JOURNAL OF VIROLOGY, Apr. 1969, p. 376-384

Vol. 3, No. 4
Printed in U.S.A.

In Vitro Synthesis of Poliovirus Ribonucleic Acid: Role of the Replicative Intermediate

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Received for publication 23 December 1968

Poliovirus ribonucleic acid (RNA) polymerase crude extracts could be stored frozen in liquid nitrogen without loss of activity or specificity. The major in vitro product of these extracts was viral single-stranded RNA. However, after short periods of incubation with radioactive nucleoside triphosphates, most of the incorporated label was found in replicative intermediate. When excess unlabeled nucleoside triphosphate was added, the label was displaced from the replicative intermediate and accumulated as viral RNA. It is concluded from this experiment that the replicative intermediate is the precursor to viral RNA. In addition, some of the label was chased into double-stranded RNA. The implications of this finding are discussed.

Three species of ribonucleic acid (RNA) which do not exist in uninfected cells have been demonstrated in poliovirus-infected cells. These species are: (i) 35*S* single-stranded RNA which is eventually found in mature virions (9); (ii) 18*S* double-stranded RNA which consists of one viral RNA strand paired to complementary RNA (2, 16); and (iii) replicative intermediate (11) which is partially single-stranded and partially double-stranded RNA (6). Although its actual structure has not been elucidated, replicative intermediate probably consists of several nascent viral RNA strands base paired, in the vicinity of their growing points, to a complementary RNA template (3). Such a model was first proposed by Fenwick, Erikson, and Franklin (11), Erikson and Franklin (10), and later confirmed (15) in the case of bacteriophage R17.

The role of double-stranded RNA or replicative intermediate, or both, in the synthesis of viral RNA has been controversial (e.g., *see* reference 24). In the case of RNA bacteriophage, it was demonstrated that, after a short pulse labeling of infected bacteria with a radioactive precursor to RNA, some of the label in ribonuclease-resistant RNA was displaced from the double-stranded state by growth in nonradioactive medium and subsequently appeared in viral RNA (11, 17). More recent experiments, with purified Qβ replicase (18), have shown that complementary RNA molecules are first synthesized on viral strands to yield double-stranded RNA. The complementary strands are then used as templates for the synthesis of new viral RNA molecules, the growth of which displaces parental viral strands from the double-stranded state and results in the formation of the replicative intermediate. The replicative intermediate, in turn, is the direct precursor to newly made viral RNA. The sequence can be summarized as follows: viral RNA → double-stranded RNA → replicative intermediate → viral RNA.

In the case of poliovirus, it was previously demonstrated that replicative intermediate (i) is preferentially labeled after short exposure of the infected cells to radioactive uridine (6); (ii) is the predominant ribonuclease-resistant species formed during the exponential increase of viral RNA (6); and (iii) is recovered in association with viral RNA polymerase in the replication complex, which is the intracellular site for viral RNA synthesis (14). These observations led to the assumption that replicative intermediate was the precursor to viral RNA, although direct evidence was not available. As previously pointed out (6), such evidence could only result from the demonstration that nascent RNA chains, found in the replicative intermediate, are completed and released as viral RNA molecules.

This could be proved by showing that label incorporated in replicative intermediate and then displaced by further growth in non-radioactive medium accumulates as viral RNA. However, no "pulse chase" experiment is feasible with HeLa cells in vivo, since intracellular uridine cannot readily be diluted by the external addition of uridine. Therefore, an in vitro system was chosen in which RNA synthesis is mediated by viral

RNA polymerase and where true chase conditions can be met.

This report is concerned with the results of a "pulse chase" experiment performed in vitro with crude poliovirus RNA polymerase.

MATERIALS AND METHODS

Preparation of crude RNA polymerase. HeLa cells grown in suspension culture were infected with 30 to 50 plaque-forming units of type I poliovirus per cell as described previously (7). Each infection was monitored on a sample of the culture by ^{14}C-uridine uptake in the presence of actinomycin D. Cytoplasmic extracts (7, 19) were centrifuged for 40 min in a Spinco titanium rotor 50 of an L2 model ultracentrifuge set at 130,000 × *g* at 3 C. Crude RNA polymerase was prepared by careful resuspension of the resulting pellets in "polymerase buffer" [0.05 M tris (hydroxymethyl) aminomethane (Tris)-hydrochloride (*p*H 7.5), 0.005 M magnesium acetate, 0.005 M β-mercaptoethanol, 0.001 M ethylenediaminetetraacetic acid (EDTA), 0.4 M KCl, 20% glycerol] to a final protein concentration of 25 mg/ml. Several such preparations were stored in liquid nitrogen over a period of weeks. They were then mixed and carefully homogenized with a tight-fitting Dounce homogenizer. The pooled mixture was frozen again in liquid nitrogen in 1.5-ml lots. One lot was thawed and used for each experiment.

Assay of RNA polymerase. The standard reaction mixture consisted of 1 ml of crude RNA polymerase, 1 mmole of Tris-hydrochloride (*p*H 7.5), 60 μmoles of magnesium acetate, 40 μmoles of β-mercaptoethanol, 200 μg of ribonuclease-free deoxyribonuclease, 60 μmoles of phosphoenolpyruvate, 60 μg of pyruvate kinase, 12 μg of actinomycin D, 4 μmoles of adenosine triphosphate (ATP), guanosine triphosphate (GTP), and cytidine triphosphate (CTP), and 2 μmoles of ^{14}C (14×10^6 counts/min)- or tritium (35×10^6 counts/min)-labeled uridine triphosphate (UTP). The final volume was adjusted to 10 ml with 50% glycerol. Duplicate samples (0.3 to 0.5 ml) were precipitated with 5 ml of ice-cold 7.5% trichloracetic acid plus 0.02 M sodium pyrophosphate, centrifuged for 5 min at 1,000 × *g*, and then washed three times with cold 7.5% trichloroacetic acid plus 0.02 M pyrophosphate. Precipitates were dissolved in 0.6 ml of concentrated NH_4OH and were counted in a Nuclear Chicago Mark I liquid scintillation counter after the addition of 10 ml of Bray's solution (8).

Analysis of in vitro labeled RNA. Deproteinization of RNA was performed by either one of the following procedures.

Method A. A 1.5-ml sample was pipetted into a chilled tube containing 5.0 ml of 88% liquefied phenol, 3.5 ml of 0.05 M sodium acetate (*p*H 5.1), 0.005 M EDTA, and 0.5 ml of 10% sodium dodecyl sulfate (SDS). Phenol extraction was carried out as already described (13, 21), except that the temperature was lowered to 37 C in order to prevent melting of the replicative intermediate (3, 14). RNA was precipitated with 2 volumes of ethyl alcohol and dissolved in 0.5% SDS buffer [0.01 M Tris-hydrochloride (*p*H 7.4), 0.1 M NaCl, 0.001 M EDTA, 0.5% SDS].

Method B. Samples of the reaction mixture were made 0.015 M with respect to EDTA, 0.008 M with respect to sodium pyrophosphate, and 1% with respect to SDS. LiCl was added to 1.9 M in order to separate double-stranded RNA from the other forms of RNA (6). Precipitates were collected after 24 hr of standing at 4 C by centrifugation at 10,000 × *g* for 40 min and were dissolved in 0.5% SDS buffer. Supernatant fluids were precipitated with 2 volumes of ethyl alcohol in the presence of carrier yeast RNA (0.2 mg/ml), and the resulting pellets were dissolved in 0.5% SDS buffer.

Gradient analysis was performed on 15 to 30% sucrose gradients in 0.05% SDS buffer. After centrifugation, these gradients were monitored for absorbance at 260 nm, fractionated, and assayed for acid-precipitable radioactivity as previously described (7, 19). For assay of the ribonuclease-resistant labeled RNA, one half of each fraction was treated with 50 μg of pancreatic ribonuclease per ml for 35 min at room temperature in 2× SSC (0.3 M NaCl, 0.03 M sodium citrate, *p*H 7.0).

Exclusion chromatography was performed as described by Baltimore (3). Beaded 2% agarose was packed into a 1.5 by 45 cm column at room temperature. RNA was eluted from the column with 0.05% SDS buffer at a flow rate of 6 ml/hr; 0.7-ml fractions were collected and assayed for acid-precipitable radioactivity.

Materials. Beaded agarose (Sepharose 2 B) was purchased from Pharmacia Pure Chemicals; ^{14}C-UTP (0.25 c/mmole) was purchased from the Commissariat á l'Energie Atomique, Saclay, France; and ^{3}H-UTP (2 to 3 c/mmole) was purchased from New England Nuclear Corp. Ribonuclease-free deoxyribonuclease was purchased from Worthington Biochemical Corp. Actinomycin D was a gift from Merck, Sharpe and Dohme Laboratories.

RESULTS

Characteristics of the crude RNA polymerase. The crude poliovirus RNA polymerase used in these experiments was prepared from cells infected for 3.25 hr, i.e., at a time when the rate of viral RNA synthesis has reached its maximum and become constant (7). Crude polymerase is bound to RNA templates and, upon treatment with detergents, can be recovered in association with the replicative intermediate in the replication complex (14). Therefore, it does not require addition of exogenous viral RNA for further nucleotide incorporation.

The experiments to be reported here were performed with frozen crude extracts. It has been reported that mengovirus RNA polymerase loses its ability to synthesize viral RNA after freezing, although it retains its ability to synthesize double-stranded RNA (20). Therefore, it was necessary to make sure that no change in the activity or specificity of the poliovirus RNA polymerase occurred after freezing and thawing and that the

stored preparations behaved identically to freshly made extracts (1, 4, 5).

Figure 1 shows the kinetics of uridine monophosphate (UMP) incorporation in a standard assay. Under the conditions employed, the optimal temperature for incorporation was 36.4 C. At this temperature, UMP incorporation proceeded at a constantly decreasing rate for at least 2.5 hr. Incorporation was slower at lower temperatures and was inhibited by high temperatures. Over a period of 6 weeks, samples from the same frozen batch preparation showed less than a 10% change in activity. Neither change in the overall kinetics of UMP incorporation nor change in the kinetics of synthesis of viral single- and double-stranded RNA was detected (*see below*).

The omission of ATP or GTP, or both, resulted in over a 90% decrease in UMP incorporation (Table 1). The reaction was markedly stimulated by the addition of Mg^{++} (Table 1), but above concentrations of 5 mM no further increase in UMP incorporation was noted. It was also

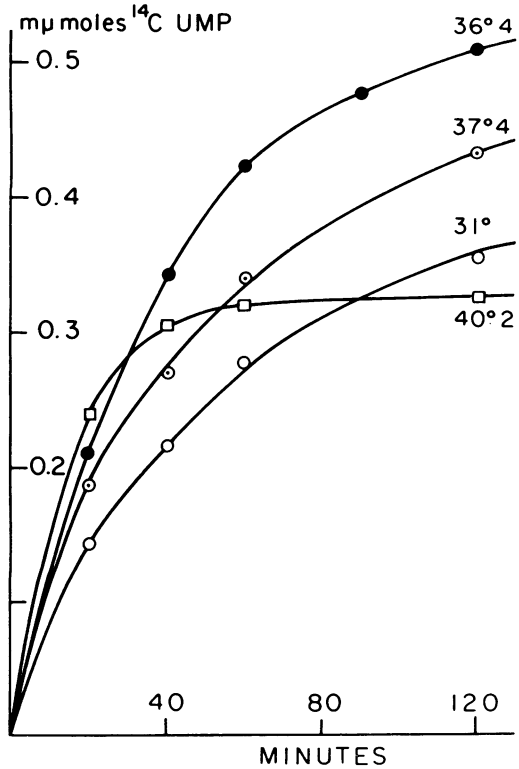

FIG. 1. *Kinetics of UMP incorporation. Crude RNA polymerase was incubated in a complete assay system at the various temperatures indicated. Each point represents the average acid-precipitable radioactivity of duplicate 0.5-ml samples (zero-time values substracted).*

TABLE 1. *Characteristics of RNA polymerase assay*[a]

Assay conditions	Amt of ^{3}H-UMP incorporated per mg of protein[b]
	counts/min
Complete system (6.5 mM Mg^{++})	5,600
−ATP	480
−ATP, −GTP	300
0.5 mM Mg^{++}[c]	1,300
1.5 mM Mg^{++}	2,400
2.5 mM Mg^{++}	3,800
12.5 mM Mg^{++}	5,400

[a] Incubation was for 100 min at 36.4 C.
[b] Average values from duplicate samples.
[c] No magnesium added in the assay; since polymerase buffer is 5 mM Mg^{++}, crude extracts contribute to 0.5 mM in the assay.

found that low concentrations of KCl had a marked stimulatory effect on the in vitro reaction (Fig. 2). In the absence of KCl, UMP incorporation halted after 30 to 40 min (curve A), whereas in the presence of 40 mM KCl it continued for at least another 2 hr (curve B). A similar effect of KCl has been reported in the case of deoxyribonucleic acid (DNA)-dependent RNA polymerase (12, 22). However, whereas DNA-dependent RNA polymerase is stimulated by 0.2 M KCl (22), poliovirus RNA replicase is inhibited by high KCl concentrations (curve C). In practice, crude viral polymerase was stored in polymerase buffer containing 0.4 M KCl and was diluted 1 to 10 in the assay.

The ability of frozen preparations to synthesize viral single- and double-stranded RNA was compared with that of freshly made extracts (1). For this purpose, samples of a reaction mixture were withdrawn after 15 and 40 min of incubation in vitro and were deproteinized with phenol according to method A. RNA was analyzed on sucrose gradients (Fig. 3). Most of the label incorporated during the first 15 min of incubation was found in material sedimenting heterogeneously from approximatively 16*S* to more than 50*S*, whereas after 40 min of incubation there was a marked peak of radioactivity at 35*S*, i.e., in the same position as viral RNA (9). All radioactivity could be hydrolyzed to acid-soluble material after 18 hr of incubation at 37 C in 0.3 N KOH. In another series of experiments, in vitro labeled RNA was deproteinized with SDS according to method B, with essentially the same results as those shown in Fig. 3.

Further evidence that the heterogeneous RNA (15-min sample) behaves like in vivo labeled replicative intermediate was obtained. Thus, it

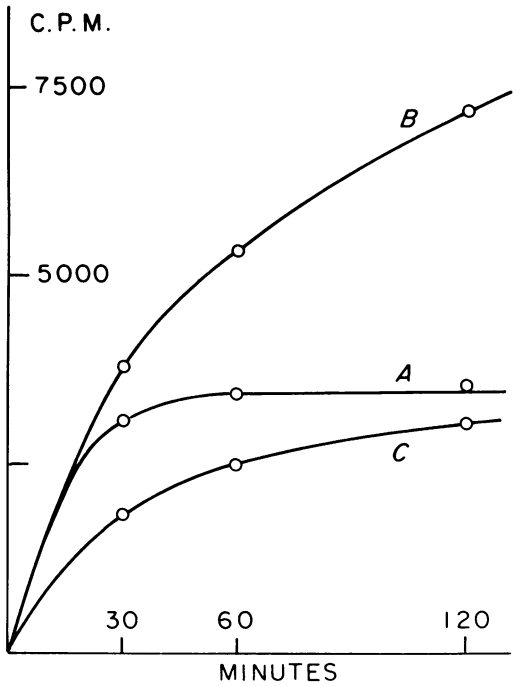

FIG. 2. *Stimulation by KCl of RNA polymerase activity. An RNA polymerase crude extract was prepared in polymerase buffer from which KCl had been omitted. It was stored frozen for 10 days in liquid nitrogen and then incubated in a complete assay system with various concentrations of KCl. Curve A, no KCl added; curve B, 30 mM KCl; curve C, 200 mM KCl. Each point represents the average acid-precipitable radioactivity of duplicate 0.5-ml samples.*

was precipitated by 2 M LiCl and excluded when chromatographed on 2% beaded agarose (*see* pulse-chase experiment). Some 15% of this LiCl-precipitable heterogeneous RNA was ribonuclease-resistant. The ribonuclease-resistant counts were distributed heterogeneously along the gradient from approximatively 16*S* to 50*S* (Fig. 5). They represent the double-stranded "core" of the replicative intermediate (6).

The radioactive material which sedimented as a sharp peak at 35*S* in the 40-min sample was ribonuclease-sensitive, LiCl-precipitable, and eluted as a broad peak from an agarose column. Its behavior was indistinguishable from in vivo labeled poliovirus RNA.

In addition, label accumulated with time in LiCl-soluble material. This material was completely resistant to ribonuclease and sedimented as a sharp peak at about 18*S* (Fig. 7); therefore, it is double-stranded RNA (2).

Synthesis of single-stranded 35*S* RNA and synthesis of double-stranded 18*S* RNA were found to proceed at relatively constant rates for more than 120 min, but single-stranded RNA was synthesized approximately four times faster than double-stranded RNA (not shown). As mentioned earlier, these kinetics were the same whether the crude RNA polymerase used had been stored for 2 to 6 weeks in liquid nitrogen.

Pulse-chase experiment. Since the major in vitro product of the crude RNA polymerase behaves like replicative intermediate after short periods of incubation but becomes viral RNA after longer incubation periods, it follows that the replicative intermediate is probably involved in the synthesis of viral RNA. Strong proof of the intermediate role of the replicative intermediate was obtained by a pulse-chase experiment.

To enhance the sensitivity of the assay, condi-

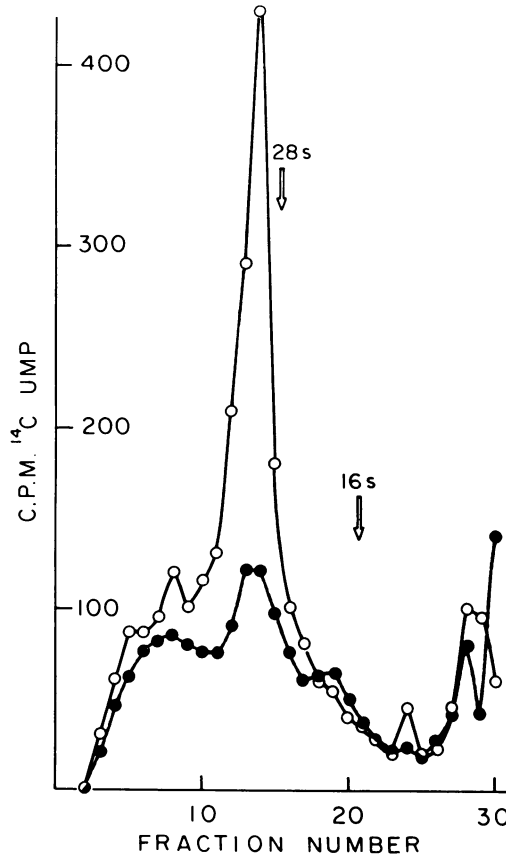

FIG. 3. *Sucrose gradient analysis of in vitro labeled RNA. RNA labeled in vitro was deproteinized by method A and was analyzed by sucrose gradient sedimentation. Centrifugation was for 17.5 hr at 24,500 rev/min and 22 C in an SW 25.1 rotor of a Spinco ultracentrifuge. Symbols: ●, 15 min of incubation; ○, 40 min of incubation. Arrows refer to the positions of various types of ribosomal RNA.*

tions were first sought which would slow down the in vitro reaction. It was found that decreasing the labeled UTP concentration in the assay mixture to 5 μM led to a much slower incorporation of UMP (Fig. 4). Under these conditions, it takes 100 min for 1 mg of protein of crude extract to catalyze the incorporation of 40 pmoles of UMP, whereas under standard assay conditions (200 μM UTP) this amount of UMP is incorporated in less than 10 min. Figure 4 also shows that when a 125-fold excess of cold UTP was added at 15.5 min, net incorporation of UMP immediately ceased.

Samples from such an experiment were withdrawn from the incubation mixture at various times before and after addition of excess cold UTP and were immediately deproteinized with SDS and precipitated with 2 M LiCl. Part of the LiCl-precipitated RNA was analyzed on sucrose gradients (Fig. 5). At 30 sec before the chase (Fig. 5*A*), labeled RNA molecules were distributed in a heterogeneous fashion from approximately 15*S* to 60*S*, with no indication of a peak in the position of viral RNA but rather a small peak at about 25*S*. Such a sedimentation pattern has been repeatedly observed with in vivo labeled purified replicative intermediate (M. Girard, Bull. Soc. Chim. Biol., *in press*). In addition, most of the label was resistant to ribonuclease digestion (hatched area). Figure 5*B* shows that, after 3 min of chase, approximately 50% of the label was in a broad 25*S* to 28*S* peak, whereas some of it was beginning to accumulate in the position of viral RNA. There was much less ribonuclease-resistant labeled material than in sample A. Three minutes later (Fig. 5*C*), a substantial amount of radio-

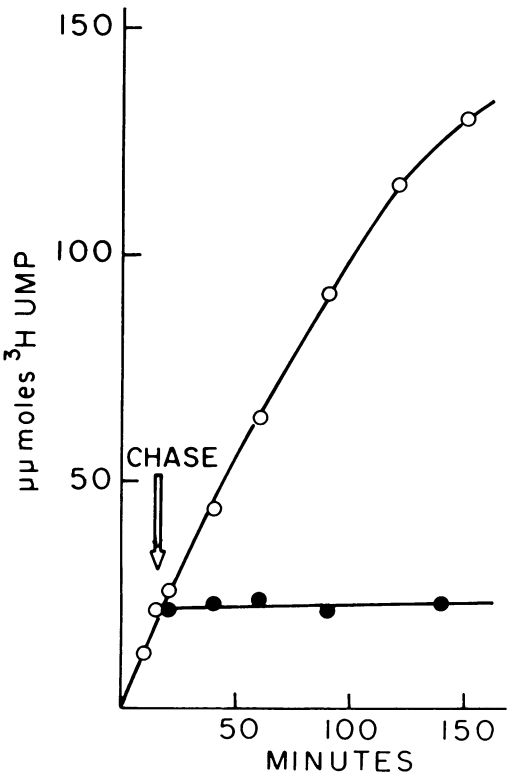

FIG. 4. *Kinetics of UMP incorporation under "pulse-chase" conditions. Crude RNA polymerase was incubated in a complete assay system, except that the concentration of tritiated UTP (500,000 counts per min per nmole) was lowered to 5 μM. Duplicate 0.3-ml samples were withdrawn and precipitated with trichloroacetic acid. After 15.5 min of incubation (arrow), a 125-fold excess of unlabeled UTP was added to a sample of the reaction mixture, and sampling was continued for an additional 2 hr. Symbols: ○, continuous labeling; ●, chased sample.*

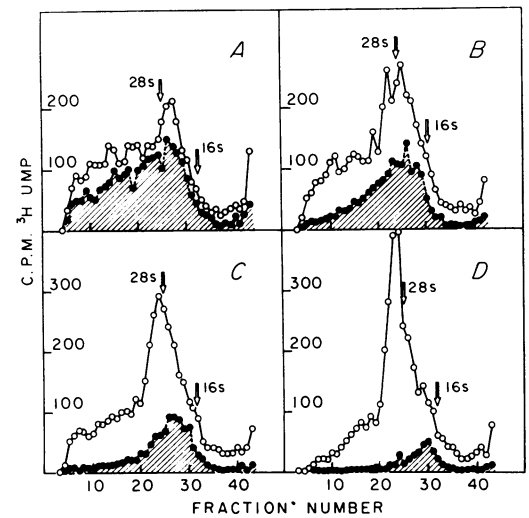

FIG. 5. *Sucrose gradient analysis of RNA labeled in vitro after a chase. Samples from the experiment illustrated in Fig. 4 were withdrawn at various times before and after addition of excess unlabeled UTP, and were immediately deproteinized with SDS and precipitated with 2 M LiCl by method B. Samples of the LiCl-precipitated material were analyzed by sucrose gradient sedimentation for 16 hr at 22,500 rev/min and 22 C in an SW 25.3 rotor of a Spinco L2 ultracentrifuge. One half of each fraction from the gradients was directly precipitated with trichloroacetic acid, whereas the other half was first incubated in the presence of 50 μg of pancreatic ribonuclease per ml for 35 min at room temperature. (A) LiCl-precipitated RNA 0.5 min before chase; (B) 3 min after chase; (C) 6 min after chase; (D) 20 min after chase. Hatched areas correspond to ribonuclease-resistant labeled material. Symbols: ●, total acid-precipitable radioactivity; ●, ribonuclease-resistant radioactivity.*

activity had accumulated in the position of viral RNA, whereas the percentage of radioactivity resistant to ribonuclease had decreased. Eventually, 20 min after the chase, the sedimentation profile of the in vitro product of the RNA polymerase was almost indistinguishable from in vivo labeled poliovirus RNA, and very little ribonuclease-resistant labeled RNA remained (Fig. 5*D*). Essentially the same results were obtained when the LiCl-precipitable types of RNA were analyzed by exclusion chromatography on agarose (Fig. 6). For brevity, only the first and last samples are shown. Whereas over 90% of the label was excluded from the agarose column at the time when the chase was done (left panel), this figure dropped to less than 20% after 20 min of chase (right panel).

LiCl supernatant fluids from the same samples were analyzed by sucrose gradient sedimentation after ethyl alcohol precipitation (Fig. 7). At the time when the chase was done, little if any label was found in double-stranded RNA. Most of the counts were found in material sedimenting faster than double-stranded RNA; they probably represent a minor part of the replicative intermediate which did not precipitate with 2 M LiCl. After the chase, however, label accumulated in a sharp peak at approximately 18*S*. This material was 100% resistant to ribonuclease, but was completely hydrolyzed to acid solubility by 0.3 N KOH. Therefore, it represents true double-stranded RNA.

Radioactivity in the replicative intermediate, viral RNA, and double-stranded RNA was corrected for optical density recoveries of ribosomal RNA in each sample. The chase of radioactive precursor was quite efficient, as judged from the fact that the total amounts of radioactivity in the various samples never differed from one another by more than 10%. Figure 8 shows the distribution of label between the various species of RNA as a function of time. At the time when the chase was done, a very large majority

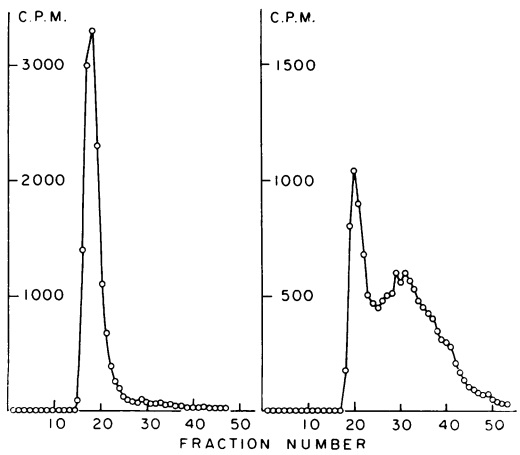

FIG. 6. *Exclusion chromatography analysis. Samples from the various LiCl-precipitable RNA shown in Fig. 5 were analyzed by exclusion chromatography on 2% agarose. Left panel: 0.5 min before chase; right panel: 20 min after chase.*

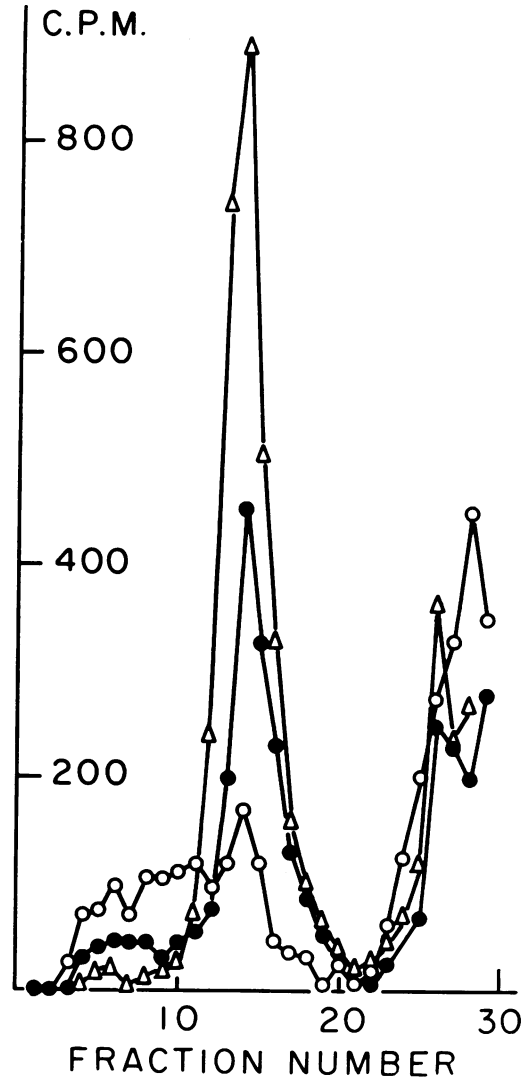

FIG. 7. *Accumulation of label in double-stranded RNA. Samples of the LiCl-soluble material from the experiment illustrated by Fig. 4 to 6 were precipitated with ethyl alcohol and then centrifuged on 15 to 30% sucrose gradients for 23 hr at 25,000 rev/min and 22 C in an SW 25.3 rotor of a Spinco L2 ultracentrifuge. Symbols: ○, 0.5 min before chase; ●, 6 min after chase; △, 20 min after chase.*

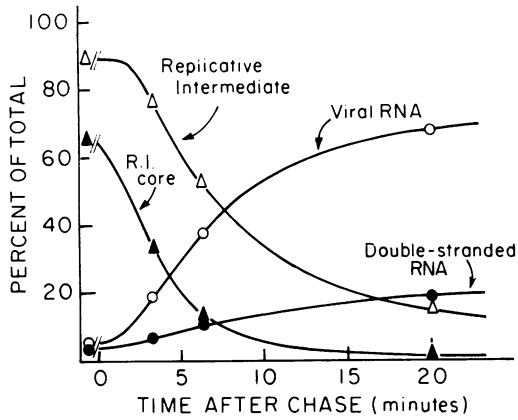

FIG. 8. *Repartition of label between the various species of RNA after a chase. Radioactivity in 35S viral RNA* (○), *double-stranded 18S RNA* (●), *total replicative intermediate* (△), *and ribonuclease-resistant portion of replicative intermediate* (▲) *from the pulse-chase experiment illustrated in Fig. 4 was computed from the results in Fig. 5 to 7 and plotted as per cent of total in function of time after chase.*

of the counts were in replicative intermediate, and most of them were in a form insensitive to ribonuclease. When excess cold UTP was added, radioactivity in the ribonuclease-resistant portion of the replicative intermediate (labeled "RI core" in Fig. 8) immediately started being chased out. Radioactivity in the replicative intermediate then decreased, whereas it progressively accumulated in both single and double-stranded types of RNA. Approximately 3.5 times more label ended up in 35*S* viral RNA than in double-stranded RNA. Since no detectable UMP incorporation took place during the period of chase, it is concluded that replicative intermediate is the precursor to poliovirus RNA.

DISCUSSION

The major in vitro product of crude poliovirus RNA polymerase preparation is single-stranded 35*S* RNA, together with a minor amount of double-stranded 18*S* RNA. After short periods of incubation, however, the major product of the polymerase is replicative intermediate. Such results are in agreement with the observations of Plagemann and Swim (20), who described, in the case of mengovirus RNA polymerase, the existence of a low-melting ribonuclease-resistant RNA which was preferentially labeled after short times of incubation in vitro and which exhibited the essential characteristics of the ribonuclease-resistant portion of replicative intermediate. However, contrary to these authors, it was found that RNA polymerase crude extracts can be stored frozen for several weeks without detectable alterations in their enzymatic activity or specificity.

The preferential in vivo labeling of replicative intermediate after short exposures of infected cells to radioactive uridine previously led to the suggestion that replicative intermediate is probably a precursor to viral RNA (6, 14). The preferential in vitro labeling of replicative intermediate after short periods of incubation of RNA polymerase with radioactive nucleoside triphosphates strengthens this conclusion. Conclusive evidence is found in the pulse-chase experiment reported here (Fig. 8). In this experiment, the label incorporated into replicative intermediate was displaced by the addition of excess unlabeled nucleoside triphosphate and appeared in completed viral RNA molecules. It is, therefore, concluded that replicative intermediate is the precursor to viral RNA, as is also the case for bacteriophage Qβ (18).

There are two features of the pulse-chase experiment which deserve some additional comment: (i) the high percentage of ribonuclease resistance in the replicative intermediate at the beginning of the chase (Fig. 5*A*); and (ii) the presence of an appreciable amount of label in double-stranded RNA at the end of the chase (Fig. 7 and 8).

The percentage of label which resists ribonuclease at the beginning of the chase (15.5 min of labeling) is much higher than previously described for replicative intermediate. This could be accounted for by the very slow rate of the reaction under the assay conditions used for the pulse labeling. Most of the labeled UMP incorporated at the end of the pulse would thus be confined to the portions of the nascent chains of viral RNA which are hydrogen-bonded to the RNA template (Fig. 9). Synthesis of RNA then proceeding in the presence of excess unlabeled UTP, the labeled hydrogen-bonded regions would be displaced from the template. The label therefore would become ribonuclease-sensitive, although still a part of the replicative intermediate (Fig. 5 and 8). It would eventually accumulate as free viral RNA molecules. In support of this hypothesis, it was found that the ribonuclease resistance of the replicative intermediate varied according to the length of the pulse labeling. Thus, after 10 min of labeling, over 90% of the label in the replicative intermediate was resistant to ribonuclease, whereas after 30 min this figure dropped to approximately 40%.

The appearance of label as double-stranded RNA during the period of chase was an unexpected result. It could be accounted for by

an immediate utilization of some of the newly synthesized viral RNA as templates for new complementary RNA molecules. It could also be explained by the following hypothesis, which is illustrated by the diagram in Fig. 9: assuming that there is no initiation of new RNA molecules in the crude in vitro system, the last nascent RNA chain on the replicative intermediate cannot be displaced by the progression of a new one, but remains hydrogen-bonded to its template. Therefore, with time, the replicative intermediate becomes smaller and smaller and finally is left as a double-stranded RNA molecule. It was previously calculated that there are four to five nascent chains of RNA to the replicative intermediate (14). If the above hypothesis is correct, there should therefore be three to four times more label in single-stranded than in double-stranded RNA at the end of the chase. The ratio found in the present experiment is 3.5 (Fig. 8).

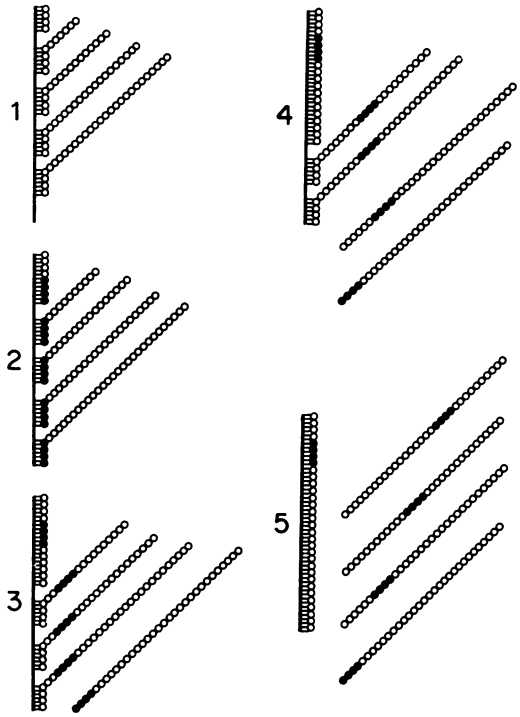

FIG. 9. *Model for the functioning of replicative intermediate in vitro. In (1), replicative intermediate is schematically representated as being made of five nascent viral RNA molecules (open circles), hydrogen bonded by their growing extremity to a complementary RNA template (solid line). RNA polymerase is assumed to be fixed to the complementary RNA molecule at the growing point of each nascent chain but has not been drawn on the diagram. In (2), upon in vitro incubation with labeled nucleoside triphosphates, radioactivity (closed circles) is incorporated into the growing strands of viral RNA, which displace each other along the template. Growth is then allowed in the presence of excess unlabeled nucleoside triphosphate. In (3), as a consequence, the previously labeled portion of each nascent chain is displaced from the hydrogen bonded state. Once completed, the partially labeled viral RNA molecules are released from the replicative intermediate (panels 3 to 5). The last viral strand, however, is not released but remains hydrogen bonded to its template. According to this hypothetical model, and as shown in (5), the end products of the in vitro incubation are one double-stranded RNA molecule to four single-stranded ones. Symbols: open circles, unlabeled portions of viral RNA strands; closed circles, labeled portions. Horizontal bars correspond to hydrogen bonded regions.*

In vivo, double-stranded RNA has been observed to accumulate in infected cells towards the end of infection, and it was previously suggested that it might then be a by-product of the replication process rather than a precursor (6). The hypothesis outlined above could offer a model for such a phenomenon. This implies the existence of two types of double-stranded RNA in infected cells, one being the immediate precursor to (18, 24, 25) and the other the "dead" form of the replicative intermediate. One type results from the synthesis of a new complementary strand on a viral RNA template, whereas the other type is formed upon completion of the last viral strand on the complementary RNA molecule of a replicative intermediate which stops functioning. The validity of this hypothesis and the reasons for the differences in the behavior of the two types of double-stranded RNA are not known at the present time. It is hoped that further insight into the mechanisms of poliovirus RNA replication will be gained through the use of purified RNA polymerase preparations.

ACKNOWLEDGMENTS

I thank A. Lwoff for continuous encouragement and many helpful discussions. J. Hurwitz's contribution in reviewing the manuscript is gratefully acknowledged. This work was supported by "Délégation Générale à la Recherche Scientifique et Technique" and by the French "Commissariat à l'Energie Atomique."

Louise Marty provided excellent technical assistance.

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