pressors act by altering the ribosome and that streptomycin acts by jamming the mechanism of advancement of the messenger, and it is suggested that this might explain the dominance of streptomycin sensitivity in heterozygotes.

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*REPLICATION OF VIRAL RNA, III. DOUBLE-STRANDED REPLICATIVE FORM OF MS2 PHAGE RNA**

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The multiplication of viruses which carry the genetic information in a single strand of RNA[1] has been the subject of detailed investigations in several laboratories. It has been established that the replication of viral RNA does not involve host DNA[2–5] and that the RNA itself can function as a messenger in a system of protein synthesis *in vitro*, directing the synthesis of coat protein[6] and possibly of other proteins[6, 7] required for virus multiplication. Infection with an RNA virus leads to the appearance of a new RNA-synthesizing enzyme in both animal cells[8, 9] and bacteria.[10–13] The enzyme induced by infection of *Escherichia coli* with phage MS2 was partially purified, and circumstantial evidence was presented that this enzyme (RNA synthetase) is involved in the replication of the viral RNA.[10, 11]

Further insight into the replication mechanism of viral RNA was provided by the observation that infection of Krebs II ascites cells with the RNA-containing encephalomyocarditis virus leads to the accumulation of virus specific double-stranded RNA (replicative form) at the end of the infection cycle.[14] The significance of this finding was underlined by our subsequent observation[15] that part of the radioactivity incorporated into acid-insoluble products on incubation of RNA synthetase with C^{14}-labeled ribonucleoside triphosphates was present in double-stranded RNA. We further reported on preliminary experiments indicating that, upon infection of *E. coli* with P^{32}-labeled MS2 phage, the infecting P^{32}-RNA strand was converted into a double-stranded form.[15] These results clearly supported the earlier suggestion[11] that replication of RNA-containing viruses involves the forma-

tion of a double-stranded replicative form and made alternative schemes,[16] in which synthesis of strands complementary to those of the viral RNA is not believed to occur, less likely.

In continuation of the above work we have found that the double-stranded RNA labeled with C^{14} by the action of RNA synthetase *in vitro* is indistinguishable from the double-stranded replicative form of MS2 RNA formed during infection *in vivo*.[17] Moreover, specific annealing tests demonstrate that the newly synthesized, C^{14}-labeled strands in the duplex are mainly "plus" or parental-type MS2 RNA strands.[18] This observation, together with the fact that RNA synthetase has been purified as a holoenzyme in association with its natural template, presumably the double-stranded replicative form of MS2 RNA, provides direct proof that RNA synthetase is concerned with the synthesis of viral RNA *in vivo*.

This paper deals with the identification of double-stranded RNA, formed in *E. coli* after infection with MS2 phage, as the replicative form of MS2 RNA and its identity with the double-stranded RNA labeled with C^{14} during incubation of RNA synthetase with C^{14}-labeled nucleoside triphosphates. The isolation and properties of the RNA synthetase holoenzyme and the enzymatic synthesis of MS2 RNA will be described in the following paper of this series.[18]

Preparation and Methods.—(1) *Materials:* Reagents were obtained as described previously,[10,15] or from the following sources: Superbrite glass beads, type 100-5005, Minnesota Mining and Manufacturing Co.; crystalline pancreatic RNAase, RNAase A, DNAase, and calf thymus DNA, Worthington Biochemical Corp.; P^{32} phosphoric acid (carrier-free), Oak Ridge National Laboratory; Macaloid (purified Hectorite), Inerto Co.; Freon-11 ($CFCl_3$), Matheson Co.; cesium chloride (purified), Fisher Scientific Co.; CM Sephadex, medium, Pharmacia Fine Chemicals; Liquifluor, Nuclear-Chicago; liquid scintillation spectrometer vials 3002-1A, Wheaton Glass Co.

(2) *General methods:* Protein was determined by a modified biuret method[19] or by the method of Lowry *et al.*[20] Nucleic acid was determined spectrophotometrically.[21] For ribosomal and cell RNA the extinction coefficient was taken[22] as 8100/mole of phosphorus or 25.0 $mg^{-1} \cdot ml^{-1}$. The corresponding values used for MS2 RNA were 8600 and 25.1.[23]

Unless specified otherwise, radioactivity was measured in $1^1/_4$-in. stainless steel planchets either with a Nuclear-Chicago gas flow planchet counting system equipped with an end-window (efficiency for C^{14}, 15%; for P^{32}, 20%) or with the Tracerlab omniguard low background counting system (efficiency for C^{14}, 11%; for P^{32}, 18%). For scintillation counting, acid-insoluble material was filtered off on Millipore filters; these were impaled on glass needles, put into vials, and dried overnight at 37°. Liquifluor diluted 1:25 with toluene was added, and the samples were counted in a Packard Tricarb liquid scintillation spectrometer. We are indebted to Dr. N. Spritz, Stroke Study Group, Bellevue Hospital, for the use of this equipment.

Sedimentation coefficients and buoyant densities in analytical Cs_2SO_4 gradients were kindly determined by Dr. R. C. Warner and Mr. F. Zaboretsky with a Spinco Model E ultracentrifuge, equipped with a monochromator, at wavelength 265 mμ.

(3) *MS2 virus:* MS2 virus and its host, *E. coli* Hfr 3000, were obtained from Dr. A. J. Clark, Department of Microbiology, University of California, Berkeley. The phage was grown and assayed as described by Loeb and Zinder[24] for the related phage f2, and the purification of the virus was based on the procedure described by them. However, no RNAase was added at any stage.

(4) *P^{32}-labeled MS2 virus:* The medium was that described by Loeb and Zinder[24] but it contained only 0.1 gm of yeast extract per liter. Two 2-liter flasks, each containing 1 liter of medium, were inoculated with 50 ml each of an overnight growth of *E. coli* Hfr 3000 in the same medium and grown at 37° with shaking to a density of about 3×10^8 bacteria per ml. Then 20 mC per liter of carrier-free phosphoric acid and 3×10^{13} p.f.u. of MS2 per liter were added. After further incubation for 4 hr, 5 mg of lysozyme per liter and 1 ml of chloroform per liter were added, and the labeled virus was purified by a modification of the method of Strauss and Sinsheimer.[23]

(5) *RNA preparations: MS2 RNA and P^{32}-labeled MS2 RNA:* RNA was prepared from purified virus by a modification of the phenol extraction procedure[25] described by Stanley[22] in which Macaloid is used to adsorb traces of RNAase. The $s_{w,20}$ of the leading boundary was 25 *S* in 0.1 *M* Tris-HCl, pH 7.0 (cf. 27 *S* found by Strauss and Sinsheimer[23]), but the presence of breakdown products led to varying amounts of trailing. The buoyant density, determined in the Spinco Model E ultracentrifuge in a Cs_2SO_4 gradient (mean density 1.620 gm/ml, containing 12 μg/ml of sodium dodecyl sulfate, and 10 μmoles/ml of potassium phosphate, pH 7.4) was 1.626 gm/ml (cf. 1.63 gm/ml found by Doi and Spiegelman[16]). P^{32}-labeled MS2 RNA was prepared from P^{32}-MS2 virus by the phenol procedure.[22] 87% of the radioactivity present in the virus was recovered; the specific activity of the RNA was 21,000 cpm/μg.

TMV-RNA and P^{32}-labeled TMV-RNA: Leaves of tobacco plants (*Nicotiana tabacum* var. Turkish) infected with wild-type TMV and grown on the P^{32}-containing medium of Hoagland were donated by Dr. K. K. Reddi. The virus was prepared following his procedure.[26] The RNA was extracted as described by Stanley.[22] Unlabeled TMV-RNA was generously donated by Dr. C. A. Knight, Virus Laboratory, Berkeley, Calif.

P^{32}-labeled total cell RNA: E. coli W, obtained from Dr. J. F. Speyer, was grown from a small inoculum in 200 ml of the medium described above with 5 mC of carrier-free P^{32} phosphoric acid added. The cells were harvested at the end of logarithmic growth and processed as described by Hayashi and Spiegelman.[27]

(6) *Other preparations:* Ribosomal RNA from *E. coli* W3101[22] was a gift of Dr. W. M. Stanley, Jr. Its buoyant density in Cs_2SO_4 was 1.654 gm/ml. Soluble RNA from *E. coli* W was a gift of Dr. A. J. Wahba. C^{14}-poly U was prepared from UDP-2-C^{14} with polynucleotide phosphorylase from *A. vinelandii*.[28] Highly purified DNA-dependent RNA-nucleotidyl transferase (RNA polymerase) was prepared[29] from *Azotobacter vinelandii* through step 6 of purification.

(7) *Assay for RNAase-resistant, acid-insoluble radioactive RNA:* Samples of not more than 1 mg of RNA in 5 ml of 0.15 *M* NaCl, 0.015 *M* sodium citrate, pH 7, were incubated with 100 μg/ml of pancreatic ribonuclease A for 30 min at 25°. After cooling to 0°, 0.75 ml of 60% trichloroacetic acid and 300 μg of acid-insoluble yeast RNA were added, the precipitate was collected on Millipore filters, washed with chilled 6% trichloroacetic acid, and its radioactivity determined as described in section 2. Addition of carrier RNA is required when only mμg amounts of radioactive RNA are to be recovered. Whereas several samples of crystalline pancreatic RNAase have proved satisfactory (cf. ref. 15), we recently obtained a batch (no. R619A, Worthington) which, under our assay conditions, would completely degrade about 3.5 mμmoles of double-stranded RNA in 30 min. We have since used chromatographically purified RNAase A (batch no. 6063) which has proved suitable for this assay.

(8) *Heat denaturation of synthetase product (and replicative form):* Since preparations of double-stranded radioactive synthetase product[15, 18] contain large amounts of endogenous double-stranded RNA and since reannealing is dependent on concentration, heat denaturation requires a commensurate dilution to prevent the reannealing which may otherwise occur even during rapid cooling. The synthetase product was diluted with distilled water to an absorbancy not higher than 8.0/ml, corresponding to about 35 μg of total double-stranded RNA/ml. The solution was boiled for 10 min and rapidly cooled by squirting into a large beaker cooled in an alcohol-ice bath. The solution may be lyophilized to the desired volume. However, spontaneous renaturation occurs if the concentrated solution is stored for a prolonged period. Less than 2% of the radioactive RNA in freshly lyophilized preparations was resistant to RNAase.

(9) *Reannealing of heat-denatured synthetase product:* Samples to be reannealed were placed in a glass tube (1-mm wall thickness, 9-mm diameter, conical bottom) and dried overnight in an evacuated desiccator (12 mm Hg) over phosphorus pentoxide. The samples were dissolved in 0.375 *M* NaCl, 0.0375 *M* sodium citrate, pH 7, to give about 300–500 μg of total double-stranded RNA/ml corresponding to an absorbancy at 260 mμ of 80–100/ml. As little as 0.02 ml of solution can be used. The tube was then sealed about 3 cm from the bottom, immersed in an oil bath at 80° for 30 min, and allowed to cool slowly to 40° for about 1 hr.

(10) *Preparative cesium sulfate centrifugation:* The procedure of Doi and Spiegelman[16] was followed with minor modifications. Unless otherwise specified, all samples containing synthetase product along with ribosomal RNA were first digested with RNAase and freed of this enzyme by phenol extraction as will be described in the following paper.[18] Artifacts arising from

coprecipitation or aggregation of radioactive material with ribosomal RNA[16] could thus be avoided. Cesium sulfate was prepared from cesium chloride.[30]

(11) *Assay for replicative form:* Five aliquots of the sample, each containing 0.1–6 μg of replicative form, were placed in tubes as described in section 9 for reannealing. To each tube was added 1.3 μg of P^{32}-labeled MS2 RNA (specific radioactivity about 6000 cpm/μg), and 1, 4, 10, and 20 μg of cold MS2 RNA, respectively, were added to tubes 2, 3, 4, and 5. After sealing, heating to 120° for 10 min, and slow cooling to about 40° for 2 hr., i.e., after thermal denaturation and subsequent reannealing, the contents of the tubes were quantitatively transferred to test tubes and assayed for RNAase-resistant radioactivity (sec. 7). A blank value was obtained by mixing 1.3 μg of the radioactive MS2 RNA with an aliquot of the sample and 20 μg of cold RNA, and subjecting the mixture to RNAase digestion without heating and cooling. The total amount of MS2 RNA that becomes RNAase-resistant through annealing is calculated from the acid-insoluble radioactivity and the specific radioactivity of the MS2 RNA. The assay gives a measure of the amount of "minus" MS2 RNA strands present either as twin-stranded complexes with complementary "plus" MS2 RNA strands or as free single strands or both, unless the sample has been pretreated with RNAase, in which case the assay is specific for the replicative form of MS2 RNA.

On the assumption that at infinite concentrations of "plus" strands the annealing efficiency is 100%, measurements were made with a constant amount of the unknown sample and increasing amounts of P^{32} MS2 RNA. The data were plotted to allow extrapolation for infinite concentrations of added MS2 RNA. Determination of the amount of "minus" strands is based on the following considerations. Let m = "minus" strands; p = endogenous, unlabeled plus strands; a^* = added, P^{32}-labeled MS2 RNA; and d = cold (endogenous) double-stranded RNA. Assuming validity of the mass action law:

$$([m] - [d])([a^*] + [p] - [d]) = k\,[d] \tag{1}$$

for $[a^*] > [d]$

$$([m] - [d])([a^*] + [p]) = k\,[d] \tag{2}$$

or

$$\frac{k + [p]}{[m]([a^*] + [p])} + \frac{[a^*]}{[m]([a^*] + [p])} = \frac{1}{[d]} \tag{3}$$

and for $[a^*] > [p]$

$$\frac{1}{[a^*]}\,\frac{k + [p]}{[m]} + \frac{1}{[m]} = \frac{1}{[d]}. \tag{4}$$

A plot of $1/[d]$ versus $1/[a^*]$ should therefore yield a straight line, and the intercept with the Y axis should be the concentration of MS2 "minus" strands present. For values of $[a^*]$ much larger than $[p]$, $[d]$ may be equated with the total amount of $[a^*]$ that has become RNAase-resistant, calculated as described above. These assumptions were borne out experimentally. With 50, 100, and 300 μg of RNAase-treated infected cell RNA and final concentrations of added P^{32}-MS2 RNA between 0.07 and 1.0 mg/ml, three straight lines were obtained which intersected the Y axis to give values of 1.1, 2.1, and 6.25 μg, respectively, of minus strands. The fact that the three lines show the same slope indicates that k is small compared to $[p]$ under the conditions employed. This assay can also be used to determine the ratio of plus to minus strands in a given preparation.

Results.—Identification of double-stranded replicative form of MS2 RNA in vivo: If replication of viral RNA involves a double-stranded intermediate, the infecting parental strand should be converted into a double-stranded complex early in the infective cycle. To test this hypothesis, *E. coli* cells were infected with P^{32}-labeled virus, and the deproteinized, radioactive RNA was examined at different times after infection. Figure 1 shows that at 6 min there was a rapid increase of resistance to RNAase which, after reaching a maximum at 15 min, again decreased.

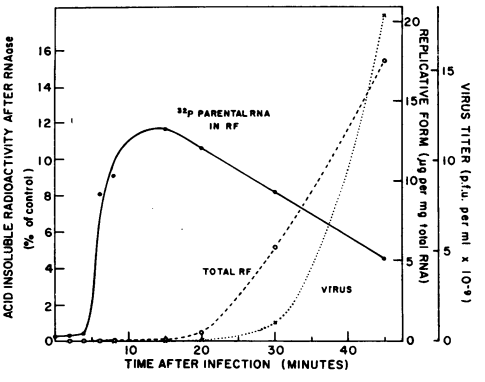

FIG. 1.—Conversion of parental, P^{32}-labeled viral RNA into a RNAase-resistant form, synthesis of replicative form of MS2 RNA, and virus synthesis as function of time elapsed after infection. *E. coli* cells were grown to a density of 3×10^8 cells in 2.1 liters of medium with shaking at 37°. P^{32}-labeled MS2 virus (3.3×10^5 cpm) was added at time zero at a multiplicity of 0.75. 400-ml samples were removed at the times indicated and rapidly cooled after adding 1 ml chloroform. 1-ml portions of each sample were incubated with 5 μg of lysozyme and 0.01 ml of chloroform for 30 min at 37° and their virus titer was determined.[24] The samples were centrifuged, the cells washed twice with 40 ml of chilled 0.02 *M* Tris-HCl, pH 7.2, containing 0.1 *M* NaCl, and the RNA was extracted by the method of Hayashi and Spiegelman.[27] Each sample yielded 12–14 mg of RNA with a radioactivity of about 1,400 cpm/mg. The RNAase resistance of aliquots containing 0.6–0.8 mg of RNA was determined as described in *Preparation and Methods*, section 7. Ten minutes after infection a 100-ml sample was cooled, the cells were harvested, washed as above, and resuspended in 100 ml of fresh medium. After shaking at 37° for 90 min the lysate was centrifuged clear of debris and intact cells. 50% of the total radioactivity was found in the supernatant. Replicative form was determined in the samples of this experiment as described in *Preparation and Methods* section 11, after the P^{32} had largely decayed.

The RNAase-resistant, radioactive material had the properties expected of double-stranded MS2 RNA. The RNAase resistance showed a sharp thermal transition at 102° in 0.15 *M* NaCl, 0.015 *M* sodium citrate, as measured by heating to different temperatures, cooling rapidly, and testing with RNAase (Fig. 2). The buoyant density of the material was 0.02 gm/ml lower than that of single-stranded MS2 RNA (Fig. 3). Lastly, by means of the specific dilution test discussed in detail in the following paper[18] it was shown that the radioactive component of the double strand was parental, "plus" MS2 RNA. By thermal denaturation and reannealing with an excess of cold MS2 RNA, 92 per cent of the radioactivity could be driven out of the duplex and rendered RNAase-sensitive. The possibility that the parental RNA had been broken down and converted to some other RNAase-resistant material could thus be excluded.

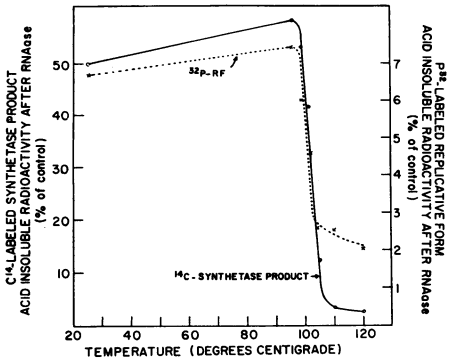

FIG. 2.—Thermal transition curves of RNAase susceptibility of P^{32}-labeled replicative form of MS2 RNA (obtained by infecting *E. coli* with P^{32}-MS2 phage) and of C^{14}-labeled, double-stranded synthetase product. A culture of *E. coli* (3 liters) was grown to a density of 3×10^8 cells, and P^{32}-labeled MS2 phage (6.2×10^5 cpm) was added at a multiplicity of 5. After 15 min the cells were processed, and the RNA was extracted as described in the legend to Fig. 1. 112 mg of RNA were obtained containing 300 cpm/mg of P^{32}-RNA. Aliquots of 4.2 mg were mixed with C^{14}-labeled synthetase product[15,18] (300 cpm), diluted to give 4.5 ml of a solution containing 0.15 *M* NaCl, 0.015 *M* sodium citrate, pH 7, and sealed in glass tubes. After heating to the temperatures indicated, followed by rapid cooling, the samples were examined for RNAase resistance. P^{32} and C^{13} were counted simultaneously in a scintillation spectrometer. The controls were similarly treated but without RNAase digestion.

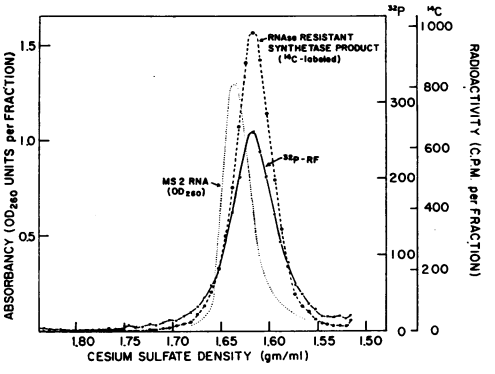

FIG. 3.—Cs_2SO_4 gradient centrifugation of P^{32}-labeled replicative form obtained by infecting *E. coli* with P^{32}-labeled MS2 phage and of C^{14}-labeled, double-stranded synthetase product. *E. coli* was infected with P^{32}-MS2 phage, the cells were harvested after 20 min, and the RNA was extracted, as described in the legend to Fig. 1. Two liters of culture yielded 47.5 mg of RNA (radioactivity, 3.9×10^4 cpm). It was dissolved in 100 ml of 0.15 *M* NaCl, 0.015 *M* sodium citrate, pH 7, and digested with 45 μg of RNAase/ml for 30 min at 25°. After addition of 0.5 ml of 2.5% sodium dodecyl sulfate, the solution was dialyzed for 14 hr against two 1-liter changes of distilled water and lyophilized. The residue was dissolved in 8 ml of water and extracted 4 times with 2 volumes of 80% phenol. After ether extration the solution was dialyzed for 10 hr against 2 liters of 0.2 *M* NaCl 0.02 *M* Tris-HCl, pH 7.2, and for three hr against three 1-liter changes of 0.005 *M* Tris-HCl, ph 7.2. After filtering through a short column of Carboxymethyl Sephadex the solution was concentrated. It had a total absorbancy at 260 mμ of 218, and its acid-insoluble radioactivity was 3,500 cpm. Most of the UV-absorbing material was acid-soduble. C^{14}-labeled, RNAase-resistant synthetase product[18] with 3,500 cpm was mixed with the P^{32}-labeled materal, and Cs_2SO_4 density gradient centrifugation was carried out as described in *Preparation and Methods*, section 10. C^{14}-labeled, RNAase-resistant synthetase product and cold MS2 RNA were mixed and centrifuged in a parallel experiment. The results of both experiments were combined in this figure.

It should be noted that not all virus, irreversibly adsorbed on its host, initiated infection. In our experiments, maximally 50 per cent but probably even less of the parental viral RNA led to replication, as shown by the fact that only 50 per cent of the radioactivity was released by lysis on prolonged incubation of a sample of infected cells. Thus, the minimal value for the conversion of parental RNA into replicative form is not 12 (cf. Fig. 1) but 24 per cent. These data are fully compatible with the idea that *all* infecting parental RNA strands go through a double-stranded state at some early time after infection. The fact that parental RNA, having become RNAase-resistant, subsequently becomes sensitive again, indicates that the parental strand is expelled from the duplex during the course of replication, which thus shows an asymmetric semiconservative character.

The question next arises, is the double-stranded RNA containing the original parental strand the only double-stranded RNA formed in the host cell? To answer this question the specific assay for nonlabeled replicative form (*Preparations and Methods*, sec. 11) was employed. Just as the specific dilution test,[18] the replicative form assay is based on the concept that only complementary base sequences can be annealed to form a stable double helix.[31–34] Table 1 demonstrates the specificity of this assay. No self-annealing could be detected in MS2 RNA obtained from purified virus, indicating absence of "minus" strands. It may be seen from Figure 1 (curve labeled total RF) that there was a continuous synthesis of replicative form leading to the accumulation of large amounts of this material by the end of the infective cycle. It may be mentioned that the replicative form labeled by heating and slow cooling with P^{32}-labeled MS2 RNA had the same thermal transition curve and T_m value as the replicative form labeled *in vivo* by infection of the cells with P^{32}-labeled virus.

TABLE 1

SPECIFICITY OF ASSAY OF REPLICATIVE FORM OF MS2 RNA*

Expt. no.	Additions	RNAase-resistant radioactivity (cpm over control)
1	P^{32}-MS2 RNA (4 μg, 11630 cpm) + MS2 RNA (400 μg)	11
	P^{32}-MS2 RNA + ribosomal RNA (400 μg)	11
	P^{32}-MS2 RNA + sRNA (400 μg)	11
	P^{32}-MS2 RNA + TMV-RNA (400 μg)	16
	P^{32}-MS2 RNA + normal *E. coli* RNA (400 μg)	0
	P^{32}-MS2 RNA + infected *E. coli* RNA (400 μg)	1095
	P^{32}-MS2 RNA + replicative form (88 μg)	3006
2	Replicative form (400 μg) + P^{32}-MS2 RNA (1.3 μg, 5450 cpm)	3455
	Replicative form + P^{32}-labeled normal *E. coli* RNA (1.3 μg, 6600 cpm)	0
	Replicative form + P^{32}-TMV-RNA (1.3 μg, 410 cpm)	0

* Samples were heat-denatured and reannealed, and their RNAase-resistant radioactivity determined, as described in *Preparations and Methods*, sections 7–9. Each sample was run simultaneously with a nonheated control. The RNAase-resistant radioactivity of controls ranged from 36 to 100 cpm in expt. 1 and 25 to 300 in expt. 2 except for the samples with P^{32}-labeled TMV-RNA (of low specific radioactivity) in which it was negligible. The replicative form used was partially purified by a procedure which will be described elsewhere.[35]

It may be further noted from Figure 1 that 45 min after infection some 1.7 per cent of the total cell RNA consisted of replicative form. Assuming that 20 per cent of the RNA was viral RNA, about 8 per cent of it would be double-stranded. Thus, 4 per cent of the total viral RNA would consist of "minus" strands complementary to the parental type "plus" strands, and each host cell would contain upward of 1000 double strands of replicative form. It has not been excluded that a small fraction of the "minus" strands may occur in a free form. Partial purification of the replicative form of MS2 RNA has been achieved.[35]

Identity of the radioactive double-stranded fraction of the product obtained enzymatically with RNA synthetase and the replicative form of MS2 RNA formed in vivo: The double-stranded, labeled product obtained by incubation of C^{14}-labeled nucleoside triphosphates with partially purified RNA synthetase[15,18] proved to be identical to the P^{32}-labeled replicative form of MS2 RNA, obtained *in vivo* by infecting *E. coli* with P^{32}-labeled MS2 phage. This statement is based on the fact that both products had identical thermal transition curves and T_m values (Fig. 2) as well as identical buoyant densities in Cs_2SO_4 (Fig. 3).

Summary and Conclusions.—Infection of *E. coli* with P^{32}-labeled MS2 phage leads within a few minutes to the appearance of an acid-insoluble, RNAase-resistant radioactive product. This material was identified as the double-stranded replicative form of MS2 RNA (*a*) by its thermal denaturation profile and T_m value, (*b*) by its buoyant density in Cs_2SO_4, and (*c*) by the demonstration that the radioactivity is present in one of the strands as parental-type MS2 RNA. From the identity of thermal denaturation curves, T_m values, and buoyant densities in Cs_2SO_4 it is concluded that the replicative form accumulating *in vivo* is identical to the RNAase-resistant, double-stranded RNA labeled with C^{14} on incubation of RNA synthetase holoenzyme with C^{14}-labeled ribonucleoside triphosphates *in vitro*. In the following paper[18] it will be shown by annealing procedures that the radioactivity newly introduced into the duplex is present as parental-type "plus" MS2 RNA.

Whereas the P^{32}-labeled replicative form, containing parental strands of viral RNA, reaches a maximum soon after infection and then decreases, it can be shown

by a new quantitative assay based on annealing techniques that there is a continuous increase of replicative form up to the end of the infective cycle. The decrease of P^{32}-labeled parental strands in the replicative form can be explained by an asymmetric, semiconservative replication mechanism whereby newly formed, cold parental-type "plus" strands expel the parental strands from the duplex as they are synthesized.

The fact that a large proportion of the infecting, parental RNA strands are converted to replicative form indicates that the formation of the double-stranded RNA is an obligatory step and not a side reaction in the replication of MS2 RNA.

The relation of the above facts to the enzymatic mechanism of synthesis of the viral RNA will be discussed in the following paper.[18]

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‡ Fellow of the Jane Coffin Childs Fund for Medical Research.

[1] Abbreviations: RNA and DNA, ribonucleic acid and deoxyribonucleic acid; ATP, GTP, UTP, and CTP, the 5′-triphosphates of adenosine, guanosine, uridine, and cytidine; UDP, uridine 5′-diphosphate; poly U, polyuridylic acid; EDTA, ethylenediamine tetraacetic acid (the trisodium salt was used throughout); Tris, tris(hydroxymethyl)aminomethane; RNAase, pancreatic ribonuclease; DNAase, pancreatic deoxyribonuclease; TMV, tobacco mosaic virus; cpm, counts per minute; p.f.u., plaque-forming units. "Plus" strands are defined as viral RNA strands of the parental type as opposed to "minus" strands which have the complementary base sequence. Absorbancy values refer to a 1.0-cm light path.

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FERREDOXIN AS A REDUCTANT IN PYRUVATE SYNTHESIS BY A BACTERIAL EXTRACT

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It is now well established that reduced pyridine nucleotides supply the hydrogen (electrons) required for the reductive steps in the cellular synthesis of carbon compounds. The oxidation-reduction potential of pyridine nucleotides (TPN or DPN) is −320 mv at pH 7. When Tagawa and Arnon[1] determined that the oxidation-reduction potential of ferredoxins isolated from spinach chloroplasts and *Clostridium pasteurianum* is about 100 mv more electronegative than that of pyridine nucleotides, it became a matter of conjecture whether ferredoxins can participate directly as reductants in carbon assimilation. Their indirect participation by way of pyridine nucleotides, with an attendant drop of about 100 mv in reducing potential, was not in doubt because ferredoxins are known to act as electron carriers in the reduction of TPN by illuminated chloroplasts (see review[2]) and by cell-free bacterial extracts.[3] However, there was no experimental evidence for the direct participation of ferredoxin as a reductant in any enzymic reaction concerned with carbon assimilation.

Evidence has now been obtained that ferredoxin (in reduced form) is required for the reductive synthesis of pyruvate from CO_2 and acetyl phosphate by a cell-free extract of *Clostridium pasteurianum* (eq. 1).

$$CO_2 + \text{acetyl phosphate} + \text{ferredoxin}_{red} \xrightarrow{CoA} \text{pyruvate} + \text{ferredoxin}_{oxid} \quad (1)$$

The "phosphoroclastic" degradation of pyruvate (in the presence of phosphate) to acetyl phosphate, CO_2, and H_2 by cell-free extracts of *C. butylicum* was first described by Koepsell, Johnson, and Meek.[4] The cofactor requirements of this reaction were found by Wolfe and O'Kane[5] to include coenzyme A, thiamine pyrophosphate, and a divalent metal. They also noted that the phosphoroclastic degradation of pyruvate differed from other oxidative decarboxylations in showing no requirement for pyridine nucleotide.