acid (DNA), nor is it mediated by DNA of the host cell (71, 72). In spite of these recent advances, information is still fragmentary on the mechanism of replication of viral RNA, on how the information coded in the viral RNA is translated into the structure of specific proteins, and on how various replicative processes operate within the framework of the regulatory mechanisms of the host cell.

Novikoff hepatoma cells infected with mengovirus have proved to be a useful system in which to investigate some of these problems. The cells are readily propagated in suspension in a relatively simple medium with a generation time of about 12 hr. Practically all of the cells are infected with mengovirus at a multiplicity of 25 ID_{50}, and the replicative cycle requires only 6.5 to 7 hr.

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The purpose of this report is to summarize the results obtained in several studies with this system, with emphasis on those relating to the replication of viral RNA. The data are discussed in relation to those of other investigations where pertinent, but no attempt has been made to review the field as a whole.

HOST-CELL SYNTHESIS OF RNA AND PROTEIN AFTER INFECTION

In one of the earlier studies of the biochemical events in the replication of RNA viruses in mammalian cells, Salzman et al. (73) found that the amount of protein, RNA, and DNA of HeLa cells decreased markedly after infection with poliovirus, and they concluded that viral infection inhibits the synthesis of macromolecules by the host cell. Subsequent studies in which the synthesis of macromolecules was determined by measuring the rate of incorporation of labeled precursors by infected cells confirmed this conclusion. The results of these studies showed that the rate of synthesis of RNA and protein by many strains of mammalian cells is depressed soon after infection with polio- (45, 85), mengo- (8, 10, 31, 66), Me (39, 75), or EMC virus (58). Inhibition of DNA synthesis, on the other hand, occurs only toward the end of the replicative cycle and, therefore, appears to represent a secondary host response. The inhibition of cellular RNA synthesis is reflected by a decline in the activity of the DNA-dependent RNA transcriptase (RNA nucleotidyltransferase) of the nucleus (8, 42). Analogous investigations on the effect of viral infection on the ability of cell-free systems to synthesize protein have yielded variable results (3, 45, 53).

The question of whether there is any direct relationship between the inhibition of RNA and protein synthesis has not been satisfactorily answered. The overall results of several studies indicate that the inhibition of protein synthesis is probably not due entirely to a depletion of messenger RNA as a result of inhibition of RNA synthesis. Infection of L cells with mengovirus inhibits protein and RNA synthesis simultaneously, but the rate of protein synthesis seems to be affected sooner and to a greater extent by infection than by treatment with actinomycin D (10, 31). It has been reported that the effect of actinomycin D on protein synthesis is accentuated in HeLa cells infected with poliovirus (45) and in L cells infected with Me virus (39). Furthermore, the rate of protein synthesis by poliovirus-infected HeLa cells declines shortly after infection with poliovirus, whereas RNA synthesis is unaffected for at least 2 hr (85).

Although inhibition of cellular RNA and protein synthesis is generally observed during the replication of many picornaviruses, it does not appear to be an essential feature of this process. For example, the rate of RNA and protein synthesis by Novikoff hepatoma cells (strain 67) infected with mengovirus is unaffected until the terminal stages of the replicative cycle (66). The DNA-dependent RNA transcriptase activity of nuclei isolated from infected cells is similarly unaffected (66). Synthesis of RNA and protein by another strain of Novikoff cells (strain 63), on the other hand, is depressed soon after infection with mengovirus (66). The two strains of Novikoff cells, nevertheless, are similar in terms of the period required for viral replication, time of appearance of viral-induced RNA polymerase, and yield of virus (66).

The mechanism of the viral-induced inhibition of RNA and protein synthesis is not understood. It has been reported (44) that the infection of HeLa cells with a multiplicity of 10,000 plaque-forming units (PFU) per cell, but not with 10 PFU/cell, inhibits cellular RNA and protein synthesis and subsequent cytopathic effects, under conditions where viral replication is inhibited by guanidine. Additional data indicate that the viral RNA is responsible for this phenomenon rather than the protein, because poliovirus grown in the presence of proflavin inhibits cellular synthesis of RNA and protein in high multiplicities in the dark but not after exposure to light. Balandin and Franklin (5), on the other hand, reported that cell-free extracts from L cells infected with mengovirus inhibit the DNA-dependent RNA transcriptase from uninfected cells and that this inhibitor is destroyed by treatment of the extracts with trypsin. Similar experiments with HeLa cells infected with poliovirus, on the other hand, failed to reveal a cytoplasmic inhibitor of RNA synthesis (42). It has been suggested that the information for the synthesis of inhibitory substances in infected cells resides in viral RNA (5). The finding that mengovirus causes inhibition of synthesis of RNA and protein by one strain of Novikoff cells but not by another (66) suggests alternatively that the host cell may contain the information for production of inhibitor(s) and that this represents a variable response to infection.

VIRAL-DIRECTED SYNTHESIS OF RNA BY INFECTED CELLS

In earlier studies, a number of investigators were unable to demonstrate the synthesis of viral RNA in infected cells by conventional biochemical techniques. For example, the RNA extracted from infected cells was found to have approxi-

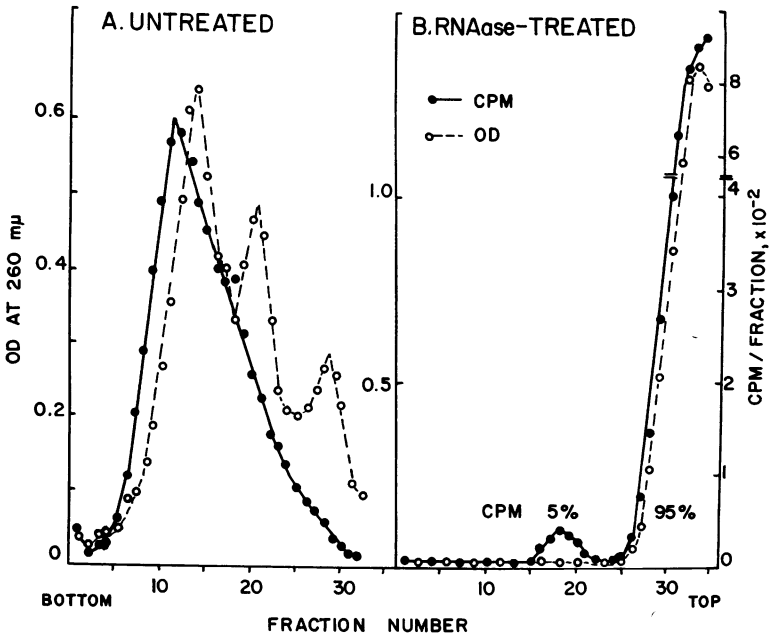

FIG. 1. *Sedimentation analysis of RNA produced by infected cells. At 2 hr after infection, 1 μg/ml of actinomycin D and 0.5 μmole/ml of FUdr were added to a suspension containing 10^6 cells per milliliter, and 1 hr later 0.025 μmole/ml of uridine-H^3 (10 μc/μmole) was added. At 7 hr the cells were harvested, and their RNA was extracted with phenol-SDS. Conditions of centrifugation: 0.15 to 0.6 M linear sucrose gradient in 10 mM tris(hydroxymethyl)-aminomethane (Tris), 10 mM ethylenediaminetetraacetic acid (EDTA), pH 7.3; SW 39 rotor; 35,000 rev/min for 5.5 hr. A = untreated RNA; B = RNA + 0.4 μg/ml of ribonuclease for 5 min at 22 C just prior to centrifugation.*

mately the same base composition as that isolated from uninfected cells (1). Viral-induced synthesis of RNA was subsequently demonstrated, however, by the use of improved techniques in a number of RNA virus-cell systems. Holland (41) pulse-labeled HeLa cells infected with poliovirus, extracted the RNA, and found that it resembled viral RNA in base composition. Later, Reich et al. (72) demonstrated that the replication of picornaviruses is unaffected by actinomycin D in concentrations which inhibit the DNA-dependent synthesis of RNA by the cell more than 90%. These data indicated that the synthesis of viral RNA is probably not mediated by a DNA intermediate. This selective inhibition of cellular RNA synthesis by actinomycin thus made it possible to study the time course of the viral-directed synthesis of RNA and to examine the products of the reaction without interference by synthetic reactions of the host. Of particular interest was the question of whether species of RNA other than viral RNA are produced which might play an important role as intermediates in the replication of viral RNA or as messengers for the synthesis of viral-specific proteins.

Nature of RNA Synthesized In Vivo

The RNA synthesized by HeLa cells infected with poliovirus in the presence of actinomycin was found to have a base composition nearly identical to that extracted from the virus (85). Sucrose density gradient analyses showed that much of the RNA synthesized by cells infected with various picornaviruses is similar to that extracted from the corresponding virus in its sedimentation characteristics (27, 45–48, 60, 85). A typical sucrose density gradient profile of RNA synthesized in the presence of actinomycin by Novikoff cells infected with mengovirus is illustrated in Fig. 1A. RNA was isolated from labeled cells by extraction with hot phenol-sodium dodecylsulfate (SDS) and centrifuged through the gradient. The absorbancy profile indicates the distribution of the 30*S* and 18*S* ribosomal RNA preparations and of transfer RNA on the gradient. The radioactivity profile illustrates that a large proportion of the newly synthesized RNA had a sedimentation coefficient of about 37*S*, which is similar to that of RNA isolated from purified mengovirus. The significance of the heterogeneity illustrated by the profile of radioactivity is not understood, and it may merely

reflect some degradation of RNA resulting from the isolation procedure.

Montagnier and Sanders (61) were the first to demonstrate that the RNA produced by Krebs ascites cells infected with EMC virus also includes a species of RNA (20S) which is highly resistant to degradation by ribonuclease. Similar ribonuclease-resistant RNA preparations have been isolated from mammalian cells infected with polio- (11, 70), Me (37), Semliki forest (33), and foot-and-mouth disease virus (18), and *Escherichia coli* (2, 28, 50–52, 62, 84) and plant cells (19, 56) infected with RNA viruses. A typical sucrose density gradient analysis for ribonuclease-resistant RNA produced by Novikoff cells infected with mengovirus is illustrated in Fig. 1B. RNA was isolated from labeled cells, incubated with ribonuclease, and centrifuged through the gradient. A comparison of the profiles for radioactivity in Fig. 1A and B illustrates that most of the RNA was hydrolyzed by treatment with ribonuclease and remained on top of the gradient. About 5% of the labeled RNA, however, was resistant to treatment with ribonuclease and sedimented as a homogeneous peak of about 20S. The physical and chemical characteristics of this RNA (69) are similar to those of ribonuclease-resistant RNA preparations isolated from a variety of cells infected with RNA viruses. In general, this type of ribonuclease-resistant RNA exhibits the following properties: (i) a sharp transition to ribonuclease sensitivity at temperatures between 80 and 100 C depending on the ionic strength of the suspension medium (2, 19, 37, 61, 69, 84); (ii) resistance to the combined action of ribonuclease and deoxyribonuclease (69); and (iii) absence of DNA from purified preparations (61). It has been reported that the ribonuclease-resistant RNA is infectious (61, 70), and that infectivity is not destroyed by formaldehyde (61). Further, the results of X-ray diffraction studies are consistent with a highly ordered structure (54). All of the foregoing data are consistent with the generally accepted view that the ribonuclease-resistant RNA produced by cells infected with certain RNA viruses, or by cell-free preparations obtained therefrom (6, 69, 80), represents a stable hybrid consisting of viral RNA paired with a strand of RNA complementary in base composition in a manner analogous to the Watson-Crick model for double-stranded DNA. Data on the base composition of ribonuclease-resistant RNA isolated from several sources are in agreement with the double-stranded structure (50, 56, 62, 82), although in one instance the results appeared to be more compatible with a triplex composed of two strands of viral RNA and one complementary strand (Bishop, Summers, and Levintow, Federation Proc. **24**:287, 1965).

Time Course of Synthesis of Viral-Directed RNA and Its Incorporation into Mature Virus

The following experiments were undertaken to determine the temporal relationships between the viral-directed synthesis of RNA and its incorporation into mature virus. Infected cells were treated with actinomycin and 5-fluorodeoxyuridine (FUdr) and subsequently labeled with uridine. Samples of the suspension were analyzed at intervals for: (i) total acid-insoluble radioactivity and (ii) acid-insoluble radioactivity resistant to treatment with ribonuclease in the presence of sodium deoxycholate (DOC). Treatment of infected cells with ribonuclease in the presence of DOC results in the degradation of all RNA except that contained in mature virus and in double-stranded RNA (66). The results of this type of

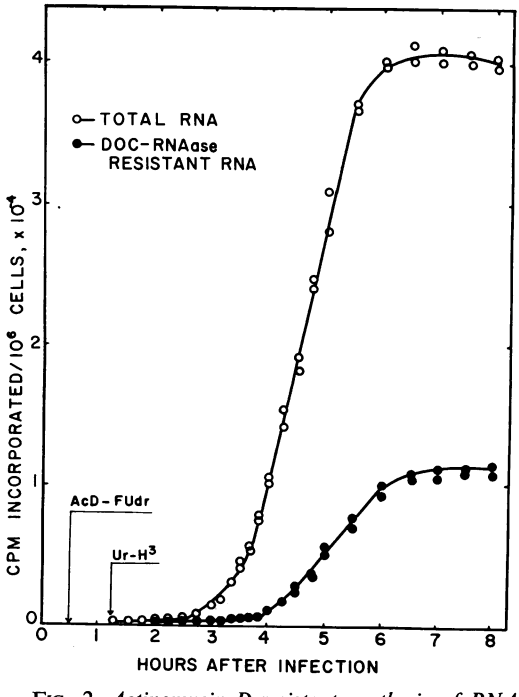

FIG. 2. *Actinomycin D-resistant synthesis of RNA as function of time after infection. One μg/ml of actinomycin D, 0.5 μmole/ml of FUdr, and 0.006 μmole/ml of uridine-H³ (350 μc/μmole) were added at indicated times to a suspension containing 1.5 × 10⁶ cells per milliliter. Cells from 1-ml samples were assayed in duplicate for total acid-insoluble radioactivity and for acid-insoluble label resistant to treatment with 1% DOC and 10 μg/ml of ribonuclease at 25 C for 30 min.*

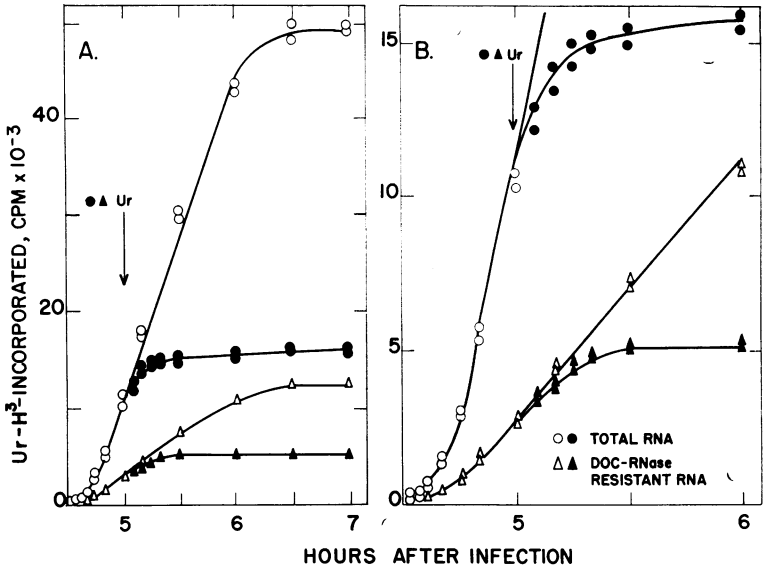

FIG. 3. *Pulse-chase analysis of RNA synthesized by infected cells. At 2 hr after infection, 2 μg/ml of actinomycin D and 0.5 μmole/ml of FUdr were added to a suspension containing 1.5 × 10⁶ cells per milliliter. At 4 hr, 0.01 μmole/ml of uridine-H³ (100 μc/μmole) was added, and 15 min later a portion of the suspension was supplemented with 3 μmoles/ml of uridine. The incorporation of label into total and DOC-ribonuclease-resistant, acid-insoluble material was determined on duplicate 1-ml samples of cells as described in Fig. 2.*

experiment (Fig. 2) indicate that most of the viral-directed synthesis of RNA occurred between 3 and 6 hr after infection. Data from other experiments (66) indicated that double-stranded RNA was produced at a relatively constant rate throughout the period of synthesis of RNA, and that it represented approximately 5% of the total. The amount of isotope in mature virus could thus be estimated from the data in Fig. 2 by subtracting 5% of the total counts from the values indicated for RNA resistant to ribonuclease in the presence of DOC. When this was done, it was evident that virus was produced only during the period between 4 and 6 hr. The temporal relationships of the production of virus measured biochemically agreed with the results obtained from infectivity titrations and from assays for hemagglutination (66). The data also illustrate another interesting feature of the replication of mengovirus in that only 18 to 20% of the total RNA synthesized as a result of viral infection was incorporated into mature virus. Data from other systems indicate that approximately 20% of the RNA synthesized in Krebs ascites cells infected with EMC virus was recovered in virus (23), whereas a value of 45% was reported for an Me virus-L-cell system (39). A total of 65% of the RNA synthesized by HeLa cells infected with poliovirus was not degraded by treatment of the cells with ribonuclease at the end of the replicative cycle (85). RNA which is not hydrolyzed by ribonuclease cannot be equated directly with that in mature virus since double-stranded RNA is also resistant to such treatment. It is also of interest in this connection that 15 to 20% of RNA synthesized by Novikoff cells infected with mengovirus is not hydrolyzed by relatively high concentrations of ribonuclease. This RNA is hydrolyzed, however, in the presence of DOC (66).

Studies of the RNA Not Incorporated into Virus

Irrespective of the variations among different systems, it appears that in general the incorporation of RNA into virus is not an efficient process or that a relatively large proportion of it is synthesized for another purpose. Pulse-chase experiments have been used to assess the extent to which the accumulated RNA represents a precursor pool for virus maturation. The results of a typical experiment are shown in Fig. 3. Cells were labeled with uridine for 30 min and then a 300-fold excess of unlabeled uridine was added to a portion of the suspension, which resulted in a sharp decline in the rate of incorporation of isotope into RNA. After 25 min, the rate of incorporation was about 8% of that of the control and remained at this level for the duration of the experiment. During the first 30 min of the chase, 35% of the isotope incorporated into acid-insoluble material was resistant to DOC-ribonuclease,

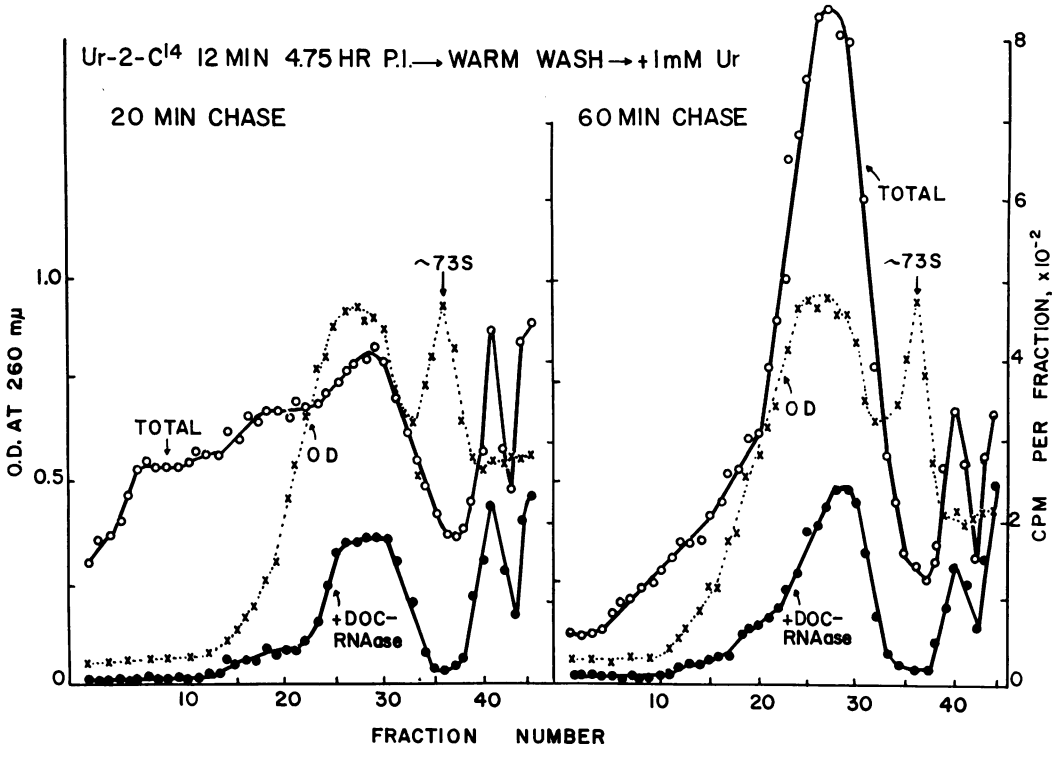

FIG. 4. *Sedimentation analysis of postnuclear fractions in pulse-chase experiment. A 2 μg/ml amount of actinomycin D and 0.5 μmole/ml of FUdr were added to a suspension containing 1.5 × 10⁶ cells per milliliter at 3 hr after infection, and 0.02 μmole/ml of uridine-2-C¹⁴ (24 μc/μmole) at 4.75 hr. After 12 min the cells were washed and resuspended in the original volume of fresh medium containing 1 μmole/ml of uridine. Postnuclear fractions were prepared after 20 and 60 min of incubation at 37 C, treated with 1% DOC, and subjected to sedimentation analysis. Conditions of centrifugation: 0.5 to 1.2 M sucrose in RSB, 25,000 rev/min, SW25 rotor, 90 min. Fractions from gradient were assayed for adsorbancy at 260 mμ and for total and DOC-ribonuclease-resistant RNA (see Fig. 2).*

whereas only 24% of the label was found in this fraction in the control. Similar results were obtained when cells were pulse-labeled, for shorter periods of time. The results of other experiments (see Fig. 14 for example) showed that the relative proportion of label in double-stranded RNA remained constant throughout the course of pulse-chase experiments. The overall data, therefore, indicate that the disproportionate increase in RNA resistant to DOC-ribonuclease during the early part of the chase reflected incorporation of label into virus and were thus consistent with the existence of a pool of RNA for viral maturation which constituted less than 10% of the total RNA synthesized. This pool appears to be somewhat larger in *E. coli* infected with an RNA phage, since it has been reported that 75% of the RNA formed during a short pulse is incorporated into virus during a subsequent chase (63).

Pulse-chase experiments were also performed in conjunction with density gradient analysis of cellular fractions as a means of learning more about the RNA which is not incorporated into virus. Cells were pulse-labeled with uridine, washed and suspended in a medium containing unlabeled uridine, and samples were incubated for 0, 20, and 60 min. Cells from each sample were suspended in a hypotonic medium (reticulocyte standard buffer, RSB, 78), disrupted with a Dounce homogenizer, and the nuclei were removed from the homogenate by centrifugation. Each postnuclear fraction (PNF) was treated with DOC and centrifuged through a sucrose gradient. Fractions from the gradient were analyzed for absorbancy at 260 mμ and for total RNA and RNA resistant to ribonuclease in the presence of DOC. The data obtained with PNF prepared from cells after incubation for 20 and 60 min in the unlabeled medium are presented in Fig. 4. The absorbancy profile indicates the relative distribution of the 73*S* ribosomes and the

more rapidly sedimenting polysomes on the gradient. The profile of tritium (20-min pulse) shows that a large proportion of the RNA was associated with particles which sedimented faster than the bulk of the polysomes. These particles appear to play a central role in the viral-directed synthesis of RNA, since they contain most of the viral-induced RNA polymerase activity (see Fig. 5 and 9). The data obtained after 60 min of chase indicate that most of the label was located in the polysome fraction. The distribution of label at the beginning of the chase was essentially the same as that shown after 20 min of chase. This finding would be anticipated from the data presented in Fig. 3, which indicate that considerable incorporation of uridine occurred during the first 25 min of the chase. That is, about 25 min of chase were required to dilute effectively the intracellular pool of labeled uridine triphosphate (UTP). The radioactivity profiles of the RNA resistant to ribonuclease in the presence of DOC in both parts of Fig. 4 demonstrate two distinct peaks. The results of other experiments (66) have shown that the peak at the top of the gradient is predominantly double-stranded RNA, whereas most of the virus is contained in fractions 15 to 35. These data also confirm the results of the experiment presented in Fig. 3, which indicate that much of the RNA which was synthesized did not represent a precursor pool for viral maturation. The accumulation of this RNA in the polysomal fraction suggests that it may function as messenger RNA for the synthesis of viral proteins. It is not known whether this RNA is identical to that which is incorporated into mature virus. If this is assumed, however, the data in Fig. 4 would be consistent with the suggestion that once newly synthesized RNA is bound to polysomes (ribosomes) it becomes inaccessible to the processes involved in viral maturation.

VIRAL-INDUCED RNA POLYMERASE

The finding that RNA synthesis in cells infected with picornaviruses is largely resistant to inhibition by actinomycin suggested that the synthesis of viral RNA is dependent on an RNA rather than a DNA template, and that these viruses induce the synthesis of a new RNA-dependent RNA polymerase in the host cell (31). Baltimore and Franklin (6, 7, 9) were first to demonstrate such enzyme activity in cytoplasmic particles isolated from L cells infected with mengovirus. Similar RNA polymerase activity has also been observed in Novikoff hepatoma cells infected with mengovirus (67), in HeLa cells and Krebs ascites cells infected with poliovirus (12, 29, 45) and EMC virus (24), respectively, and in *E. coli* infected with RNA phage (4, 35, 36, 81, 83). Comparable RNA polymerase activity has not been observed in cytoplasmic fractions from uninfected cells. The formation of RNA polymerase in infected cells appears to require the de novo synthesis of protein, since it is not produced in cells treated with puromycin (9, 25, 67). It is generally assumed that viral RNA per se or some RNA formed as the result of viral infection serves as messenger for the synthesis of polymerase. The possibility that this enzyme activity reflects the activation of a host-cell enzyme, possibly by acquiring an RNA template, has not been rigorously excluded. Studies in this laboratory have been directed toward establishing the significance of the viral-induced RNA polymerase in the replication of mengovirus in Novikoff hepatoma cells, and the results of some of these experiments are summarized in the following sections.

General Characteristics of the Viral-Induced RNA Polymerase

Cell-free preparations were prepared as follows. The cells were suspended in a hypotonic medium (RSB), disrupted with a Dounce homogenizer, and the nuclei were removed from the homogenate by centrifugation. All of the RNA polymerase activity of the PNF was sedimented at 105,000 $\times$ *g* in 1 hr (68). The resulting pellet containing the mitochondria and microsomes was suspended in RSB and referred to as the mitochondrial-microsomal fraction, or MMF. The RNA polymerase of the MMF requires all four nucleoside triphosphates, Mg^{++}, and an adenosine triphosphate (ATP) generating system at *p*H 8.2 for maximal activity (67).

The RNA polymerase activity of the MMF is readily distinguished from the nuclear DNA-dependent RNA transcriptase of the host cell, since it is not affected by deoxyribonuclease or actinomycin D (67) but is inactivated by ammonium sulfate (67). The activity of each of the enzymes can thus be estimated in preparations containing both, by assaying for viral RNA polymerase in the presence of actinomycin D and for DNA-dependent RNA transcriptase in the presence of ammonium sulfate (67).

Solubilization of RNA Polymerase

Studies have also been directed toward solubilizing the RNA polymerase from the MMF. A variety of chemical and physical procedures were unsuccessful, but it was subsequently found (68, 69) that when cells were fractionated in the absence of added Mg^{++} (such as in sucrose-KCl), the polymerase activity of the mitochondrial-microsomal fraction was not sedimentable at

105,000 × *g* after treatment with 1% DOC. The results of sucrose density gradient analyses showed that the "DOC-solubilized polymerase" (DSP) has a sedimentation coefficient of approximately 40*S* (68, 69). The DSP appears to represent an enzyme-template complex since its activity, like that of the MMF, was not stimulated by the addition of either viral RNA or that extracted from infected cells (69). Similarly, such preparations can be centrifuged through sucrose density gradients without significant loss of activity (68). The requirements for the DOC-solubilized preparation are the same as those described already for the MMF, except that an ATP-generating system is not required because this preparation is free from adenosine triphosphatase (69).

Requirements of the Viral-Induced RNA Polymerase for Template

The RNA polymerase of crude cytoplasmic fractions was found to be relatively resistant to inactivation by ribonuclease (Table 1; see Fig. 10 for additional data). The dependence of the enzyme on an RNA template is suggested, however, by the finding that the polymerase was inactivated by ribonuclease in the presence of DOC (Table 1). The RNA template thus appears to be protected in some unspecified manner from nuclease attack, in the untreated MMF. The finding that polymerase activity was abolished by ribonuclease in the presence of DOC under conditions which do not hydrolyze double-stranded RNA (69) is probably not adequate evidence that the template is single-stranded. For example, the sedimentation coefficient of double-stranded RNA isolated from Novikoff cells infected with mengovirus is altered by treatment with 1 μg/ml of ribonuclease for 10 min at 25 C (*unpublished data*). It is possible, therefore, that template activity of double-stranded RNA could be destroyed by minor modifications in structure resulting from treatment with ribonuclease. Attempts to free the RNA polymerase of template so that direct tests for the effect of various RNA preparations on enzyme activity could be performed have been unsuccessful. Weissmann et al. (81) similarly were unsuccessful in attempts to resolve the RNA polymerase isolated from *E. coli* infected with MS2 phage. Haruna et al. (35, 36), on the other hand, have isolated RNA polymerases from *E. coli* infected with MS2 or Qβ phages which are specifically activated by the homologous viral RNA, and August et al. (4) reported the isolation of an RNA polymerase from *E. coli* infected with f2 phage which is activated by a variety of heterologous RNA preparations. Collectively, the foregoing data indicate that more than one RNA polymerase may be involved in the replication of viral RNA, although the question of whether the specificity of RNA polymerase for template may vary among different systems has not been adequately investigated.

TABLE 1. *Effect of ribonuclease on RNA polymerase activity of MMF*

Treatment*	GTP-H^3 incorporated		Inactivation
	count/min		%
None	1,830,	1,900	
Plus 10 μg/ml of ribonuclease	1,200,	1,290	33
Plus 10 μg/ml of ribonuclease + 0.5% DOC	55,	68	98
Plus 0.5% DOC	1,430,	1,530	21

* Samples containing 1 mg of protein of MMF from 6-hr infected cells were supplemented with ribonuclease and DOC, allowed to stand at 0 C for 15 min, and were then assayed for viral RNA polymerase activity.

If the main function of the double-stranded RNA is that of a template, one might anticipate that it would be located in the cellular fraction containing the RNA polymerase activity. The viral-induced RNA polymerase from Novikoff hepatoma cells can be centrifuged through sucrose density gradients without significant loss in activity, and the particulate fraction containing the enzyme-template complex can be partally resolved by this procedure (see section on *Intracellular Location of Viral-Directed Synthesis of RNA*). These characteristics made it possible to test experimentally the extent to which double-stranded RNA is associated with the polymerase activity. In the experiment presented in Fig. 5, infected cells which had been labeled for 10 min with tritiated uridine were disrupted with a Dounce homogenizer. The nuclei were removed from the homogenate by centrifugation, and the postnuclear supernatant fraction was treated with DOC and centrifuged through a sucrose gradient. Each fraction from the gradient was divided into two equal portions; one was treated with ribonuclease and DOC and assayed for tritium remaining in acid-insoluble material, and the other was tested for RNA polymerase activity employing α-P^{32}-guanosine triphosphate (GTP) as the labeled substrate. The RNA in mature virus is not hydrolyzed by ribonuclease in the presence of DOC (66) and thus is represented along with double-stranded RNA in the profile of tritium in Fig. 5. To establish the distribution of double-stranded RNA on the gradient, fractions from an identical gradient were pooled into several

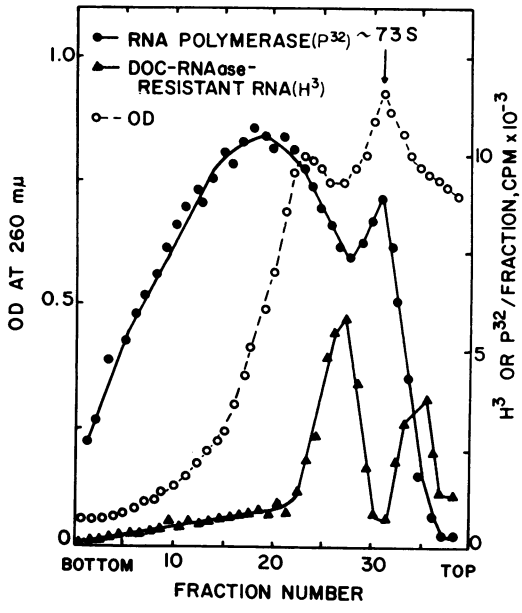

FIG. 5. *Sedimentation analysis of postnuclear fraction and RNA-polymerase activity. Infected cells were labeled with 0.02 μmole/ml of uridine-H³ (100 μc/μmole) in the presence of actinomycin D and FUdr for 30 min beginning at 4.75 hr. The cells were fractionated in RSB, the nuclei were removed, and the postnuclear supernatant fractions were treated with 1% DOC at 0 C for 10 min and sedimented through a gradient. Conditions of centrifugation as in Fig. 4. Fractions from the gradient were divided into two equal portions, and one was assayed for DOC-ribonuclease-resistant RNA. The other was incubated at 37 C for 30 min with 2 volumes of reaction mixture containing per milliliter: 12.5 μmoles of magnesium acetate, 125 μmoles of Tris-HCl (pH 8.2), 10 μmoles of phosphoenol pyruvate, 20 μg of pyruvic kinase, 25 μg of actinomycin D, 0.15 μmole each of ATP, CTP, UTP, and 0.5 μc of α-P³²-GTP (130 μc/μmole). The P³² incorporated into acid-insoluble material was determined with a two-channel liquid scintillation spectrometer.*

groups and each was extracted with hot phenol-SDS and analyzed for double-stranded RNA by a procedure illustrated in Fig. 1. The proportion of the total double-stranded RNA located in various parts of the gradient was estimated from these data. Fractions 1 to 30 (Fig. 5) contained 80% of the polymerase, but only 25% of the double-stranded RNA was located in this portion of the gradient. Fractions 31 to 39, on the other hand, contained 75% of the double-stranded RNA, but only 15% of the polymerase activity.

Analogous experiments were also performed with untreated postnuclear fractions, and the data failed to demonstrate any correspondence between the distribution of RNA polymerase activity on gradients relative to that of double-stranded RNA. It should be noted, however, that in none of these experiments was polymerase observed in a fraction free from double-stranded RNA. Interpretation of the overall data is subject to uncertainties, such as artifacts which may have been introduced by the procedures used to isolate various cellular fractions. Although the data suggest that double-stranded RNA does not function directly as a template, it will be necessary to obtain preparations free from double-stranded RNA to establish this point unequivocally.

Relationship Between RNA Polymerase Activity of Cell-Free Extracts and Uridine Incorporation by Intact Cells

It is generally assumed that synthesis of RNA by infected cells in the presence of actinomycin,

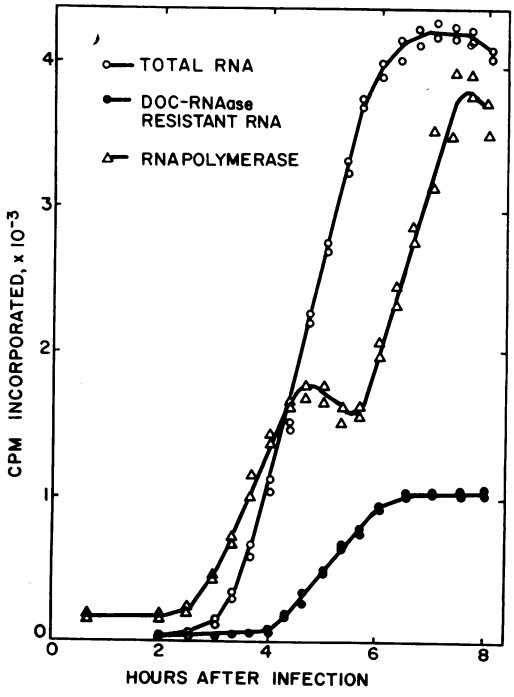

FIG. 6. *Time course of formation of RNA polymerase in relation to uridine incorporation by intact cells in presence of actinomycin D. A suspension of cells at 2 × 10⁶ per milliliter infected with a multiplicity of 30 was divided into 2 parts. Cells from 5-ml samples were assayed for RNA polymerase activity. The second part of the suspension received 2 μg/ml of actinomycin D and 0.5 μmole/ml of FUdr at 1.5 hr after infection, and 0.01 μmole/ml of uridine-H³ (100 μc/μmole) at 2 hr. Acid-insoluble radioactivity was determined in duplicate on samples with and without DOC and ribonuclease treatment (see Fig. 2).*

as measured by the incorporation of labeled uridine into acid-insoluble material, reflects the activity of the viral-induced RNA polymerase. This relationship has been studied in some detail, and the results of a typical experiment are presented in Fig. 6. The data indicate that uridine incorporation by intact cells began with the appearance of the polymerase as measured in cell-free extracts. It will be noted, however, that the rate of uridine incorporation was not a true index of the polymerase activity. For example, the rate of uridine incorporation remained constant between 3 and 6 hr, whereas the polymerase activity continued to increase from 3 to 4 hr, remained relatively constant between 4 and 6 hr, and then increased again at the end of the replicative cycle. The failure of cells to incorporate uridine after 6.5 hr probably resulted from loss of ability to synthesize or to retain nucleoside triphosphates. Evidence has been presented elsewhere (67) that the biphasic curve for polymerase activity is probably not due to the sequential production and destruction of an inhibitor. Further, limited conversion of uridine to UTP does not appear to account for the failure of uridine incorporation to keep pace with polymerase activity between 3 and 4 hr (67). It is considered more likely that the rate of synthesis of viral-directed RNA is under some form of metabolic control and thus not directly related to the amount of polymerase per se.

Temporal Relationship Between Polymerase Activity and the Production of Mature Virus

Figure 6 is also of interest with regard to the level of polymerase relative to production of mature virus. The production of virus can be estimated from data on the incorporation of uridine into RNA which is resistant to DOC-ribonuclease as described earlier. About 70% of the DOC-ribonuclease-resistant RNA synthesized after 4 hr is located in mature virus, and the remainder is double-stranded RNA. The data in Fig. 6 (see Fig. 2 also) demonstrate that, during the period of maximal production of virus (4 to 6 hr), the polymerase activity remained relatively constant. The secondary rise in polymerase activity occurred only after the rate of production of virus began to decline, and enzyme activity continued to increase after viral maturation had ceased. The data suggest some relationship between the production of polymerase and viral maturation. For instance, the data could be explained if the polymerase is incorporated into mature virus in an inactive form. If production and utilization of polymerase occurred at the same rate between 4 and 6 hr, the level of enzyme would be expected to remain constant during this period. Similarly, if synthesis of enzyme continued after viral maturation ceased, the observed secondary rise in activity would be anticipated. The enzyme would also accumulate between 3 and 4 hr when little virus is produced. The foregoing hypothesis has not been verified experimentally, and the data are also subject to the interpretation that the biphasic curve for the production of polymerase simply reflects the operation of some unspecified control mechanism or fluctuation in the level of template for the enzyme.

Effect of Puromycin on RNA Polymerase

The results of studies of the effect of puromycin on polymerase activity also imply some relationship between the level of enzyme activity and the production of virus. The data presented in Fig. 7 illustrate that when puromycin was added at 4 hr there was no further increase in polymerase, and that thereafter the activity gradually declined. Similar findings have been reported from studies of HeLa cells infected with poliovirus (12, 25) and have been attributed to instability of the polymerase. Further examination of the data

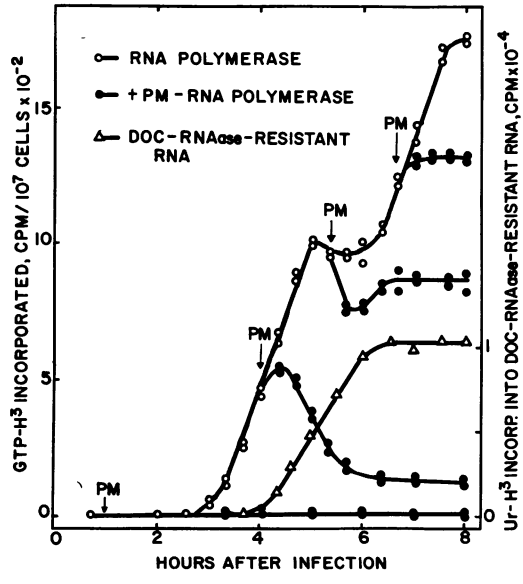

FIG. 7. *Effect of puromycin on the formation of viral-induced RNA polymerase. Samples of a suspension of infected cells (2 × 10⁶ cells per milliliter) received 100 μg/ml of puromycin at indicated times. Cells from 3-ml samples were assayed in duplicate for RNA polymerase activity. Incorporation of uridine-H³ into DOC-ribonuclease-resistant RNA was determined as described in Fig. 6.*

in Fig. 7 will reveal that the enzyme appeared to be unstable in cells treated with puromycin only during the period between 4 and 6 hr. Puromycin also prevented the secondary rise in polymerase activity when added at 6.5 hr, but under these conditions the enzyme appeared to be relatively stable. Utilization of polymerase in viral maturation does not explain the apparent metabolic instability of the enzyme after treatment with puromycin at 4 hr, because viral maturation appears to cease within 30 min of its addition (*unpublished data*). This may indicate that the RNA polymerase is inactivated by combination with a viral precursor or some cellular constituent which accumulates in the presence of puromycin. This model would require that synthesis of the inactivating material ceases late in the replicative cycle, or that it is lost from the cells to account for the stability of the polymerase after 6 hr. Although a precise mechanism to explain the data in Fig. 6 and 7 is not available, it is anticipated that this system will be useful in future studies of regulatory processes involved in the replication of mengovirus.

Intracellular Location of Viral-Directed Synthesis of RNA

Studies of the distribution of viral-induced RNA polymerase in mengovirus infected Novikoff cells were initiated as a means of establishing conditions for its isolation and to obtain information on the nature of the enzyme relative to other intracellular components. The initial phase of the investigation was concerned primarily with the question of whether the polymerase is located in the nucleus or in the cytoplasm. The results of an experiment which aided in resolving this question are illustrated in Fig. 8. Cells were disrupted in RSB and fractionated into nuclei, microsomal-mitochondrial fraction, and cell sap by differential centrifugation (68). Cells were fractionated at various times after infection, and the fractions were assayed for viral-induced RNA polymerase activity. The data demonstrate that the polymerase was located almost exclusively in the MMF between 2.5 and 4 hr. Thereafter, the relative proportion of enzymatic activity associated with the nuclear fraction increased steadily. Evidence that cytoplasmic contamination of the nuclear fraction increased progressively after 3.5 to 4 hr is presented elsewhere (68). The polymerase, however, appears to remain preferentially associated with the nucleus when cells are fractionated during the terminal stages of the replicative cycle, since the degree of cytoplasmic contamination was found to be insufficient to account for the enzyme activity in this fraction after

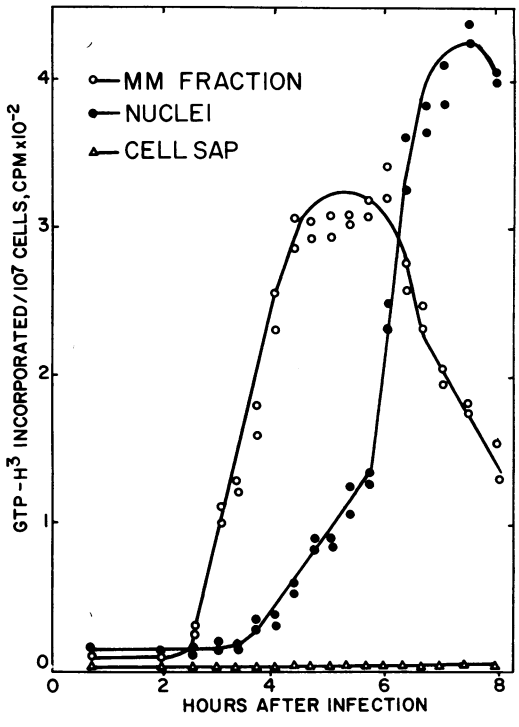

FIG. 8. *Distribution of viral-induced RNA polymerase among various cellular fractions as a function of time after infection.*

5.5 hr (68). Changes in the appearance of the nuclear membrane is a typical cytopathic response of cells to infection with mengovirus (13, 21, 22, 59). These alterations may be responsible for the increased affinity of the nuclei for certain cytoplasmic components. The results of cytochemical studies of L cells infected with mengovirus indicate that the viral-directed synthesis of RNA occurs in the perinuclear region of the cytoplasm (31, 32). The cytoplasm has been also implicated as the site of synthesis of poliovirus RNA, since actinomycin-resistant synthesis of RNA occurs in anucleate fragments of infected HeLa cells (20). It has been shown that nuclei isolated from HeLa cells infected with poliovirus do not contain significant amounts of newly formed infectious RNA or acid-insoluble radioactivity from labeled uridine, provided the nuclei are freed from cytoplasmic components by centrifugation through 2 M sucrose (44). A report (29) which states that the polymerase from HeLa cells infected with poliovirus is associated with the nuclear fraction is difficult to interpret, since the cells were fractionated after storage at −20 C. Recent data (23, 24) on the distribution of viral-directed synthesis of RNA by Krebs ascites cells

infected with EMC virus also indicate that it occurs in the cytoplasm. Earlier findings (15, 58) which implicated the nucleus as the site of synthesis of viral RNA probably resulted from undetected cytoplasmic contamination of the nuclei (16, 23).

On the basis of the data in Fig. 8, subsequent experiments were performed with various cytoplasmic fractions. When the PNF was treated with DOC to disrupt the mitochondria and subjected to sucrose density gradient analysis, the RNA polymerase was found to be located in a heterogeneous fraction which sedimented faster than the bulk of the polysomes. Experiments of the type illustrated in Fig. 9 were performed to investigate the problem of whether these particles had significance in the viral-directed synthesis of viral-directed RNA in the intact cell. Infected cells were pulse-labeled with uridine-H^3 in the presence of actinomycin and 5-fluorodeoxyuridine and then homogenized in RSB. The PNF was isolated and treated with DOC and sedimented through a sucrose density gradient. Each fraction from the gradient was incubated with polymerase reaction mixture containing α-P^{32}-GTP as labeled substrate and then analyzed for absorbancy at 260 mμ and for acid-insoluble H^3 and P^{32}. The absorbancy profile illustrates the position of the 73*S* ribosomes and the more rapidly sedimenting polysomes, and the profiles of tritium and P^{32} demonstrate the distribution of RNA synthesized during the pulse in vivo and the position of the polymerase on the gradient, respectively. Most of the RNA synthesized in vivo was found in the same fraction which contained the bulk of the polymerase activity (fractions 1 to 31). A PNF was also prepared in 0.3 M sucrose and sedimented through a sucrose density gradient in the absence of DOC, and the fractions were assayed for polymerase activity. Most of the polymerase was again found in the rapidly sedimenting but heterogeneous fraction (Fig. 10A), and, as in the previous experiment (Fig. 9), a relatively small amount of activity was located in the ribosomal region (73*S*) of the gradient. The absorbancy profile in Fig. 10A indicates that most of the polysomes had been destroyed by the isolation procedure employed. The data in Fig. 10B demonstrate that treatment of the PNF with ribonuclease prior to centrifugation did not result in a significant loss of polymerase activity, but that the polysomes were effectively eliminated by this procedure. The data also demonstrate that relatively little polymerase was associated with the mitochondria which are located in the pellet (Fig. 10).

The high degree of correspondence between

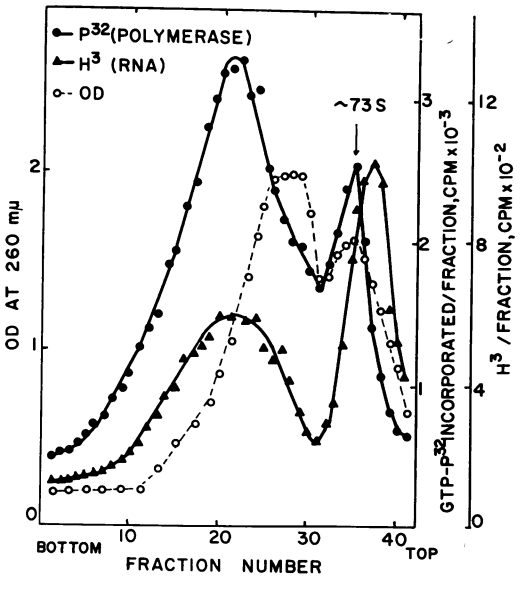

FIG. 9. *Relationship between intracellular location of viral-induced RNA polymerase and RNA synthesized by intact cells. At 2 hr after infection, 2 μg of actinomycin D and 0.5 μmole of FUdr were added per ml of suspension (1.5 × 10⁶ cells per milliliter). The cells were labeled with 0.003 μmole/ml of uridine-H³ (100 μc/μmole) for 10 min at 5 hr and fractionated in RSB. The PNF was supplemented with 1% DOC and allowed to stand at 0 C for 10 min prior to centrifugation. Conditions of centrifugation as described in Fig. 4. Fractions from the gradient were assayed for RNA polymerase with α-P³²-GTP as labeled substrate (see Fig. 5).*

the distribution of RNA synthesized in vivo and the RNA polymerase (Fig. 9) suggests that the rapidly sedimenting particles may represent the site of viral-directed synthesis of RNA. The precise nature of these particles cannot be specified at present. They appear to be distinct from polysomes in that their sedimentation characteristics are not changed significantly under conditions which eliminate the polysomes (Fig. 10). The polymerase activity of cytoplasmic particles is resistant to ribonuclease (Fig. 10), but it should be recalled that the enzyme is inactivated by ribonuclease in combination with DOC (Table 1). The question of whether resistance to ribonuclease indicates that the template is bound in such a manner that interaction with ribonuclease does not occur, or whether it is physically protected and thus inaccessible to the nuclease, remains to be answered.

The viral-induced RNA polymerase activity of L cells infected with mengovirus has been detected in a crude particulate fraction (7, 9), and

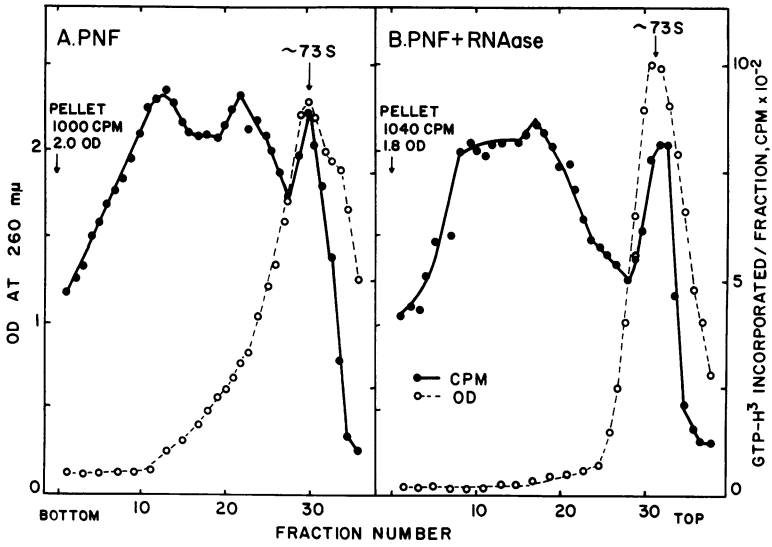

FIG. 10. *Sedimentation characteristics of RNA polymerase of the postnuclear fraction (PNF) prepared in sucrose. Cells were fractionated in 0.3 M sucrose at 5 hr after infection. Conditions of centrifugation: 0.5 to 1.2 M sucrose in 0.01M Tris-HCl (pH 7.3); SW 25 rotor, 25,000 rev/min, 90 min. A = untreated PNF; B = PNF incubated with 10 μg/ml of ribonuclease at 22 C for 10 min just prior to centrifugation.*

that of HeLa cells infected with poliovirus was found to be associated with particles which sediment for the most part at 10,000 × *g* in 30 min (6). A large proportion of the RNA and protein synthesized by infected HeLa cells is also associated with these particles, and the latter are considered to be viral-specific since their counterpart is not observed in uninfected cells (14, 65). Earlier electron microscopic studies revealed large cytoplasmic aggregates in HeLa cells infected with poliovirus which appeared to be enclosed by a membrane (49). On the basis of the foregoing observations, Penman et al. (65) suggested that the synthesis of viral RNA and protein, as well as viral maturation, occurs in viral-induced structures bounded by a membrane. Although these "virus-synthesizing bodies" share certain features in common with the polymerase containing particles from Novikoff hepatoma cells, they are clearly distinct in several important respects. For example, the particles in HeLa cells are much larger than those found in Novikoff hepatoma cells and are disrupted by treatment with DOC even in the presence of Mg^{++}. Furthermore, structures equivalent to the large bodies in HeLa cells infected with poliovirus were not observed in L cells infected with mengovirus (21). It is of interest in this connection that recent electron microscopic studies of cells infected with poliovirus (22, 59) and other picornaviruses (21) have revealed a large number of small vesicles, some of which appeared to contain virus whereas others did not. Although the overall data obtained in several systems on the nature of the particles containing the viral-induced RNA polymerase are fragmentary, and in part controversial, there is more general agreement that they play a central role in the synthesis of viral RNA.

RNA SYNTHESIZED IN VITRO BY VARIOUS CELL-FREE PREPARATIONS

It has been shown that both the particulate MMF and the DOC-solubilized preparations of RNA polymerase from infected cells synthesize mixed nucleoside polymers containing all four bases (67, 69). The following series of experiments was undertaken to determine whether the cell-free preparations of polymerase were capable of synthesizing RNA which resembles that extracted from virus in its physical properties. The RNA extracted from mengovirus (37*S*) is readily distinguished from the cellular RNA preparations by sedimentation analysis. RNA was extracted from a reaction mixture containing the MMF and labeled GTP and centrifuged through a sucrose density gradient (Fig. 11A). The absorbancy profile represents the microsomal RNA preparations and transfer RNA present in the MMF. A significant portion of the labeled RNA was similar to that isolated from virus (37*S*) or that extracted from infected cells (Fig. 1) as shown by the profile of radioactivity. Most of the remaining isotope (polymerase product) was contained in a peak which sedimented slightly

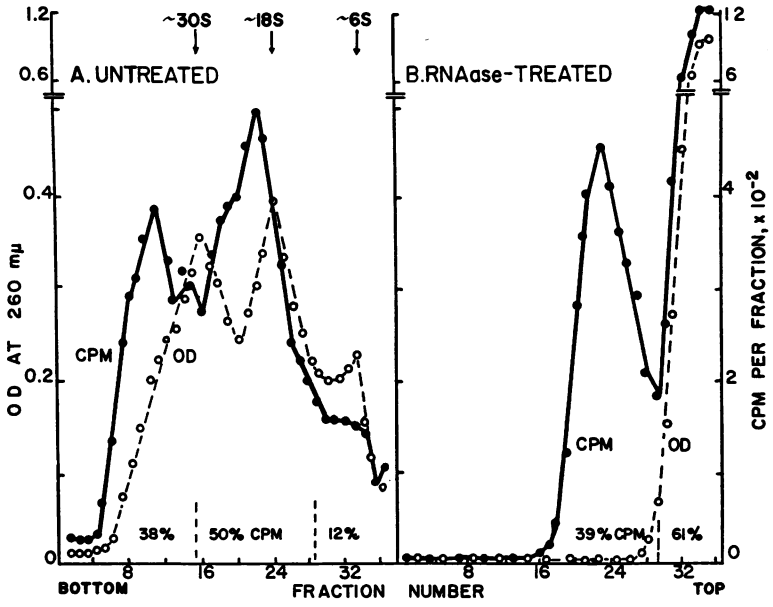

FIG. 11. *Sedimentation analysis of RNA synthesized by mitochondrial-microsomal fraction. An MMF prepared from cells 6 hr after infection was incubated with polymerase reaction mixture containing GTP-H³ as labeled substrate. RNA was isolated by extraction with phenol-SDS, and 0.2 ml (absorbancy at 260 mμ = 40) was centrifuged through a linear sucrose gradient. Conditions of centrifugation: 0.15 to 0.6 M sucrose in 10 mM Tris, 10 mM EDTA (pH 7.3); SW 39 rotor, 35,000 rev/min, 5.5 hr. A = untreated sample; B = sample treated with 0.4 μg of ribonuclease at 22 C for 5 min just prior to centrifugation.*

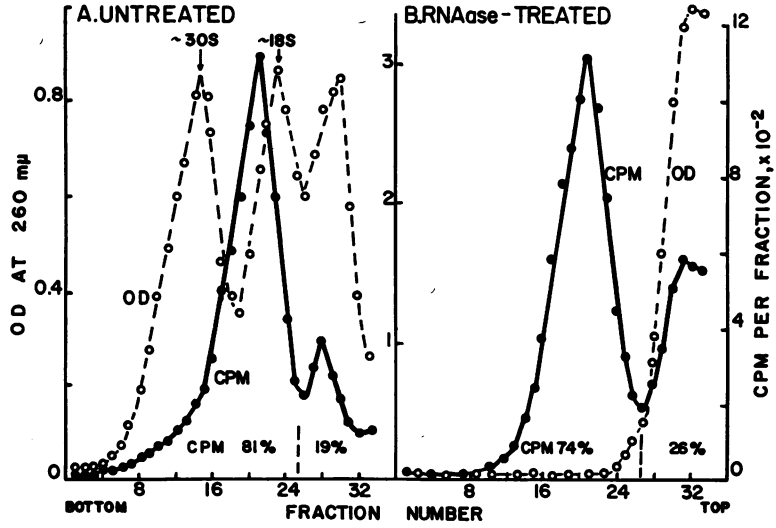

FIG. 12. *Sedimentation analysis of RNA synthesized by DOC-solubilized preparation. A DOC-solubilized preparation was prepared from cells 6 hr after infection and incubated with polymerase reaction mixture containing GTP-H³. RNA was isolated and analyzed as described in Fig. 11.*

faster than the 18S species of microsomal RNA. A portion of the RNA was also treated with ribonuclease prior to centrifugation, and the data in Fig. 11B demonstrate that about 40% of the RNA synthesized by the MMF was resistant to ribonuclease.

Products were also isolated from reaction mixtures containing the DOC-solubilized prepa-

ration and analyzed on sucrose density gradients. The results of a typical experiment are presented in Fig. 12. It will be noted that the DSP differed from the MMF in that it failed to yield viral-like (37S) RNA. The principal product in this instance was ribonuclease-resistant RNA along with some material of relatively low molecular weight. The ribonuclease-resistant RNA synthesized by both the DSP and MMF is not hydrolyzed by deoxyribonuclease alone or in combination with ribonuclease, but becomes sensitive to ribonuclease when heated to 95 C and rapidly cooled (69), and thus appears to be double-stranded RNA which was discussed earlier.

The effect of DOC on the ability of the MMF to synthesize viral-like RNA was also investigated in view of the results obtained with the DSP. It should be recalled that the polymerase activity of the MMF prepared in the presence of Mg^{++} remains associated with relatively large particles even after treatment with DOC (Fig. 9). When the MMF was treated with DOC, however, it failed to synthesize viral-like RNA, and the profiles obtained by sucrose density gradient analysis were similar to that illustrated in Fig. 12A for the product of the DSP (69). Similarly, when the MMF was stored at −20 C for a month, the product of the in vitro reaction was primarily double-stranded RNA (69).

It was noted earlier (Fig. 10) that the polymerase activity of the MMF (or PNF) is relatively resistant to ribonuclease. About one-half of the single-stranded RNA synthesized by such preparations is also resistant to ribonuclease. This RNA is not double-stranded since it becomes sensitive to ribonuclease upon treatment with DOC or upon extraction with phenol-SDS (69). These data suggested that the DSP or the DOC-treated MMF may synthesize viral-like RNA, but that the latter is destroyed by endogenous nucleases and thus is not found in the reaction mixture. To investigate this possibility, the DSP was partially purified and was found to be free from detectable nuclease activity (69). This "nuclease-free" preparation failed, nevertheless, to yield viral-like RNA, and the products were essentially the same as those produced by the crude DSP (69). These data suggest that the action of nuclease does not account for the finding that viral-like RNA is absent from reaction products of the DSP. These experiments do not rigorously exclude the possibility, however, that the partially purified DSP contains a nuclease which is bound to the enzyme in such a manner that it hydrolyzes the product but is inactive on exogenous RNA.

The presence of two polymerases in the MMF would account for the data if one of these synthesizes viral RNA and the other double-stranded RNA. The results of the experiments would be accounted for if the polymerase which synthesizes viral RNA is inactivated by DOC or by storage at −20 C. Definitive evidence that more than one RNA polymerase is operative in the MMF, however, is lacking. The foregoing experimental observations will be discussed subsequently in terms of a potential mechanism of RNA synthesis.

SIGNIFICANCE OF DOUBLE-STRANDED RNA AS AN INTERMEDIATE IN THE SYNTHESIS OF RNA

The double-stranded RNA produced by cells infected with EMC virus has been referred to as the "replicative form," implying that it represents an intermediate in the replication of viral RNA (61). The results obtained by Fenwick et al. (28) in pulse-chase experiments with *E. coli* infected with f2 phage and similar experiments by Hausen (37) with L cells infected with Me virus suggest that a small portion of the double-stranded RNA synthesized by infected cells turns over during the synthesis of single-stranded RNA. Evidence has been presented by Weissmann et al. (83) which suggests that the functional RNA polymerase from *E. coli* infected with MS2 phage is a complex of double-stranded RNA and enzyme. Further, it was proposed that viral RNA is synthesized by a semiconservative mechanism whereby the newly formed viral strand of the template duplex and the complementary RNA is conserved (79–82).

Pulse-Chase Analysis of Double-Stranded RNA In Vitro

If double-stranded RNA is an intermediate in the synthesis of RNA, it should not only turn over but should be more rapidly labeled during the course of a short pulse. The data presented in Fig. 13A illustrate the time course of synthesis of single- and double-stranded RNA preparations by the MMF in vitro. In this experiment, double-stranded RNA was estimated by treating samples of the reaction mixture with DOC-ribonuclease. The residual acid-insoluble material after such treatment was found to be exclusively double-stranded RNA. This was demonstrated by extracting the residual RNA with hot phenol-SDS and analyzing it on sucrose density gradients by the procedure illustrated in Fig. 1 and 11. The results in Fig. 13A indicate that most of the RNA synthesized during the first 10 min was double-stranded. Synthesis of single-stranded RNA (hydrolyzed by DOC-ribonuclease) increased sharply

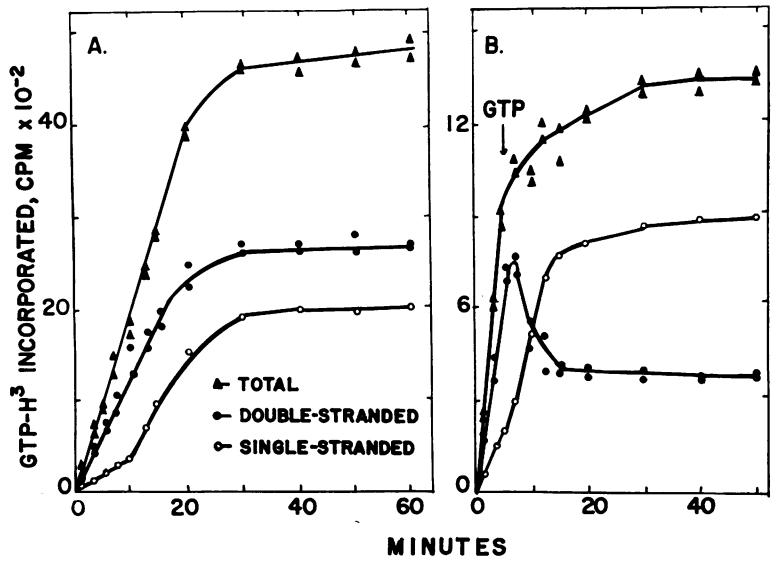

FIG. 13. *Pulse-chase analysis of RNA synthesized by mitochondrial-microsomal fraction. (A) An MMF prepared from cells at 5.5 hr after infection was incubated at 37 C with an equal volume of reaction mixture containing, per milliliter: 12.5 μmoles of magnesium acetate, 125 μmoles of Tris-HCl (pH 8.2), 10 μmoles of phosphoenol pyruvate, 20 μg of pyruvic kinase, 25 μg of actinomycin D, 0.15 μmole each of ATP, CTP, UTP, and 0.1 μc of GTP-H³ (1,700 μc/μmole). (B) After 5 min of incubation, 0.15 μmole/ml of unlabeled GTP was added to part of the suspension. Samples were analyzed at intervals for total acid-insoluble radioactivity and that resistant to 10 μg/ml of ribonuclease for 30 min at 25 C (double-stranded RNA). The amount of single-stranded RNA was calculated by subtracting the values for double-stranded RNA from the total.*

after this time and accounted for 40% of the total after 30 min. It will also be noted that the reaction proceeded linearly for 20 min, and then the rate declined rapidly and little RNA was synthesized after 30 min.

The results of a pulse-chase experiment employing the MMF are presented in Fig. 13B. The reaction mixture contained labeled GTP-H³, and after 5 min of incubation a 100-fold excess of unlabeled GTP was added. This resulted in a sharp decrease in the rate of incorporation to about 8% of that of the control. During the first 15 min of the chase, about 50% of the tritium in double-stranded RNA was lost, with a concomitant increase of label in the single-stranded RNA. The data indicate that at least a portion of the double-stranded RNA turns over and thus satisfies a basic requirement for its consideration as an intermediate in the synthesis of RNA in vitro.

Pulse-Chase Analysis of Double-Stranded RNA In Vivo

Cells were pulse-labeled for periods ranging from 3 to 60 min, and the RNA was extracted and analyzed for double- and single-stranded RNA. The data indicated that double-stranded RNA was not preferentially labeled even during the 3-min pulse (*in preparation*). Infected cells were also labeled with uridine for short periods and then washed and resuspended in a medium containing an excess of unlabeled uridine. At intervals thereafter, samples of cells were analyzed for double-stranded RNA relative to the total. The data in Fig. 14A demonstrate that the washing itself did not affect the incorporation of labeled uridine. Figure 14B illustrates the results of the chase in more detail, and the figures beneath the curve indicate the relative proportion of double-stranded RNA which was present at various times during the chase. The data indicate clearly that double-stranded RNA did not turn over during the 2-hr chase. Pulse-chase experiments performed under a variety of conditions with different periods of labeling yielded results similar to those shown in Fig. 14. In spite of the limitations of pulse-chase experiments in this system, the data indicate clearly that, if turnover of double-stranded RNA occurred, only a small fraction of the total participated in this process. An attempt was made to increase the sensitivity of pulse-chase experiments by preparing an MMF from cells labeled with uridine in vivo for 10 to 20 min and incubating it in vitro with polymerase reaction mixture containing unlabeled substrates. The results of analyses performed at

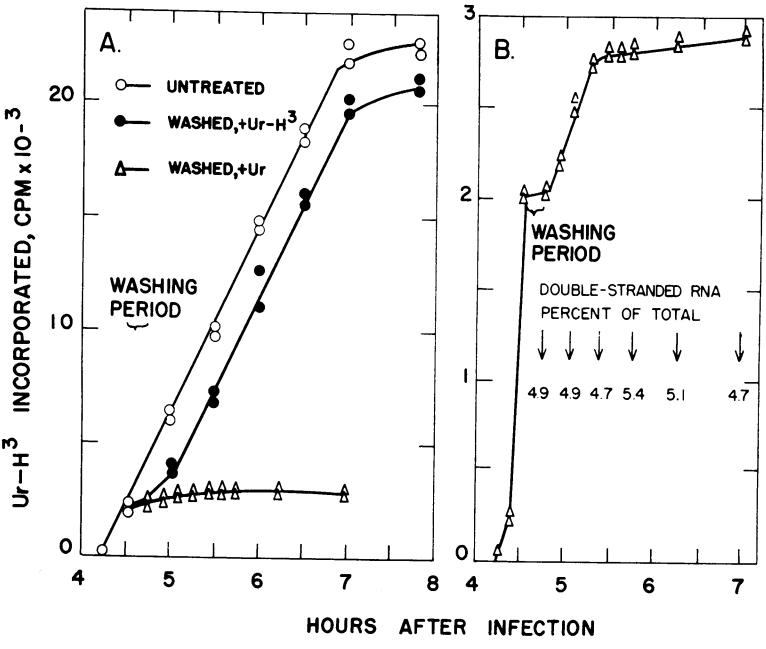

FIG. 14. *Pulse-chase analysis of double-stranded RNA synthesized in vivo. At 2 hr, 2 μg/ml of actinomycin D and 0.5 μmole/ml of FUdr were added to a suspension containing 1.5 × 10⁶ cells per milliliter. At 4.25 hr, 0.01 μmole/ml of uridine-H³ (100 μc/μmole) was added, and 15 min later a portion of the cells was washed in fresh medium. One-half of the washed cells was suspended in the original volume of labeled medium, and the remainder was suspended in medium containing 2 μmoles/ml of uridine. The incorporation of uridine into acid-insoluble material was determined on samples of cells at suitable intervals. At intervals during the chase (indicated by arrows), samples were removed and the RNA extracted from cells with phenol-SDS was analyzed for radioactivity in double-stranded RNA relative to the total (see Fig. 1).*

intervals indicated that no further incorporation of label into acid-insoluble material occurred under these conditions and that there was no detectable loss of isotope from double-stranded RNA. The methods employed in these studies are not sufficiently precise to permit the statement that none of the double-stranded RNA turns over, but they are sufficiently sensitive to detect turnover if more than a few per cent of the total double-stranded RNA participates as an intermediate.

A comparison of the data obtained from the pulse-chase experiments performed in vivo with those done in vitro reveals an interesting paradox. About one-half of the double-stranded RNA synthesized in 5 min by the MMF in vitro turns over, whereas the double-stranded RNA synthesized in vivo appears to be metabolically stable. These data imply that a pool of double-stranded RNA which turns over may exist in the intact cell, but that it is too small to be detected by the methods available. If this reasoning is correct, it is apparent that the double-stranded RNA which appears to be an intermediate on the basis of turnover is somehow different from its metabolically stable counterpart which accumulates throughout the period of synthesis of RNA. A large proportion of the double-stranded RNA may be displaced from the enzyme and thereafter be incapable of participating in the reaction as suggested by data presented in an earlier section. An alternate explanation might be that the RNA which turns over is not a stable hybrid (duplex) in situ, but exists in a form readily converted to double-stranded RNA by the isolation procedures employed.

CONCLUDING REMARKS

Consideration of the overall data presented in the foregoing suggests the basic outline of a mechanism for the synthesis of RNA by the viral-induced RNA polymerase. This model is presented here as a means of coordinating many of the experimental findings and is summarized in equations I and II, where E = polymerase; R^T = template-RNA; R^P = product-RNA; and X and Y = unspecified cytoplasmic components:

$$\text{(I)}\quad E - R^T + X + \text{n(ATP, GTP, CTP, UTP)} \rightarrow E - R^T \cdots R^P - X + 4\text{n (PPi)}$$

(II) $E - R^T \cdots R^P - X + Y \rightarrow E - R^T + X + R^P - Y$

It is proposed that the product of the polymerase is synthesized by complementary base copying of a single-stranded RNA-template (R^T). The newly synthesized RNA is prevented from hybridizing with the template by complexing with X as illustrated in equation I. Y is proposed in the second equation to indicate that the newly synthesized RNA (R^P) is not released in a free form, but is rather transferred to some unspecified structural element of the cytoplasm with concomitant liberation of X and $E - R^T$. $E - R^T \cdots R^P - X$ depicts a structure in which the R^T and R^P are held in close proximity but are complexed with E and X, respectively, in such a manner that a stable $R^T - R^P$ hybrid is not formed. The reaction summarized in equation III occurs if the supply of X is inadequate, or if X is absent or inactivated:

(III) $E - R^T + n(ATP, UTP, CTP, GTP) \rightarrow E + R^T - R^P + 4n(PPi)$

That is, the template is copied and the newly synthesized strand forms a stable hybrid which tends to dissociate from the enzyme. Double-stranded RNA ($R^T - R^P$) is considered to be an end product of the reaction under these conditions and has not been assigned any specific function. It is further proposed that X is modified by treatment with DOC or phenol, which results in the conversion of $E - R^T \cdots R^P - X$ to $R^T - R^P$ and other unspecified products.

The data obtained with fractions from infected cells can be inserted into the foregoing model as follows. Reactions I, II, and III are performed by the mitochondrial-microsomal fraction in vitro to yield both single and double-stranded RNA. The results of pulse-chase experiments performed in vitro with the MMF indicate that a portion of the double-stranded RNA turns over. According to the model, these data indicate that $E - R^T \cdots R^P - X$ is labeled in R^P during the pulse, and that this isotope is subsequently passed on to $R^P - Y$ during the chase. When the reaction mixture is treated with DOC or extracted with phenol, the $E - R^T \cdots R^P - X$ is converted to $R^T - R^P$ and is thus analyzed along with the pre-existing $R^T - R^P$ arising via reaction III. Experimentally, this results in the finding that a portion of the double-stranded RNA turns over and thus behaves as an intermediate in the synthesis of single-stranded RNA.

Reaction III proceeds at a relatively rapid rate in vitro, which results in the conversion of all the available template-RNA (R^T) to double-stranded RNA ($R^T - R^P$) in 20 to 30 min, and the reaction is terminated. X is thought to be modified by treatment of the mitochondrial-microsomal fraction with DOC or by storage at −20 C. Reaction III prevails under these conditions, and the major product, therefore, is double-stranded RNA. The events visualized in the synthesis of RNA by intact cells are the same as those indicated for the in vitro system, except that reactions I and II are highly coordinated, and thus a detectable amount of the intermediate ($E - R^T \cdots R^P$) does not accumulate. The supply or availability of X appears to be limiting, however, since double-stranded RNA accumulates throughout the period of synthesis of RNA. Although the proposed scheme is compatible with most of the data obtained with the Novikoff hepatoma-mengovirus system, it is evident that additional information is required to establish its true merit. Many aspects of the model are accessible, however, to direct testing, and it is anticipated that it will be of value in the design of future experiments.

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