outlined here serves well for such studies and provides the possibility of distinguishing the actions of various growth regulators on the enlargement and differentiation of immature sieve cells.

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12 January 1966

Replication of the RNA of Bacteriophage f2

Abstract. *Events occurring after infection of bacteria with wild-type and a temperature-sensitive mutant phage indicate that there are two enzymatic activities necessary to replicate phage RNA. One converts single strands into double strands, while the other uses double strands to synthesize viral RNA. The mutant is deficient in the first activity, probably because the mutation is in the gene specifying the requisite enzyme. On the basis of these and other results, a model is presented for the replication of phage RNA.*

A central problem presented by the growth of a bacteriophage with an RNA genome (*1*) is the mechanism of replication of the viral nucleic acid. There are numerous reports of double-stranded RNA in cells infected by RNA phages (*2–5*). Although the evidence is incomplete, this material may play a critical role in RNA replication. One of the most cogent points for the involvement of this RNA is that after infection some of the parental phage RNA is converted to a double-stranded form (*2, 3, 6*). To carry out such a conversion, an enzyme capable of using single-stranded RNA as template for the synthesis of the complementary RNA strand is required. If double-stranded RNA is an intermediate in RNA replication, a second enzymatic step is required to synthesize the viral RNA.

We now report that these two enzymatic steps are probably catalyzed by different enzymes, and that enzyme I, catalyzing the first step—the synthesis of double-stranded RNA—is synthesized under the direction of the viral genome. We consider in detail a class of temperature-sensitive phage mutants that are blocked primarily in the conversion of single-stranded RNA to double strands (function of enzyme I) and only secondarily in the synthesis of single-stranded viral RNA. This phage function is required throughout infection for the production of phage particles (*7*). Since the mutation is in the phage genome, presumably in the gene specifying enzyme I, these effects indicate that double-stranded RNA is an intermediate in virus replication.

Our experiments are based on the following model for the replication of an RNA bacteriophage. The parental phage RNA molecule, immediately upon entering the cell, becomes attached to a ribosome (*6*) and directs the synthesis of all the necessary enzyme I. Enzyme I now uses the parental RNA as template for the synthesis of the complementary RNA strand, forming a double-helical RNA structure. This double-stranded RNA then becomes a template for the synthesis of single-stranded viral RNA molecules, which is perhaps accomplished by a second enzyme. A small fraction of the newly made single-stranded RNA is converted by enzyme I into double strands that form new templates for the second step. This cyclical process continues throughout infection, and the number of double-stranded RNA molecules increases in parallel with the number of viral single-strands.

Double-stranded RNA was measured in infected cells as follows: 2.0 ml of the labeled cells were added to 0.5 ml of a 5 percent sodium dodecyl sulfate solution and immediately frozen in a mixture of dry ice and acetone. The samples were frozen and thawed twice, mixed with 0.2 mg of yeast RNA (Calbiochem) as carrier, and extracted with an equal volume of water-saturated phenol. The aqueous layer was made 0.3*M* in sodium acetate, and three volumes of ethanol were added. After at least 2 hours at −20°C, the nucleic acids were collected by centrifugation and resuspended in 2.0 ml of buffer (0.10*M* NaCl, 0.001*M* $MgCl_2$, 0.05*M* tris, *p*H 7.4). Deoxyribonuclease (*8*) was added (50 μg/ml) for 30 minutes at 25°C. A portion was added to a solution of 5 percent trichloroacetic acid (TCA) containing 0.1 mg of serum albumin as carrier. The precipitate was collected on fiberglass filters, and the radioactivity was determined (*9*). Another portion was added to 2 ml of buffer, and ribonuclease (50 μg/ml) was added for 30 minutes (*8*). Trichloroacetic acid (to 5 percent) was added, and the precipitate was collected as above. In most experiments portions were also treated with ribonuclease in water (*10*), conditions which degrade double-stranded RNA, to insure that there was no contaminating label not

Table 1. Viral RNA polymerase induced by temperature-sensitive mutants. *Escherichia coli* strain K37 was grown in broth (*24*) at 37°C to a density of 2×10^8 cells/ml. The cells were incubated at the appropriate temperature (second column) for 5 minutes before infection with phage. The cells were harvested for polymerase assay (*12*) at 40 minutes after infection. One unit of enzymatic activity is defined as the incorporation of 100 $\mu\mu$mole of UTP-C^{14} per milligram of protein per 15 minutes at 25°C.

Phage	Temp. of infection (°C)	Polymerase (units)
None	34	0.10
f2	34	2.80
	43	3.20
ts-4	34	2.20
	43	0.10
ts-5	34	.96
	43	.11
ts-6	34	1.70
	43	0.10

in RNA. In accord with the experiments of Weissmann and Borst (*10*), we define double-stranded RNA as material resistant to pancreatic ribonuclease in 0.15*M* NaCl but not in water.

In order to compare the effects of mutation on phage growth, we shall first consider the elements found in f2-infected cells.

A growth curve of f2 is shown (Fig. 1). Intracellular phage appear at 15 minutes; half of the virus has been made by 40 minutes (*1*). Ten to fifteen minutes after infection, extracts of infected cells contain a new RNA polymerase activity (*11, 12*) which increases for 10 minutes and then remain constant until the cells lyse.

Within 10 minutes after infection, a part of the parental phage RNA becomes double-stranded (Fig. 2) (*2, 3, 6*). This part, perhaps only coincidentally, corresponds to the amount of viable phage in the preparation (*13*). About half of the labeled double-stranded RNA slowly becomes single-stranded again (*2, 6*). In sucrose gradients, viral single-stranded RNA sediments in a single peak at 27*S* (Fig. 3). Double-stranded RNA derived from parental phage RNA sediments in a broad band (Fig. 3) with one peak at 14*S* and another at about 18*S*.

Double-stranded RNA is synthesized for the most part between 20 and 50 minutes after infection (Fig. 1) at about the same time that the viral single-stranded RNA is synthesized, namely, 10 minutes before the synthesis of mature phage (*14*). About 1.5 to 3 percent of all of the RNA synthesized after 20 minutes is double-stranded. This RNA has the same sedimentation profile as double-stranded RNA derived from parental phage with similar peaks at 14*S* and 18*S*.

Horiuchi, Lodish, and Zinder (*7*) described the isolation and properties of temperature-sensitive mutants of f2; these mutants grow at 35°C but not at 43°C. The mutants were divided into three classes. The first class, which contains mutants ts-4, ts-5, and ts-6, is defective in a phage function required both early and late in infection; the other classes are defective in functions needed only late in infection.

Consideration of the possible functions determined by the small genome of f2 leads to the postulate that mutations of the first class are in a gene controlling the structure of a phage RNA polymerase. Two kinds of experiments support the predictions of this hypothesis. With cultures kept continuously at high termperatures, the synthesis of elements usual in infected cells and the fate of the parental RNA can be measured. Infected cells can be grown initially at low temperatures and then shifted to the high temperature; the effect of the shift on the synthesis of various phage components can be studied.

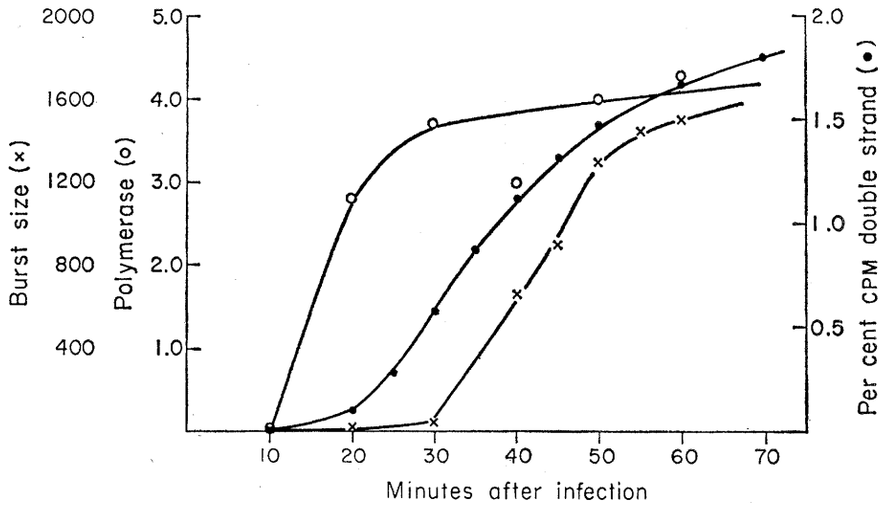

Fig. 1. Properties of f2-infected cells. *Escherichia coli* strain K38 (*23*) infected with wild-type f2 were assayed for viral RNA polymerase and for intracellular phage (*12*). In a separate experiment, bacteria were grown for 2.5 hours in broth (*24*) containing 0.5 μc of $P^{32}O_4$ per milliliter and infected with five viable f2 phage per bacterium. At the indicated times portions were assayed for P^{32} in double-stranded RNA. The amount of this labeled RNA is recorded as a percentage of the total incorporated P^{32}. A background of 0.3 percent of ribonuclease-resistant material found in uninfected cells has been subtracted; cpm, counts per minute.

When cells infected with ts-6 are kept at 43°C they synthesize none of the components found in normal infected cells: viral RNA polymerase (Table 1); double-stranded RNA (Table 2); single-stranded viral RNA, measured either by the production of infectious phage RNA (*7*) or by isotope-labeling techniques (Fig. 4); or phage antigen (*7*).

Although about 2 percent of the RNA from P^{32}-labeled f2 is converted to a double-stranded form after infection at both low (34°C) and high (43°C) temperatures, RNA from parental ts-6 was converted to a double-strand only after infection at the low temperature (Fig 2). This is not the result of a failure of the mutant phage to absorb at 43°C, for when cells infected with mutant were incubated at 43°C for 15 minutes, washed, and

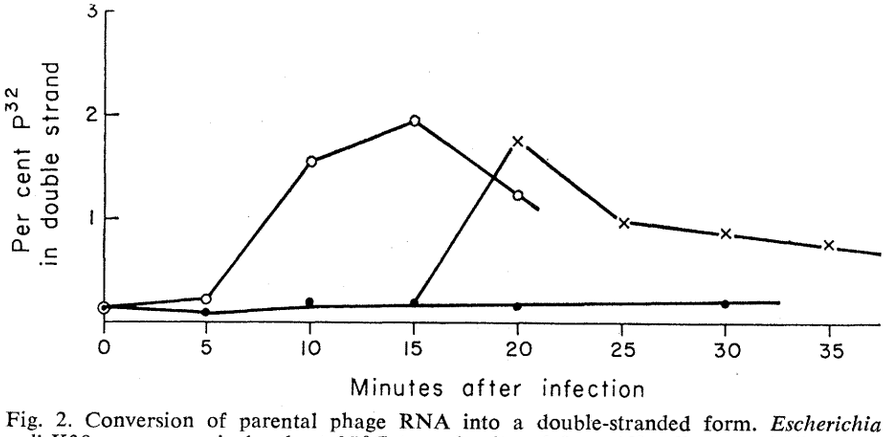

Fig. 2. Conversion of parental phage RNA into a double-stranded form. *Escherichia coli* K38 was grown in broth at 37°C to a density of 2 × 10^8 cells per milliliter and infected with five viable P^{32}-labeled ts-6 phage (*9*) per bacterium. Portions of the infected cells were immediately placed at 34°C (open circles) and at 43°C (closed circles). At 15 minutes a portion of the culture at 43°C was washed, resuspended in warmed fresh medium, and incubated further at 34°C (crosses). At intervals the amount of P^{32} in double-stranded RNA was measured as described.

Table 2. Effect of temperature shift on synthesis of double-stranded RNA. *Escherichia coli* K37 was grown in supplemented minimal medium (*26*) at 37°C to a density of 2×10^8 cells/ml, and infected with five of the indicated phages. Portions were immediately placed and incubated at the indicated temperatures. The samples labeled "shift-up" were incubated for 28 minutes at 34°C and then at 43°C. All samples received uracil-C^{14} (0.5 μc/ml, 4 μg/ml) at 30 minutes. At the indicated times RNA was isolated, and the amount of radioactivity in double-stranded RNA was determined. All samples incorporated approximately the same amount of uracil-C^{14} into total RNA at the indicated times. The number in the last column is the average percentage of incorporated uracil in double-stranded RNA, less the background of 0.30 percent found in uninfected cells.

Temperature (°C)	Incorporated uracil-C^{14} in double-stranded RNA (%)		
	45 min	60 min	Average
	No phage		
34	0.30	0.36	
43	.36	.25	
	Phage f2		
34	1.56	1.72	1.34
(Shift up)	2.13	3.09	2.31
43	10.98	13.98	12.18
	Phage ts-6		
34	1.70	2.00	1.55
(Shift up)	0.34	0.32	0.03
43	.33	.24	.00

then incubated at 34°C, a maximum conversion of P^{32}-labeled parental RNA into double-stranded form occurred within 5 minutes after the shift (Fig. 2). Penetration of mutant phage RNA at high temperatures is shown by the fact that mutant RNA can be converted to double-stranded form at 43°C if the cells are first infected with f2 host-dependent mutant sus-11 (*15*).

The conversion of parental phage RNA into double strands is the earliest detectable biochemical event occurring in f2-infected cells. Probably when this process is blocked none of the phage-specific induced events can occur. We postulate that this mutant loses enzyme I function at high temperatures.

Further support for this hypothesis comes from a series of experiments in which cells infected with ts-6 are incubated at 34°C for 28 minutes and then shifted to 43°C. If enzyme I itself is temperature-sensitive, then this "temperature shift-up" should inhibit first the synthesis of double-stranded RNA, and only later should it inhibit the synthesis of the single strands.

The synthesis of double-stranded RNA after a temperature shift-up with cells infected with f2 and ts-6 is shown in Table 2. Uracil-C^{14} was added 2 minutes after the shift, and the radioactivity in double-stranded RNA was measured after 15-minute intervals. After a shift-up, ts-6–infected cells synthesized less than 2 percent of the amount of double-stranded RNA at 34°C. The f2-infected cells, on the other hand, synthesized more double-stranded RNA at 43°C than at 34°C (Table 2). This excess double-stranded RNA is smaller than that found in f2 cells at 34°C and similar to the RNA produced in cells infected with amber mutants in the coat-protein cistron (*15*).

In experiments measuring synthesis of single-stranded viral RNA the results were significantly different (Fig. 4). Cells were labeled with radioactive uracil for 10 minutes, washed free of unincorporated isotope, and then incubated further for 1 hour. The amount of labeled RNA which can be incorporated into phage particles is taken as a measure of single-stranded viral RNA synthesized during the labeling period (Fig. 4) (*14*).

Cells infected with f2 synthesized the same amount of single-stranded viral RNA regardless of temperature. With both f2- and ts-6–infected cells, regardless of the conditions used for the uracil-C^{14} labeling, almost the same amount of isotope can be incorporated into phage particles at 43° as at 35°C. This result demonstrates that, once a single-stranded viral RNA is synthesized, it is incorporated into phage particles equally well at both temperatures. Cells infected with ts-6 synthesize 5 to 15 minutes after a temperature shift from 35°C to 43°C almost the same amount of single-stranded viral RNA as cells kept continuously at 35°C (Fig. 4*A*).

In similar experiments (not shown), 15 to 25 minutes after a shift-up ts-6–infected cells synthesized only 30 percent of the amount of single-stranded viral RNA as the control cells did. The amount of single-stranded RNA synthesized decreased thereafter; little was synthesized after 40 minutes.

In addition, we have also used the sensitive annealing and isotope dilution techniques developed by Weissmann and co-workers (*2, 11, 16*) to measure viral RNA. Cells infected with ts-6, shifted from 35° to 43°C 30 minutes after infection, synthesized, during the next 5 minutes, 70 percent of the amount of viral RNA as cells

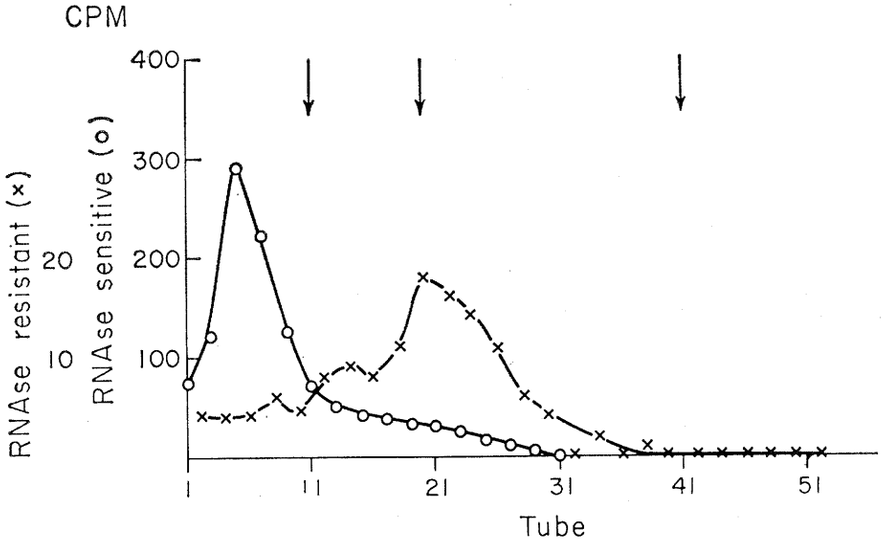

Fig. 3. Sucrose gradient of double-stranded RNA derived from parental phage. A broth culture (in the log phase of growth) of *E. coli* K38 (40 ml) was infected with five viable P^{32}-labeled f2 phage per bacterium. Ten minutes after infection the cells were chilled. RNA was isolated (*9*) and resuspended in 0.4 ml of buffer (0.05*M* tris, *p*H 7.4, 0.10*M* NaCl, 0.001*M* $MgCl_2$). Three percent of the labeled RNA was double-stranded. Of the RNA solution, 0.1 ml was layered on a 4.6-ml sucrose gradient (5 to 20 percent) in the same buffer. The tubes were centrifuged for 270 minutes at 37,500 rev/min in the SW-39 rotor of the Spinco model L ultracentrifuge. Alternate one-drop (odd number) and two-drop (even numbers) fractions were collected from the bottom of the tube and diluted with 2.5 ml of buffer. The optical density at 260 mμ was read on each fraction; the arrows represent (from left to right) the peaks of the 23*S*- and and 16*S*-ribosomal RNA and 4*S*-soluble RNA. All samples were treated with deoxyribonuclease (20 μg/ml) and even-numbered tubes were also treated with ribonuclease (100 μg/ml) for 30 minutes at 25°C. The radioactivity in the acid-insoluble fraction was determined as described. Sedimentation coefficients were calculated by the method of Martin and Ames (*25*).

incubated continuously at the lower temperature (*17*).

Thus, ts-6–infected cells shifted from 35° to 43°C immediately stop synthesizing appreciable amounts of double-stranded RNA. Soon after the shift the cells make a normal amount of single-stranded viral RNA. However, the amount of viral RNA made decreases with time after the temperature is raised. Once a single-stranded RNA is made at 43°C, it is incorporated into phage particles equally well at 35°C and at 43°C. Mutant ts-6, therefore, probably does not contain a second temperature-sensitive mutation required for the production of phage particles.

The total amount of ts-6 single-stranded viral RNA made 1 hour after a temperature shift-up is only about 10 to 15 percent of that synthesized by control cells kept constantly at 35°C. Similarly, Horiuchi (*7*) found that, after a shift-up at 30 minutes, ts-6–infected cells synthesize over the next 50 minutes only 10 percent of the amount of infective phage synthesized by cells incubated throughout at 33°C. The ts-6 function is therefore required during the entire course of infection for the production of viral RNA. These results indicate a role for double-stranded RNA in the synthesis of single-stranded viral RNA. Labeling periods much shorter than 10 to 15 minutes can elucidate some further details. Weissmann and co-workers (*16*) demonstrated that, after a pulse of radioactive guanosine of less than 20 seconds most of the radioactive viral RNA strands are found in double-stranded form. During a subsequent "chase" period (incubation in the presence of a large excess of nonradioactive precursor) about 70 percent, but not more, of the radioactivity in these viral RNA strands is displaced from the double strand and appears as free single-stranded radioactive viral RNA molecules. After the short pulse about 10 percent of the label in the double strands is in the complementary RNA strand. This label is not displaced during the chase period.

To account for the fact that not all of the radioactive viral RNA strands can be chased from the double strands, we assume that a fraction of the free single-stranded radioactive viral RNA molecules is converted back into double strands by enzyme I. Therefore, all of the pulse-labeled RNA which remains double-stranded after a 10-minute chase is due to the conversion, by enzyme I, of single-stranded RNA into double strands as a result of the synthesis of the radioactive complementary strands during the 10-second pulse, and by the conversion of labeled viral RNA strands back into double strands during the chase period.

This hypothesis makes two critical predictions concerning RNA metabolism in ts-6–infected cells after a temperature "shift-up." (i) When the temperature of growth is increased from 34°C to 43°C, almost the same amount of radioactivity should be incorporated into double-stranded RNA during a 10-second pulse as in cells incubated continuously at 34°C. This radioactivity would be entirely in the newly synthesized viral RNA strands still in double-stranded form. (ii) All of the radioactivity in double strands after a pulse must be displaced during the subsequent chase period. Enzyme I in these cells should be inactivated at 43°C, and therefore would be unable to convert viral RNA strands to double-stranded form either during the 10-second pulse or during the chase.

These predictions were fully realized (Fig. 5). When cells infected with ts-6 were subjected to an increase in temperature and then given a 10-second pulse of uracil-C^{14}, over 2 percent of the incorporated radioactivity was in a double-stranded form. Over 95 percent of the label was displaced from double-stranded RNA during a 10-minute chase. The level of sensitivity is limited by the background of 0.3 percent of RNA radioactivity in a ribonuclease-resistant core in both ts-6–infected cells incubated continuously at 43°C (not shown) and in uninfected cells. The f2-infected cells, after an increase in temperature from 34° to 43°C incorporated 4 percent of the uracil-C^{14} during the pulse period into double-stranded form, but little, if any, of the radioactivity was displaced during the chase period. This result may be related to the fact that, at 43°C, f2-infected cells synthesize large amounts of a small double-stranded RNA (Table 2). Cells infected with ts-6 and f2 kept continuously at 34°C gave results essentially like those of Weissmann

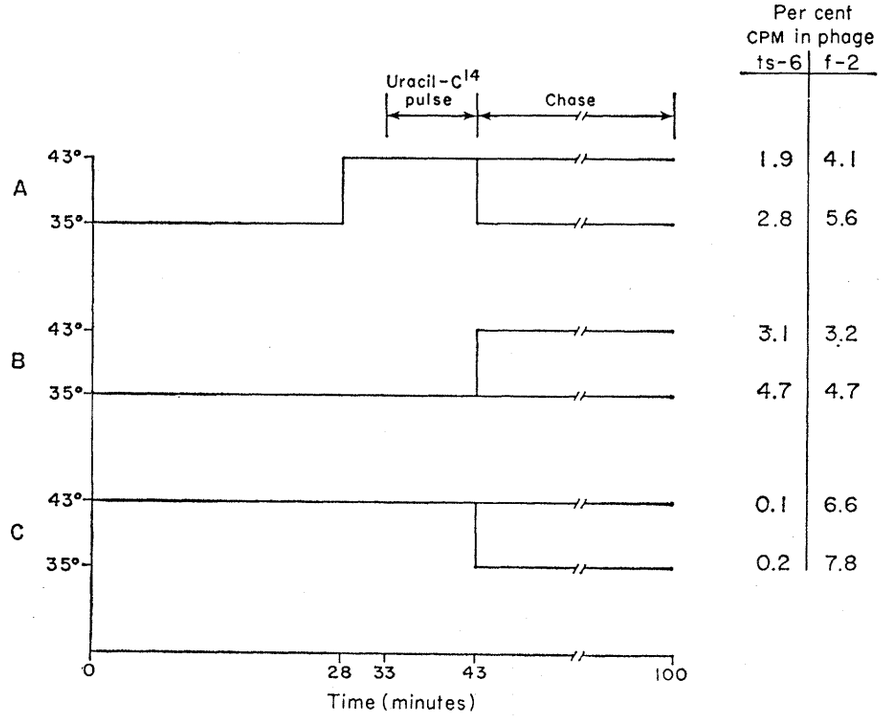

Fig. 4. Synthesis of single-stranded viral RNA. *Escherichia coli* K38 was grown in minimal medium (*26*) to a density of 2×10^8 cells/ml and infected with five viable f2 or ts-6 phage. Portions of the infected cultures were incubated at 35°C (samples A and B) or 43°C (sample C). At 28 minutes, sample A was shifted to 43°C. At 33 minutes all samples were treated with uracil-C^{14} (0.5 μc/ml, 2 μg/ml). Ten minutes later, the samples were centrifuged, washed once with medium containing uracil-C^{12} (100 μg/ml), and resuspended in fresh medium containing uracil. Half of each sample was incubated at 35°C and half at 43°C. At 100 minutes all samples were lysed and the radioactivity in phage determined (*27*). In the last column is the percentage of incorporated uracil-C^{14} in acid-precipitable phage particles; a background of 0.06 percent from uninfected cells has been subtracted.

et al. (*16*). At the end of the 10-second pulse 4 to 5 percent of the incorporated isotope was in a double-stranded form, and 60 percent but not more of this label was displaced from the double-stranded RNA during the chase period.

Our experiments indicate that newly synthesized viral RNA strands are made in such a way as to be initially resistant to ribonuclease. As synthesis proceeds they become nuclease-sensitive. This is best explained by a form of asymmetric semiconservative replication (*18*) of a double-stranded RNA wherein newly synthesized viral RNA strands each in turn displace the previously synthesized RNA molecule. The replicative double-stranded RNA would consist of one complementary RNA molecule paired in part to one (or more) viral strands in the process of being synthesized. Fenwick *et al.* (*4*) and Weissmann *et al.* (*11*) have proposed similar models for phage RNA replication.

Our experiments also suggest that one can label preferentially with a 10-second pulse of radioactive uracil the newly synthesized viral RNA still bound in ribonuclease-resistant form to its RNA template. A sucrose gradient of uracil-H^3 pulse-labeled double-stranded RNA is shown in Fig. 6*a*. Present during the growth cycle also was adenine-C^{14}, in order to provide a marker for the RNA elements being synthesized. Most of the H^3-pulse-labeled double-stranded RNA sediments at about 18*S*. There is also a smaller peak at 15*S*. The H^3-pulse-labeled RNA which remains double-stranded after a 3-minute chase sediments predominantly at 14*S* (Fig. 6*b*) (*19*). The sedimentation constant for the bulk (labeled with adenine-C^{14}) double-stranded RNA found in infected cells is also mostly 14*S* (Fig. 6). In similar experiments (not shown), ts-6–infected cells were shifted at 30 minutes from 34° to 43°C and then given a 10-second pulse of uracil-H^3. Again, most of the labeled double-stranded RNA sedimented at 18*S*. However, as described previously, no detectable labeled double-stranded RNA remained after a 10-minute chase.

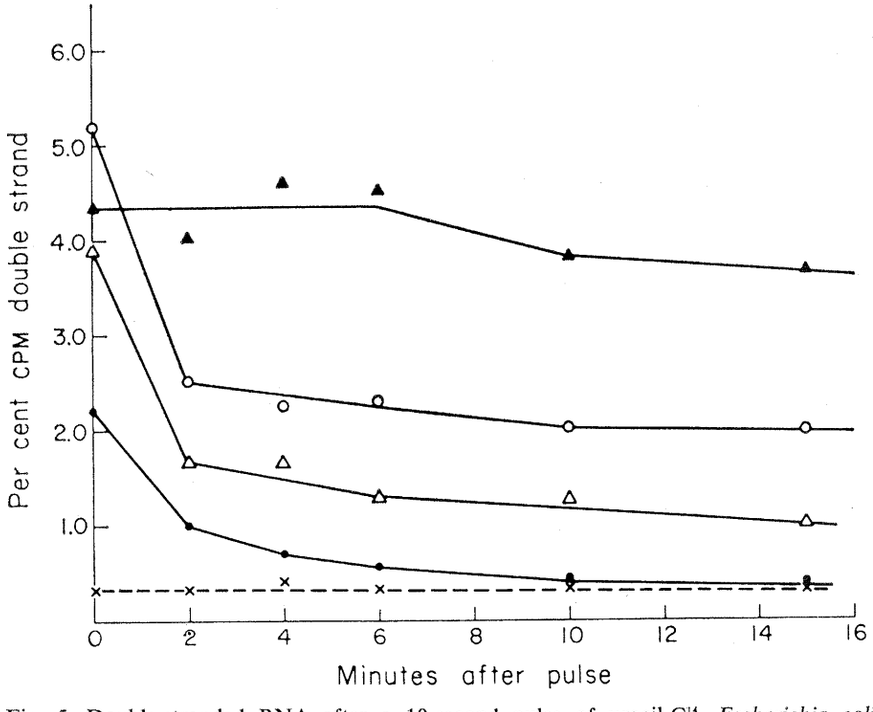

Fig. 5. Double-stranded RNA after a 10-second pulse of uracil-C^{14}. *Escherichia coli* K38 was grown in supplemented minimal medium (*26*) at 37°C to a density of 2×10^8 cells/ml, infected with five of either f2 or ts-6 phage, and incubated at 34°C. At 28 minutes, half of the cultures were shifted to 43°C and incubation was continued. Thirty-three minutes after the infections, uracil-C^{14} (0.05 μc/ml, 0.2 μg/ml) was added to all cultures. Ten seconds later the pulse was terminated by the addition of uracil-C^{12} (100 μg/ml), and the first sample was withdrawn. Additional samples were taken at the indicated times after the pulse. Samples (2 ml) of labeled cells were pipetted into 0.5 ml of 5 percent sodium dodecyl sulfate and immediately frozen in a bath containing dry ice and acetone. As in Fig. 2, the RNA was isolated, and the percentage of radioactivity in double-stranded RNA was measured. After the 15-minute chase period, all cells contained 65 percent more radioactive RNA than immediately after the 10-second pulse. Uninfected cells, crosses; f2 at 34°C, open triangles; f2 at shift-up, solid triangles; ts-6 at 34°C, open circles; ts-6 at shift-up, solid circles.

Because we cannot follow in detail the fate of all of the isotope in the different peaks we can draw no definite conclusions concerning the metabolic relationships of the material in the fractions to each other. Fenwick *et al.* (*4*) have presented evidence that the 18*S* RNA consists of a double-stranded RNA core with attached single-stranded viral RNA, and that the 14*S* RNA consists almost entirely of double-stranded regions. It appears that most of the active viral RNA synthesis gives rise to material sedimenting at 18*S*. The 14*S* material probably consists of whole viral RNA and complementary strands in completely double-helical form. It appears to be double-stranded RNA, either inactive in RNA synthesis or just beginning or completing viral RNA synthesis.

Analysis of some of the physiological abnormalities of the mutant ts-6 leads us to postulate that it contains a mutation in a gene specifying a viral RNA polymerase (*7*). At high temperatures ts-6 does not initiate any of the events that normally ensue after RNA phage infection. In particular, the parental RNA is not converted to a double strand. When cells grown at low temperatures are shifted to high temperatures, synthesis of double-stranded RNA ceases. Single-stranded viral RNA continues to be synthesized normally for some time after the shift. Thus there is an enzyme (enzyme I) that converts single-stranded viral RNA to double strands, and it is this enzyme which is now thermolabile. Since the synthesis of single-stranded RNA proceeds normally for at least 10 minutes after the shift-up, we believe that a second enzyme (enzyme II) is responsible for this synthesis. This actor could be either the normal cellular RNA polymerase or a second phage-induced enzyme. The fact that, in time, after the increase in temperature the synthesis of single-stranded is also inhibited, implies that this synthesis is coupled to the synthesis of double-stranded RNA. We envisage a cyclical process in which a fraction of the viral RNA strands are continuously converted (by enzyme I) to double strands; these double strands are essential for continued synthesis of single-stranded RNA.

The details of the structure of the replicative double-stranded RNA intermediate remain obscure. As noted by others, a number of species of double-

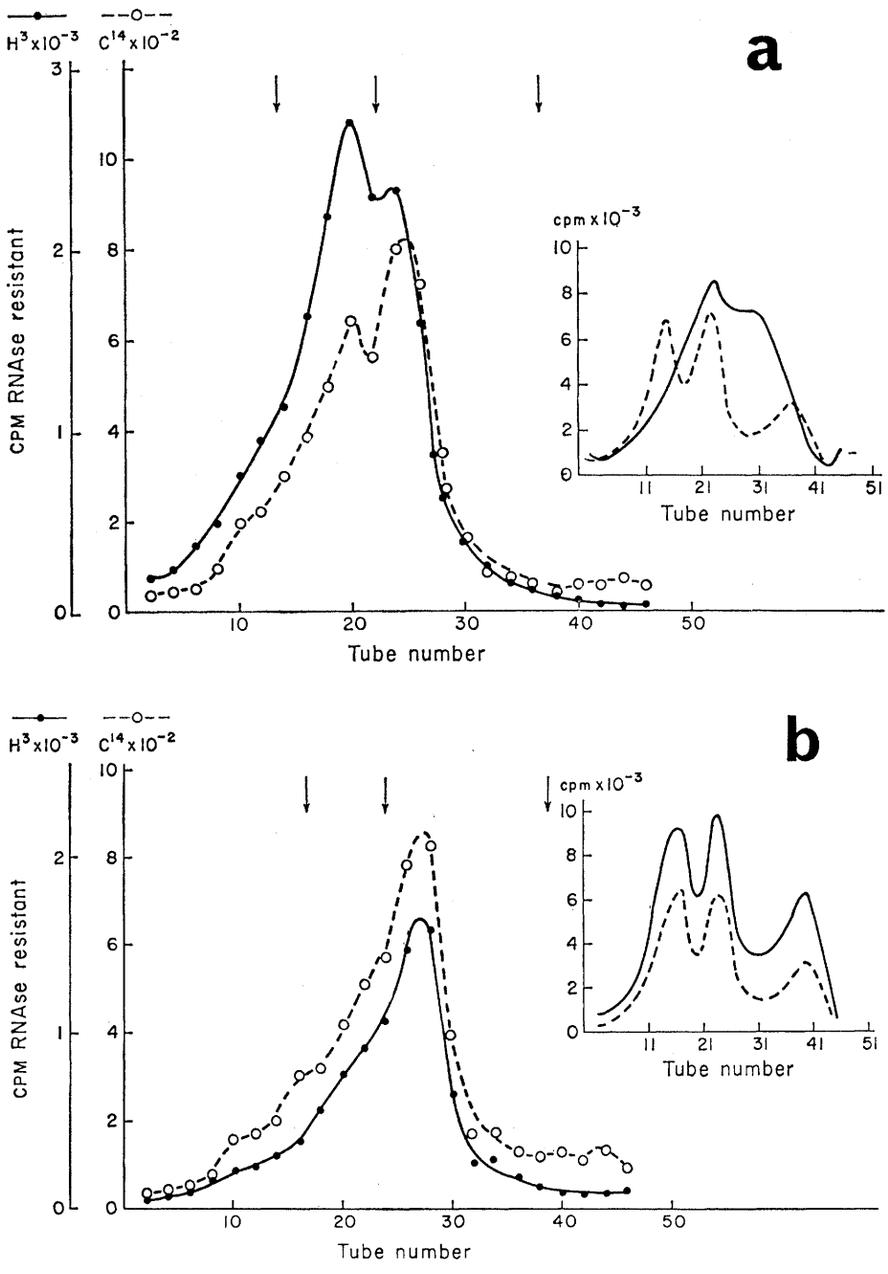

Fig. 6. Sucrose gradient analysis of pulse-labeled RNA. (*a*) Immediately after the 10-second pulse; (*b*) after a 3-minute chase. *Escherichia coli* K19 was grown at 37°C in minimal medium to a density of 2×10^8 cells per milliliter and infected with 10 viable f2 per bacterium. Adenine-C^{14} (0.1 μc/ml, 4 μg/ml) was added at 20 minutes after infection. At 33 minutes uracil-H^3 (5 μc/ml, 0.2 μg/ml) was added. The pulse was terminated 10 seconds later by pouring half of the culture (*a*) onto twice its volume of finely crushed frozen (−20°C) medium (containing 100 μg of uracil per milliliter). At the same time uracil (100 μg/ml) was added to the other half of the culture (*b*). After a 3-minute incubation it was also poured onto frozen medium. The cells were harvested by centrifugation, and RNA was extracted as before, except that Macaloid (0.2 percent) was added before the phenol extraction. RNA was dissolved in 0.5 ml of buffer; deoxyribonuclease (5 μg/ml) was added, and the solution was incubated for 30 minutes at 25°C. In both samples, 4.0 percent of the C^{14}-labeled RNA was double-stranded; in (*a*) 12.4 percent and in (*b*) 4.2 percent of the H^3-labeled RNA was double-stranded. Portions (0.2 ml) of each RNA solution were layered on a 4.6-ml sucrose gradient (5 to 20 percent) in the same buffer. Centrifugation was for 285 minutes at 37,000 rev/min in the SW-39 Spinco rotor. Alternate one-drop (odd-numbered) and two-drop (even-numbered) samples were collected from the bottom. Odd-numbered fractions were precipitated with TCA directly (inset). Even-numbered fractions were diluted with buffer, treated with ribonuclease (100 μg/ml) for 30 minutes at 25°C, and then precipitated with acid (main part of figure). The C^{14}-labeled total-RNA profile (inset) is that of normal cellular RNA. After the 10-second labeling period most of the H^3-labeled RNA (inset *a*) has a profile typical of cellular messenger RNA; after the 3-minute chase (inset *b*) most of the H^3 radioactivity has the normal three-peak cellular RNA profile.

stranded RNA can be differentiated by sedimentation constant and by nuclease sensitivity. Our data indicate an active role for the 18*S* double-stranded RNA.

There has yet been no separation of the RNA polymerase activity in extracts of phage-infected cells. However, the observations reported on this polymerase activity are at least consistent with the postulated two-enzyme model. Weissmann (*20*) has shown that 90 percent of the product synthesized in vitro is the viral strand, while 10 percent is the complementary strand. This is approximately the ratio of the products found in the infected cells (*21*). We thus postulate that 90 percent of the in vitro RNA polymerase is enzyme II, and 10 percent is enzyme I. Spiegelman *et al.* have isolated an enzyme that uses single-stranded viral RNA as template for the synthesis of identical single-stranded molecules (*22*). According to our model, this activity would be a complex or mixture of enzymes I and II.

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double-stranded RNA (Table 2). Half of the label in double-stranded RNA is in viral RNA and half is in the complementary strand. Therefore, the ratio of viral to complementary RNA molecules is as 8.4 : 1.
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22 December 1965

Zonal Ultracentrifuge for the Separation of Ribosomal Subunits

Abstract. *Ribosomal subunits were prepared by means of the Anderson zonal ultracentrifuge. With this technique it is possible to separate more than 300 milligrams of subunits with complete resolution in a single run, as compared with about 2 milligrams with conventional rotors. Superior resolution is achieved in the zonal ultracentrifuge, affording possibilities for the detection and preparation of minor ribosomal components.*

The quantity of fractionated ribosomal subunits which may be conveniently prepared by the conventional method of zone centrifugation in swinging buckets or in fixed-angle rotors imposes a severe limitation to the study of the structure and the function of ribosomes. Our results show that with the Anderson zonal ultracentrifuge (*1*) the number of subunits prepared in a single run may be increased by a factor of at least 100, with resolution of remarkable quality.

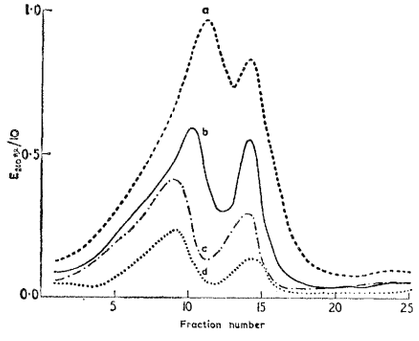

Fig. 1. The effect of loading on the fractionation of ribosomal subunits by zone centrifugation. The gradients of 10 to 25 percent sucrose with 0.001*M* tris, *p*H 7.6, were centrifuged for 19 hours at 25,000 rev/min (Spinco SW25 rotor) at 0°C. The direction of sedimentation is from right to left: (*a*) 2 mg; (*b*) 6 mg; (*c*) 8 mg; and (*d*) 16 mg.

Figure 1 shows how the quantity of dissociated reticulocyte ribosomes (*2*, *3*) centrifuged in a single bucket of the Spinco SW25 rotor affects the quality of the separation between the subunits. Under these conditions it is apparent that mutual contamination of the two subunits occurs with a loading of more than 2 mg on the gradient. The conventional rotors of somewhat greater capacity which attain similar or greater centrifugal fields (such as Spinco SW 25.2, Spinco 30, MSE 40) do not substantially facilitate the preparation of subunits.

The capacity of the Anderson zonal ultracentrifuge rotor is 1760 ml. On this basis one would expect to be able to separate larger quantities of material. A second important difference between the zonal and conventional rotors is that, in the former, loading the sample at the beginning and unloading the gradient at the end of the run occur while the rotor is centrifuging at 5000 rev/min. Diffusion of the sample which may cause appreciable band broadening when the rotor is at rest (*4*) is therefore minimum.

A typical fractionation of 200 mg of reticulocyte ribosomal subunits is shown in Fig. 2. The sample (4 mg/3 ml) was applied in 0.1*M* sodium ethylenediaminetetraacetate (EDTA), *p*H 7.6, to a gradient, linear with respect to radius, of 10 to 25 percent sucrose containing 0.01*M* sodium acetate. The gradient was layered over a cushion of 35 percent sucrose, and the sample was layered with water. Centrifugation was carried out at 40,000 rev/min for 6 hours at 5°C. The gradient was then displaced by a concentrated sucrose solution and monitored at 290 mμ in a 1-cm flow cell with a Beckman DB recording spectrophotometer. Fractions (40 ml) were collected, and the refractive indexes were measured to determine the density. The recovery of ribosomes was found to be 182 mg as judged by the optical density pattern. Over 300 mg of dissociated ribosomes have been fractionated with complete resolution of the two subunits by this method.

The purity of the isolated subunits has been established within the limits of the analytical centrifuge (Spinco model E), ultraviolet optics being used. Furthermore, the RNA which may be isolated from the subunits is undegraded, the larger subunit yielding only 30*S* RNA and the smaller subunit 19*S* RNA, the values which have previously been attributed to the two species of reticulocyte ribosomal RNA (*2*).

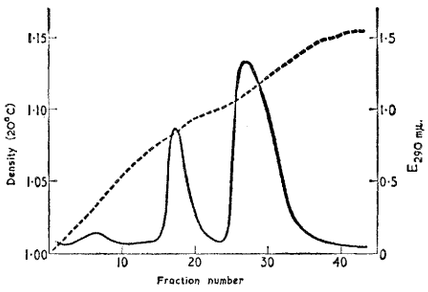

Fig. 2. Fractionation of 200 mg of rabbit reticulocyte ribosomal subunits. First a cushion of 35 percent sucrose and then the gradient was pumped into a Spinco B-IV zonal rotor at 3000 rev/min (modified Spinco Model L). The 200-mg sample of dissociated ribosomes was then layered onto the gradient at 5000 rev/min, and the sample was layered with water. The rotor was accelerated to 40,000 rev/min and the run was continued for 6 hours. The rotor was then decelerated to 5000 rev/min; the gradient was pumped out through a flow cell and the optical density was measured. All operations were carried out at 5°C. The direction of sedimentation is from left to right.

It is anticipated that the superior resolution of the subunits which may be obtained will facilitate the search for minor components (such as messenger RNA, and complexes of messenger and transfer RNA bound to the ribosomal subunits) which may be revealed by biological assays.

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