*THE KINETICS OF PRODUCT APPEARANCE AND TEMPLATE INVOLVEMENT IN THE IN VITRO REPLICATION OF VIRAL RNA**

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We have previously reported the synthesis of biologically competent[1-3] RNA by a purified Qβ-replicase[4] (an RNA-dependent RNA-polymerase induced by the bacteriophage Qβ). This enzyme, like its MS-2 counterpart,[5] requires intact[6] homologous[4, 5] RNA as a template. Fiers and his colleagues confirmed[7] this selectivity with MS-2 replicase. The demonstration[1-3] that the purified enzyme was in fact generating infectious copies of the template provided an acceptable experimental system for the *in vitro* examination of the replicating mechanism.

An unambiguous analysis required extensive involvement of the initiating templates. Further, a method was needed for examining the products of the reaction which avoids phenol purification, a procedure which can lead to selective loss of particular constituents. The first condition was met by the use of freshly prepared viral RNA and the second by the finding that prior treatment with sodium dodecyl sulfate permitted direct inspection of template and product with complete recovery of both. The use of these procedures and sucrose gradients revealed[8] the existence of a six-minute latent period which precedes the appearance of the first new infectious strands. Further, this lag is accompanied by an apparent loss of the initiating RNA as infectious entities, an event accompanied by the appearance of noninfections complexes containing both product and template and capable of yielding infectious material on heat denaturation.

It became apparent that sucrose gradients did not possess the resolving power required to identify with precision all the possible components and stages of the reactions. Electrophoretic separation in acrylamide gels[9, 10] proved to be far superior.[11] Employing this technique, it was possible to show[12] that two classes of complexes containing the initiating templates materialized in the latent period. The first complexes (HS) to appear correspond to the structures found *in vivo* by Francke and Hofschneider[13] and one minute later a second class (FS) is synthesized which resembles the "replicative intermediate" identified by Franklin[14] in cells infected with R-17. The temporal order of their appearance is consistent with a mechanism which suggests that HS structures give rise to FS which, in turn, generate viral RNA.

The experiments described focused attention on the initiating templates. It was clearly necessary to extend our information by a simultaneous examination of the early products and their physical relation to the templates. This required the synthesis of H^3-Qβ-RNA labeled at high specific activity and the use of P^{32} to identify the product. The necessary experiments have been done and it is the purpose of the present paper to describe the results.

Methods.—(a) *Enzyme, substrates, and assays:* Purification of the Qβ-replicase through the stages of CsCl and sucrose sedimentation,[2] synthesis of radioactive substrates,[5] assay of enzyme under standard conditions,[6] and liquid scintillation counting on membrane filters have been detailed previously. High specific activity H^3-Qβ-RNA was extracted from Qβ virus purified from a 25-ml lysate of *Escherichia coli* K-38 in 3XD medium[15] containing 15 mc H^3-adenosine (3.1 c/mmole), 15 mc H^3-cytidine (18.0 c/mmole), and 20 mc H^3-uridine (22.0 c/mmole). The purified Qβ-RNA possessed a specific activity of 1.2×10^6 cpm/μg.

(b) *Kinetic analysis of RNA and infectious unit synthesis:* A 2.5 ml standard reaction mixture[6] containing 7.5 μmoles Mg ethylenediaminetetraacetate (EDTA), 1.2 mg replicase protein, and UTP labeled with P^{32} (1.14×10^9 cpm/μm) in the α position was warmed to 38°. To the warmed reaction mixture was added 1.0 μg H^3-Qβ-RNA (1.2×10^6 cpm/μg), and the incubation continued at 38°. At intervals, aliquots were withdrawn and added to 0.1 volume of 2.5% SDS. A 0 time sample (0.125 ml) was identical in composition to the 2.5-ml reaction, except that 0.01 ml 2.5% sodium dodecyl sulfate (SDS) was added prior to addition of the H^3-Qβ-RNA. To determine the titer of infectious RNA in each aliquot 0.02 ml of each sample was mixed with 0.02 ml of 0.006 *M* EDTA-Na, pH 7.4, 1 mg/ml pronase (Calbiochem, B grade, predigested for 2 hr at 35°), 0.4% SDS, and allowed to digest for 20 min at 35°. After digestion, the sample was diluted with 0.76 ml of 0.003 *M* EDTA-Na, pH 7.4, and assayed directly for infectious units as previously described.[2] A second 0.02-ml aliquot was precipitated and washed on a cellulose nitrate membrane with cold 10% trichloroacetic acid for liquid scintillation counting.

A third sample (0.06–0.18 ml) from each original aliquot was adjusted to approximately 10% sucrose and layered over 0.9 × 9 cm, *bis*-acrylamide cross-linked 2.4% unswollen polyacrylamide gels prepared as described previously.[10] Electrophoresis was carried out for 3 hr at 10 ma per gel (approximately 6 v/cm gel). After electrophoresis, gels were frozen in aluminum troughs on dry ice and sliced sequentially using a CO_2-cooled microtome. Each 0.5-mm slice was soaked overnight at 4°C in 5 ml of 10% TCA and washed on cellulose nitrate membrane filters with 15 ml of cold 10% TCA to remove acid-soluble radioactive materials, dried, and dissolved in 0.5 ml 30% H_2O_2 by overnight incubation at 65°. Samples were assayed for H^3 and P^{32} counts in Kinard's scintillation fluid as described previously.[10] All counts were corrected for background and cross channel overlap.

Results.—A replicase reaction was carried out as detailed in the legend of Figure 1 and analyzed as described in *Methods* (*b*). As noted, the initiating templates are identifiable by the H^3-label and the product by the P^{32} in P^{32}-UTP. Figure 1 summarizes the progress of the synthesis in terms of the new RNA and infectious units synthesized. The curves exhibit the usual feature of a latent period accompanied by an initial loss (64%) in the infectivity of the input templates. This is followed in six to seven minutes by an emergence from the latent period signalled by the onset of a net increase in the number of infective units. At the termination of the experi-

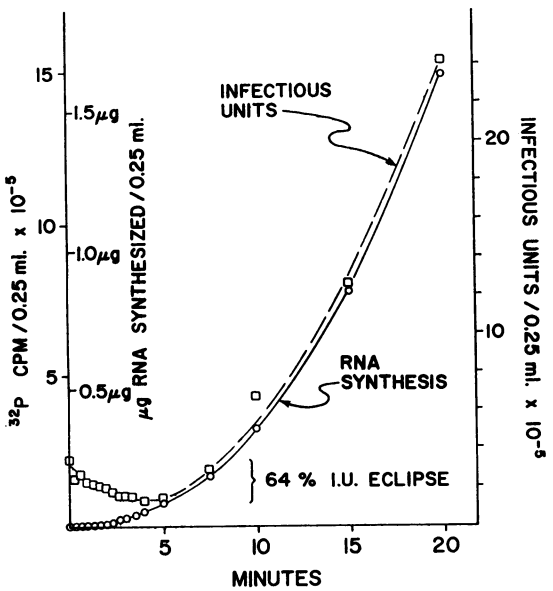

FIG. 1.—Synthesis of RNA and infectious units. A tenfold (2.5-ml) standard reaction as detailed in *Methods* was carried out at 38° with H^3-labeled template and P^{32} incorporation signifying product. From the reaction, aliquots were withdrawn at intervals for examination of acid-insoluble radioactivity (RNA product) and infectious units as described in *Methods*.

ment, an amount of biologically active RNA has been synthesized corresponding to 17 times the starting material.

An aliquot of each sample in Figure 1 was submitted to electrophoresis through acrylamide gels with results of some of the profiles shown in Figures 2 and 3. The

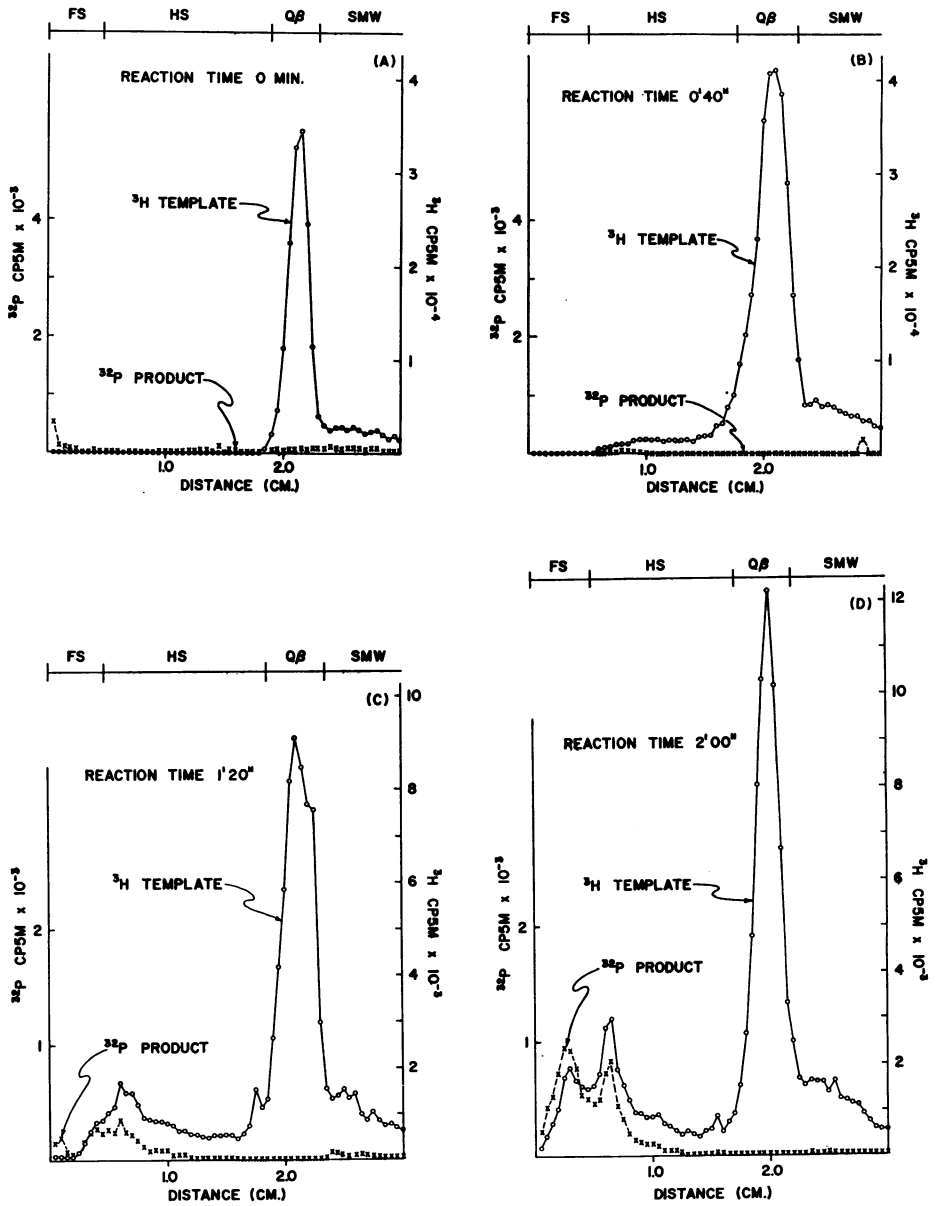

FIG. 2.—Acrylamide gel analysis of reactions. The 0- to 2-min samples drawn from the reaction detailed in Fig. 1 were submitted to electrophoresis through 2.4% unswollen *bis*-acrylamide gels as detailed in *Methods*. As described in *Methods*, 0.5-mm slices of the gels were washed with TCA to remove soluble radioactivity and monitored for H^3 and P^{32} radioactivity.

gels are defined on the basis of our earlier investigations into regions which contain the Franklin structures (FS), Hofschneider structures (HS), mature 28*S* viral RNA (Qβ), and small molecular weight (SMW) fragments. It should be noted that although the entire gel is cut and counted in every case, to conserve space, not all of the SM*W* regions are shown.

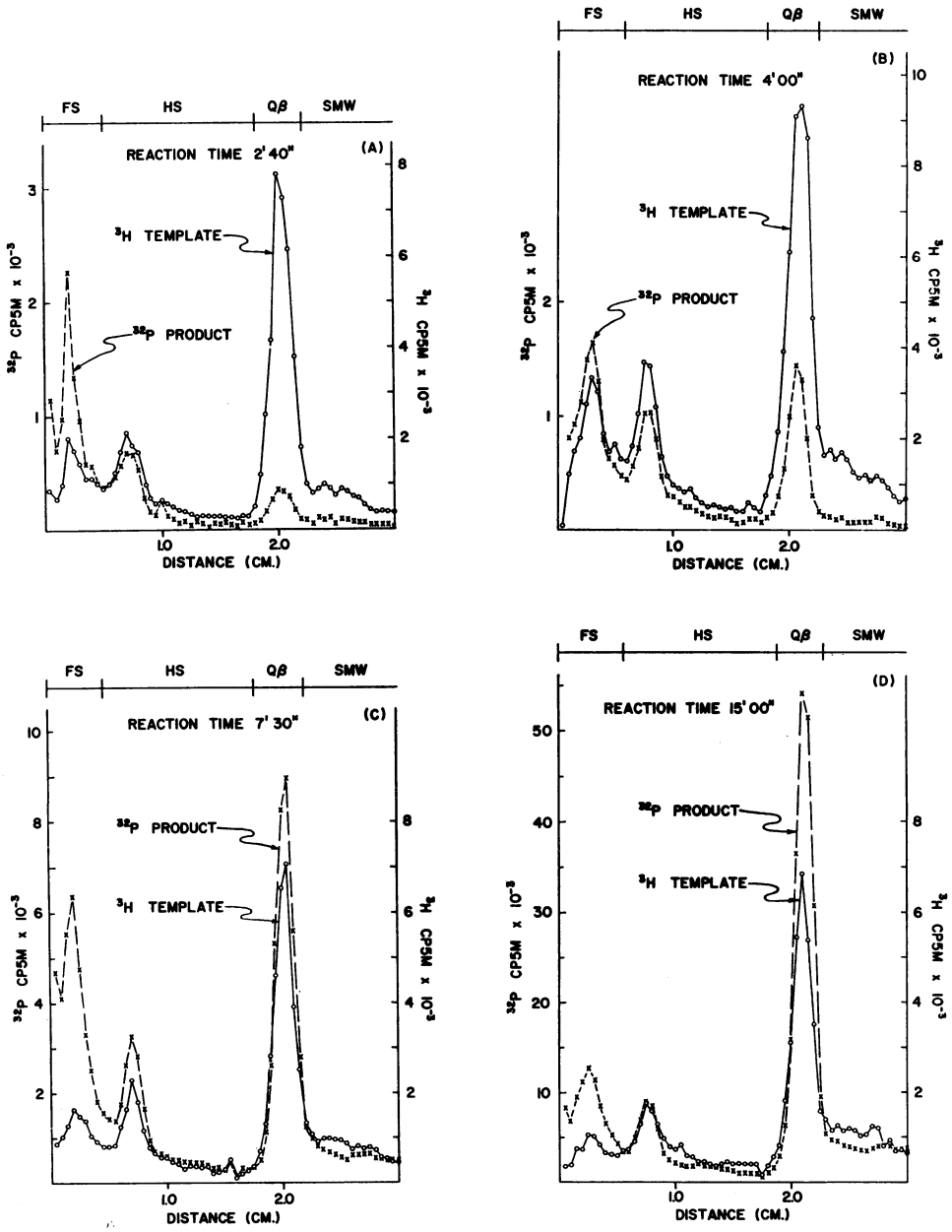

FIG. 3.—Acrylamide gel analysis of the reaction. The 2-min 40-sec to 15-min samples drawn from the reaction detailed in Fig. 1 were analyzed by gel electrophoresis as described in *Methods*.

In the zero time sample (Fig. 2A) 80 per cent of the H^3 template falls within the mature Qβ-RNA region. The remainder is found in the small molecular weight portion, a situation usually observed with Qβ-RNA purified through sucrose gradients. By 0 minutes 40 seconds (Fig. 2B) a significant portion (14%) of input template has moved into the HS region. Note that no P^{32} product is as yet observed in this region, a fact probably ascribable to the difficulty of detecting the low level of synthesis attained in the first 40 seconds. Forty seconds later (Fig. 2C) P^{32}-product is seen mostly in the HS region in association with H^3 template. In the two-minute sample (Fig. 2D) FS and HS complexes are evident, containing both template and new product, but without any evidence of mature viral RNA. Slight indication of the appearance of 28S is first seen at two minutes 20 seconds and clear evidence is seen 20 seconds later (Fig. 3A). In the subsequent samples examined (Fig. 3B, C, D) the 28S variety accumulates rapidly.

To provide an over-all picture of the flow pattern among components, P^{32} and H^3 radioactivities were summed in each of the defined regions of the gel for every sample. With regard to the fate of the H^3-labeled initiating templates and in agreement with our earlier findings[12], we found that templates appear first in the HS structures and this is followed by their involvement in FS complexes.

We now focus our attention on the behavior of the product and its distribution among HS, FS, and Qβ-RNA as a function of time. Figure 4 graphically summarizes the net accumulation of P^{32}-product in the various components as the replicase reaction proceeds. The inset to Figure 4 magnifies by ordinate expansion the results obtained with samples taken in the first four minutes of the synthesis, the interval of greatest interest. As is apparent, the earliest detectable P^{32}-product is found in the HS region, clear evidence of accumulation being observed at one minute and before any signs of entry of product into the FS complexes. It will be noted (inset of Fig. 4) that as FS synthesis becomes appreciable, the formation of HS is temporarily diminished. Similarly, the onset of Qβ-RNA synthesis at about 2.5 minutes is accompanied by a temporary diminution in the rate of accumulation of FS.

Figure 5 gives the relative quantities of each species at each point of time. Included are the small molecular weight regions of the gel in order to illustrate their relation to the other components.

The HS structures containing product appear first and increase to a maximum at about two minutes. As the HS decrease, the Qβ-FS appear and reach their maximal proportion at a little over two minutes, soon after which 28S Qβ-RNA is synthesized. It should be noted that the RNA in the SMW regions begins to increase simultaneously with the production of mature RNA molecules. The fragments do not therefore play a role in the events occurring in the latent periods. The fact that fragmentation of intact H^3-28S of the input templates does not occur suggests that the accumulation of the P^{32} fragments is due primarily to aborted syntheses rather than nucleolytic degradation.

Discussion.—In evaluating the significance of the experiments reported, it is important to note that all samples were analyzed directly after treatment with SDS. There was no prior fractionation by procedures which can lead to selective loss or enrichment of particular components. The full spectrum of heterogeneity of the HS and FS complexes is represented as is evident from the marked polydispersity seen in the earlier phases of the synthesis (Figs. 2B–D). This is not unexpected since the

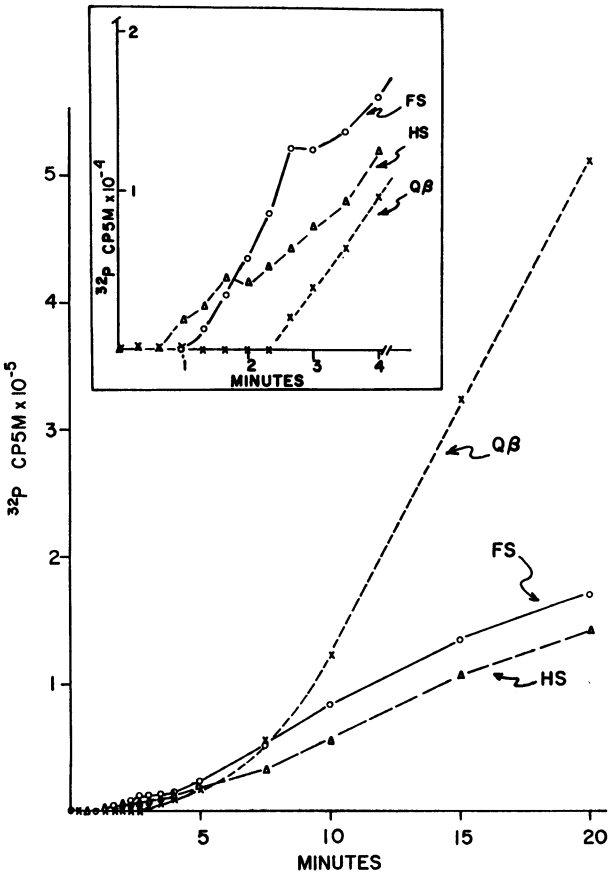

FIG. 4.—Kinetic accumulation of product. P^{32} Cp5m in all the gel profiles were summed over the regions as indicated in Figs. 2 and 3. Summations over profiles were normalized to unit recovery on the basis of H^3 recovery in each gel, relative to H^3 recovery in the zero time gel. Product cp5m are presented as *accumulated* materials. The inset magnifies the first 4 min of the reaction course.

HS and FS structures are classes of heterogeneous complexes caught at various stages of completion.

The data described provide precise information relevant to an understanding of the replicative process. It is clear that the sequence of events which emerges is the same whether attention is focused on template involvement or product synthesized. The most revealing events occur in the eclipse and latent periods (Fig. 1) and the salient features may be summarized as follows: (*a*) Initiating templates leave the 28*S* region and appear first as noninfectious complexes in the HS region. (*b*) *All* of the first product synthesized appears initially as material complexed to H^3-templates in the HS region. (*c*) *After* the HS structures are formed, complexes containing both template and product appear in the FS region of the gel. (*d*) The emergence of the HS and FS structures is paralleled by a concomitant loss of the input RNA as infectious entities. (*e*) New 28S-Qβ-RNA is synthesized *after* the FS structures have appeared.

Our observations are not readily explained by the claims of Weissmann and his collaborators[16, 17] that *free* negative strands are produced initially and that duplexes are artifacts of protein removal. In the first place, as we shall show elsewhere, free negatives move somewhat slower on gels than positives and we have thus far not de-

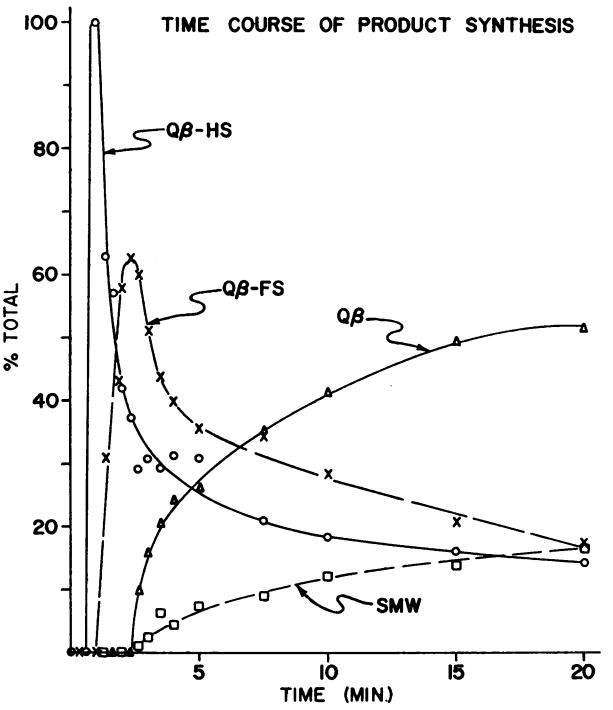

FIG. 5.—Relative quantities of product in the reaction. The data from Fig. 4 for each species are considered as a per cent of the total P^{32}-product recovered from respective gels.

tected any evidence of their existence in our reactions. In addition, since *all* of our early product is complexed to template, one would have to invoke complete and perfect annealing simply on adding SDS to our reaction. A plausible explanation of their findings has not been eliminated by Weissmann *et al.*, and stems from the presence of *active* replicase during their assay for ribonuclease sensitivity and sucrose gradient analysis of early product. The active replicase could by its ability to open up the duplex slowly make it available to ribonuclease digestion, or similarly release free negatives during a long sucrose gradient centrifugation.

The temporal order of their appearance and the fact that the complexes contain both initiating template and newly synthesized product are *consistent* with a mechanism of synthesis which involves the following sequence of steps:

$$\text{Template} \dashrightarrow \text{HS} \dashrightarrow \text{FS} \dashrightarrow \text{Q}\beta\text{-RNA}.$$

It is generally held[18–22] that the conversion of initial template (plus strand) to HS requires the synthesis of a complementary (negative) strand to form a hydrogen-bonded double-stranded duplex. The latter is then used to generate new plus strands, and in the process the HS structures are converted to the multistranded FS structures.

This generally accepted model makes certain predictions concerning the detailed chemistry of the HS and FS structures and their behavior in "pulse-chase" labeling

experiments. Thus, the first strands synthesized should be complementary in base composition to the templates. Further, a nearest-neighbor analysis should enable one to decide whether the complement synthesized is parallel or antiparallel to the templates. The ease with which the complexes can be synthesized *in vitro* with suitable labels on the two components and subsequently isolated on gels made it possible to perform the necessary experiments. These and other relevant results will be detailed in subsequent publications.

Summary.—The early stages in the synthesis of infectious viral RNA by purified Qβ-replicase has been examined in detail by gel electrophoresis. The present study employed H^3-template and the synthesized product was labeled with P^{32}, permitting a simultaneous examination of the behavior of both. The data obtained may best be summarized as follows: (*a*) All of the first product synthesized is found complexed to H^3-template possessing the properties of Hofschneider[13] structures (HS). (*b*) Subsequently Franklin[14] structures (FS) appear containing template and early product. (*c*) Soon after the formation of FS, mature Qβ-RNA is synthesized. The temporal sequence is *consistent* with a mechanism involving the following series of events:

Template ---------〉 HS---------〉 FS---------〉 Qβ-RNA.

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