*AN ANALYSIS BY GEL ELECTROPHORESIS OF Qβ-RNA COMPLEXES FORMED DURING THE LATENT PERIOD OF AN IN VITRO SYNTHESIS**

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Communicated March 30, 1967

We have previously reported the synthesis of infectious[1, 2] RNA by a purified "replicase"[4] (a viral-induced RNA-dependent RNA polymerase). It was further established[3] by the use of a temperature-sensitive mutant that the RNA is the instructive agent in the replicative process. Examination of the early events in the reaction revealed[5] the existence of a six-minute latent period that precedes the appearance of the first new infectious strands. Further, this lag is accompanied by an eclipse as plaque-forming units of the input RNA, an event paralleled by the formation of a noninfectious complex having a sedimentation coefficient of 15*S*. The complex is partially (50%) ribonuclease resistant, and contains both initiating template and newly synthesized product. Finally, the complex gives rise to infectious strands on heat denaturation. These properties coincide with those of the structure (hereinafter referred to as Qβ-HS) found *in vivo* by Hofschneider and his colleague.[6]

The experiments cited employed sucrose gradients to follow the fate of the template and the early product. It became apparent as the investigation progressed that centrifugal analysis does not possess the resolving power necessary to establish unambiguously the relation between Qβ-HS and the structure recently reported by Franklin[7] (hereinafter referred to as Qβ-FS). We consequently explored the use of the electrophoretic separation on acrylamide gels[8] and found that viral RNA molecules, ranging in molecular weights from 3×10^5 to 2.3×10^6, could be resolved with ease and recovered from the gels with full retention of biological activity.[9] It was further established that suitably labeled Qβ-HS and Qβ-FS isolated from cells infected with Qβ-phage are readily separated from each other and from Qβ-RNA on acrylamide gels.[10] Further, the electrophoretic separation exhibited a precision and resolution far superior to that attainable in sucrose gradients.[11]

The present paper describes experiments which employ gel electrophoresis to examine the early fate of the input templates during *in vitro* synthesis of infectious Qβ-RNA. Particular attention is focused on the events in the latent and eclipse periods. The experiments establish that the first structure to appear involving template is Qβ-HS, which is then followed by the formation of Qβ-FS. Both complexes occur in the latent period which precedes the synthesis of new infectious units.

Materials and Methods.—(a) *RNA, enzyme, substrates, and assays:* Purification of Qβ-replicase[12] through the stages of cesium chloride and sucrose sedimentation,[2] synthesis of radioactive substrates,[4] assay for enzyme activity under standard conditions,[12] and liquid scintillation counting of acid-precipitable product on membrane filters[4] have all been detailed previously. P^{32}-labeled viral RNA (8×10^5 cpm per μg) employed as the initiating template was extracted from suitably labeled[13] Qβ-virus and further purified by centrifugation through sucrose gradients. Assay for infectious RNA with spheroplasts was as detailed previously.[2]

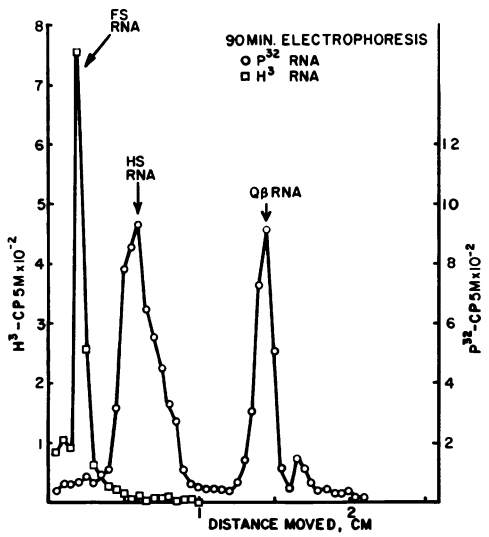

FIG. 1.—Separation Qβ-FS, Qβ-HS, and Qβ-RNA. H^3-labeled Franklin-structure RNA (Qβ-FS, 7×10^3 cpm, 0.1 μg) and P^{32}-labeled Hofschneider-structure RNA (Qβ-HS, 1.4×10^3 cpm, 0.005 μg) were mixed with P^{32}-labeled Qβ viral RNA (7×10^2 cpm, 0.001 μg) and subjected to electrophoresis on a polyacrylamide gel (0.7-cm diameter) for 90 min under standard conditions. The gels were sliced, dried, and counted. (The apparent low recovery of H^3 is due to quenching by the gel slice.)

(b) *Electrophoresis of purified Qβ-HS and Qβ-FS:* H^3-Qβ-FS and P^{32}-Qβ-HS were prepared from *E. coli* Q-13 infected with Qβ according to the procedures of Franklin[7] and Hofschneider,[6] respectively.

The methods of preparing the polyacrylamide 2.4 per cent gels, their polymerization, and handling in Plexiglass tubes have all been detailed elsewhere.[9, 11] After electrophoresis, gels were frozen in a right-angle aluminum trough on powdered dry ice and sequentially sectioned, using a CO_2 freezing microtome.

Mixtures of H^3-FS, P^{32}-Qβ-HS, and P^{32}-Qβ-RNA were subjected to 90-minute electrophoresis, using 0.7-cm diameter gels under standard conditions. It is apparent from Figure 1 that Qβ-HS and Qβ-FS separate from each other as well as

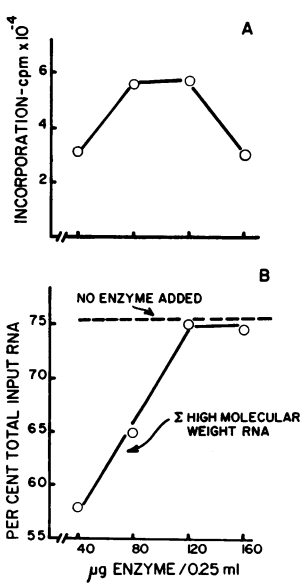

FIG. 2.—Optimal conditions for enzyme reactions. Standard reaction mixtures were prepared in which the concentration of enzyme varied from 40 μg to 160 μg per 0.25 ml. For measuring enzyme activity (*A*) the unlabeled template Qβ-RNA to protein ratio in each reaction mixture was (in μg) 1 RNA to 40 protein, and activity was measured by the incorporation of P^{32}-labeled UTP into acid-insoluble material after 20 min incubation at 38°. All other details as in legend of Fig. 3. To determine the preservation of template in high-molecular-weight RNA under similar experimental conditions, the P^{32}-labeled Qβ-viral RNA (8×10^5 cpm per μg) to protein ratio in each reaction mixture was (in μg) 0.1 RNA to 40 protein. The recovery of template in high-molecular-weight material (*B*) after 10 min incubation at 38° was determined by gel electrophoresis.

from Qβ-RNA. There is no evidence of any tendency for the Qβ-RNA to aggregate with either one of these complex structures. Qβ-HS has a relative electrophoretic mobility (REM) approximately 40 per cent that of Qβ-RNA, while the REM of Qβ-FS is 15 per cent that of Qβ-RNA. We have found that the distances traversed by Qβ-FS, Qβ-HS, and Qβ-RNA in *bis*-acrylamide cross-linked 2.4 per cent gels are proportional to the time of electrophoresis.

(c) *Optimal conditions for preservation of input primer and replicase activity:* The design of the experiments to be described required maximal involvement of the input RNA as templates in the reaction. Consequently, reactions were required at levels of initiating RNA well below saturation of the enzyme (1 γ of RNA for 40 γ of enzyme protein). Since our primary concern was with the fate of the template RNA, freedom from nuclease activity was even more important than in our previous experiments. It was found that increasing either the amount of replicase or the concentration of $(NH_4)_2SO_4$ suppressed the residual nuclease. However, the high levels of $(NH_4)_2SO_4$ also led to some loss of activity. Since the enzyme is normally stored in 10 per cent $(NH_4)_2SO_4$, the effect of adding increasing levels of stock solution of enzyme was examined with results as shown in Figure 2. It is evident that a mixture containing 120 γ of replicase protein per 0.25 ml provides the optimal conditions required. At this level of protein, the enzyme showed maximum activity (Fig. 2*A*), while no detectable fragmentation of input template (0.3 γ or less per 0.25 ml) was observed on gel electrophoresis (see Fig. 2*B*)—an extremely sensitive test. It has been observed that the replicase reaction proceeds approximately two times as fast at 38°C as at 35°C. To maximize template involvement and synthetic rate, all reactions here were performed at 38°C.

Results.—It was first desirable to examine the synthesis and biological activity of the product under the conditions in which the ratio of template to enzyme is far below saturation. Figure 3 shows the outcome in terms of the incorporation of P^{32}-uridine triphosphate (P^{32}-UTP) into insoluble material and the appearance of

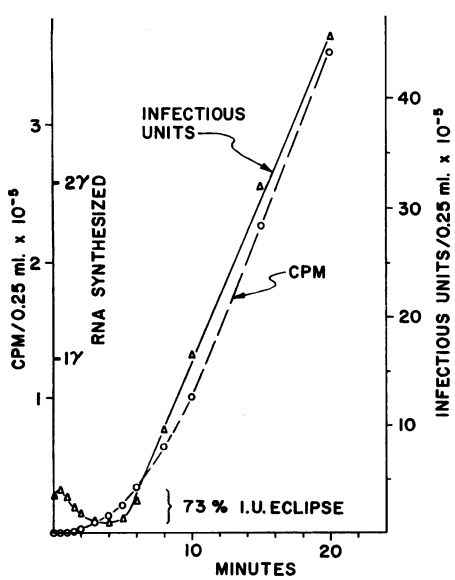

FIG. 3.—Enzyme activity and the synthesis of infectious RNA. A standard reaction (0.75 ml) containing 0.6 μmoles each of GTP, CTP, and ATP, 0.6 μmoles of P^{32}-labeled UTP (1.6×10^8 cpm per μmole), 9 μmoles $MgCl_2$, 60 μmoles tris-HCl buffer pH 7.4, 2.2 μmoles Mg-EDTA, 360 μg enzyme preparation were incubated at 38° with 0.3 μg unlabeled Qβ-RNA. Aliquots (0.05-ml) were removed at intervals to determine the acid-insoluble radioactivity and total infectious RNA by the pronase-SDS procedure.[5]

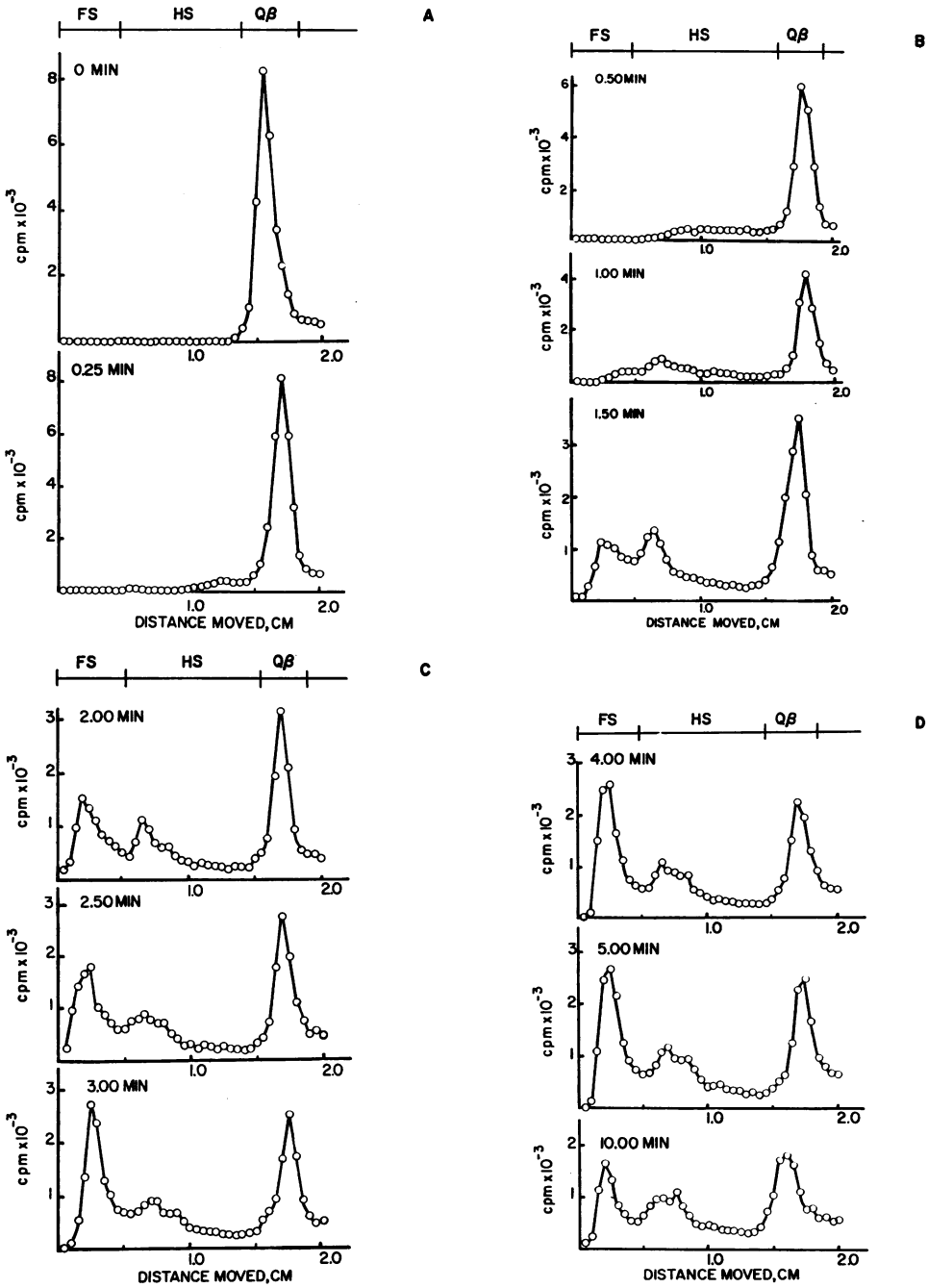

FIG. 4.—Involvement of template RNA. A 0.75-ml reaction mixture (as detailed in legend for Fig. 3) containing unlabeled triphosphates was incubated at 38° with P^{32}-labeled Qβ-RNA (0.9 μg, 8 × 10^5 cpm per μg). Aliquots were removed at the indicated time intervals, mixed with sucrose (10% final concentration) and SDS (0.2% final concentration), and subjected to polyacrylamide electrophoresis (0.9-cm diameter gels) under standard conditions. The gels were frozen, sliced, dried, and counted to determine the distribution of radioactivity. The regions of gel in which Qβ-FS, Qβ-HS, and Qβ are situated are indicated at the top of each panel.

infectious RNA. An initial eclipse of infectious RNA is evident which corresponds to 73 per cent of the input at four minutes. The latent period extends to about six minutes, subsequent to which synthesis of new infectious material begins.

Our primary concern here is the detailed fate of the input RNA. Consequently, P^{32}-labeled Qβ-RNA (8×10^5 cpm per μg) was used as a template in a reaction similar to that of the experiment described in Figure 3, except that here the product was not isotopically labeled. Aliquots were removed at the indicated time intervals, mixed with sodium dodecylsulphate (0.1% final concentration) to dissociate RNA from protein, and made 10 per cent with respect to sucrose. The samples were then loaded on *bis*-acrylamide cross-linked 2.4 per cent gels (0.9 cm diameter) and subjected to electrophoresis for 180 minutes under standard conditions. The gels were then frozen and sliced into 0.5-mm sections, which were then dried on filter paper and counted in a liquid scintillation counter. The profiles obtained from the various samples are given in the panels of Figure 4, which record only the high-molecular-weight regions of the gels.

The zero time sample shows that 67 per cent of the RNA primer was recovered in the Qβ-RNA region, situated about 1.7 cm from the origin. It has been our experience that viral RNA preparations purified by the commonly used sucrose gradient method may be contaminated to the extent of 10–40 per cent with biologically inactive fragments which are easily detected as fast-moving components on gels.

Comparison with the zero time sample reveals that after 0.25 minutes of incubation, some of the primer RNA moves more slowly than the original Qβ peak. This trailing is more pronounced by 1 minute, and by 1.50 minutes two main slow-moving complexes containing the initiating RNA are already apparent. The REM's of these two complexes are 14 per cent and 39 per cent that of the original Qβ-RNA peak and correspond, therefore, to FS and HS, respectively (Fig. 1). Between 1.50 minutes and 3.0 minutes, more primer RNA is found associated with the FS material and the ratio of the amounts of P^{32}-template in these two peaks does not substantially change between 3 and 5 minutes. However, by 10 minutes there is some loss from the FS region.

It must be recalled that P^{32}-RNA distributed in the three regions of Figure 4 represent only the high-molecular-weight components, constituting 70 per cent of the input material. Summation of the counts found in each region over the entire gel provides a complete kinetic picture of the progress of the reaction. Figure 5 shows the behavior of the initiating template in the Qβ region and also the amount of P^{32}-RNA found in the fragment region which corresponds to the gel segment extending from 2 cm to the end. Several features are immediately evident. RNA is lost from the Qβ region exponentially during the first few minutes and by three minutes reaches a plateau when about 69 per cent of the Qβ-RNA has left to become involved in either HS or FS. Note that this is in rather good agreement with the 73 per cent loss in original PFU as determined in the experiment of Figure 1 as well as in previous experiments.[5] Further, the percentage of the total RNA recovered as small-molecular-weight fragments is essentially constant and equal to the amount found in the input sample, indicating that there was little or no degradation of high-molecular-weight RNA during the course of the experiment.

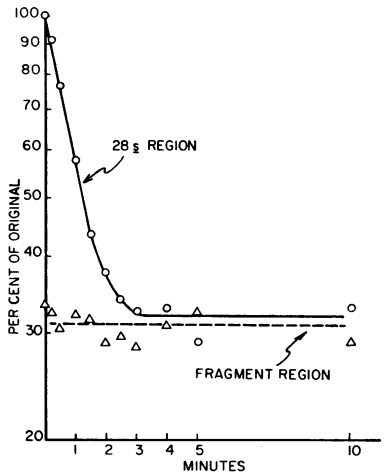

FIG. 5.—Recovery of template in viral RNA and fragments. The amounts of P^{32}-RNA recovered in Qβ-RNA and fragment (SMW) regions of the gels of Fig. 4 plotted as the percentages of the initial found in the 28S region for Qβ-RNA and as percentages of the *total RNA* applied to each gel for the fragment fraction.

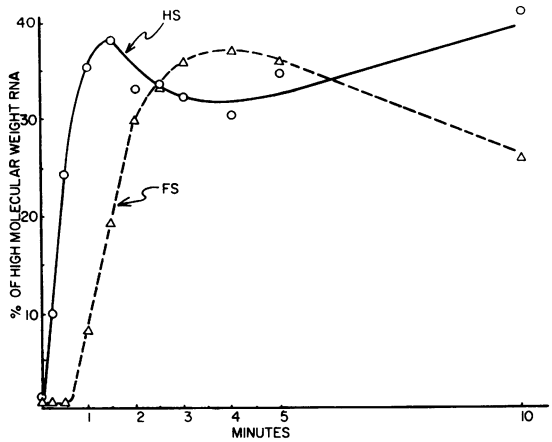

FIG. 6.—Recovery of template in Qβ-FS and Qβ-HS. The amounts of P^{32}-RNA recovered in the Qβ-FS and Qβ-HS regions of the gels of Fig. 4 are plotted as the percentages of high-molecular-weight RNA recovered from each gel. High-molecular-weight RNA is the sum of the Qβ-FS, Qβ-HS, and Qβ viral RNA species. Fragments represent the sum of the counts found to the left of these regions in the remainder of the gel.

Figure 6 provides a similar summation in terms of the amount of P^{32}-RNA to be found in the FS and HS regions. It is evident that initiating template enters into the Qβ-HS region immediately, whereas none is to be found in the FS region until 1 minute has elapsed. A maximum is reached in the HS region at 1.5 minutes followed by a slight decline. The FS region attains its maximum of P^{32}-RNA at about 3.5 minutes. From the fourth to the tenth minute, there is a slight loss of initiating template from the Qβ-FS region.

Discussion.—The FS and HS regions of Figure 4 reveal a much greater heterogeneity than the corresponding regions of Figure 1. This is not surprising since in the case of Figure 4 there is no prior fractionation; all the components found in the reaction are represented in various stages of completion. In contrast, the preparative and chromatographic procedures[6, 7] necessary to purify HS and FS from infected cells for the profile of Figure 1 could well have preferentially enriched for a particular class.

We have demonstrated elsewhere[9] that the log of the molecular weight of a single-stranded RNA molecule is linearly related to its REM. This suggests that the electrophoretic separation observed in the gels is based on molecular sieving in which the specific volume determines relative mobility.

The initial event observed (Figs. 3 and 4) in the latent period of synthesis is the involvement of the initiating template in a noninfectious complex having a lower REM than Qβ-RNA, suggesting the formation of an enlarged structure. It is clear that these observations are consistent with a duplex intermediate implied by experiments with mutants[14] and suggested by the presence of partially ribonuclease resistant structures first observed[15] with an animal virus and also found in cells

infected with RNA bacteriophages.[16-21] The experiments described here are also in agreement with our previous[5] examination of the Qβ-replicase reaction using sucrose gradients. The gel electrophoresis method is, however, obviously more informative since it clearly identifies the early appearance of two classes of complexes involving template and permits their ready separation.

The summary of the early events shown in Figure 6 indicates that both complexes are formed in the latent period prior to the appearance of new infectious units. It is also evident that HS is the first structure to be formed, followed by FS. Further, the total amount of initiating template involved in both agrees with the decrease in original PFU observed in the eclipse period.

The facts reported here and previously[5] on the *in vitro* synthesis are all *consistent* with a mechanism[17, 20, 22] that postulates that HS is the first replicative intermediate and FS the second, the latter being used to generate new viral RNA. Nevertheless, it must again be emphasized that this plausible mechanism has not yet been established. We need detailed chemical information on the nature of the complexes synthesized by the replicase. In particular, we must know the size, base composition, and nearest-neighbor frequency of the strands complexed to the initiating templates in both kinds of structures. With this information available, one can decide if full complements are present and whether they are parallel or antiparallel. Further, a detailed analysis of the flow of material among the three components in "pulse-chase" experiments is requisite for a complete delineation of the mechanism. The use of double-labeling to identify negative and positive components of the structures, combined with electrophoretic separation on gels, makes many of these experiments technically feasible.

With such information available, necessary rather than suggestive conclusions on the chemistry of the replication can be drawn. Many of the relevant experiments have been completed and the results will be reported in subsequent publications.

Summary.—Electrophoretic separation on acrylamide gels was used to follow the progress of infectious viral RNA synthesis by purified Qβ-replicase. Particular attention was focused on the fate of the initiating templates during the latent and eclipse periods[5] that precede the appearance of the first complete strands. It has been shown that two classes of complexes containing initiating template materialize in the latent period. Their emergence is paralleled quantitatively by a concomitant loss of the input RNA as infectious entities. The first complex to appear (HS) corresponds in its properties to the structure found *in vivo* by Hofschneider[6, 22] and his colleague. The second complex (FS) makes its appearance about one minute later and resembles the "replicative intermediate" isolated from infected cells by Franklin.[7] Although not as yet conclusive, the temporal order of their appearance is consistent with a mechanism which suggests that HS gives rise to FS which, in turn, generates viral RNA.

The ease with which these components can be detected and separated by gel electrophoresis makes it technically feasible to perform the experiments required to delineate definitively the chemical details of the RNA-replicating mechanism.

* This investigation was supported by U.S. Public Health Service research grant no. CA-01094 from the National Cancer Institute and grant no. GB-4876 from the National Science Foundation.

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