postmicrosomal fraction contained rapidly labeled mRNA, which was also found to exist in a sedimentable form; newly made mRNA might thus be transported to its site of function in association with subcellular particles.

Summary.—RNA from spleen and lymph nodes of the rat has been characterized by a study of its sedimentation properties, kinetics of radioactive labeling, nucleotide composition, and template activity. Immunization results in an unusually rapid rate of synthesis of rRNA and rRNA precursors, which account for a large part of newly synthesized RNA of these tissues. The first newly made RNA to reach the microsomes is a fraction of low molecular weight with the characteristics of mRNA. There is also evidence for the existence in the cell of mRNA of higher mol wt, which probably corresponds to mRNA as it is synthesized in the nucleus.[1] Newly made rRNA is transferred in the cytoplasm as subcellular particles; it appears very rapidly on the microsomes, 18*S* RNA preceding 30*S* RNA.

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Abbreviations used are rRNA, ribosomal RNA; mRNA, messenger RNA; sRNA, soluble RNA; mol wt, molecular weight.

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*REPLICATION OF VIRAL RNA, VIII. STUDIES ON THE ENZYMATIC MECHANISM OF REPLICATION OF MS2 RNA**

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Evidence was presented in the preceding paper[1] that the bulk of the RNA[2] synthesized from radioactive nucleoside triphosphates by RNA synthetase[3] consists of parental type, "plus" MS2 RNA strands, showing that the enzyme is concerned with replication of the viral RNA. Previously it had been found that after the usual isolation procedure, involving extraction with phenol and precipitation with ethanol, about half of the newly formed radioactive RNA was resistant to RNase[4] and had properties identical to those of the MS2-specific double-stranded RNA

found in infected *Escherichia coli*.[5, 6] Most of the radioactivity was located in the "plus" strands; the nonradioactive complementary "minus" strands were apparently derived from endogenous template. This was thought to be the double-stranded MS2 RNA that in purified preparations of RNA synthetase had undergone a similar degree of purification as the enzyme.[6]

It will be shown in this paper that when the phenol extraction is omitted, particularly with use of crude enzyme and low incubation temperatures, nearly all of the product formed by RNA synthetase is as sensitive to RNase as single-stranded MS2 RNA. Treatment with phenol or sodium dodecylsulfate for a few minutes at room temperature, or heating to 50°, renders 50–65 per cent of the product resistant to RNase. These observations suggest the formation of an RNase-sensitive complex involving the enzyme protein, the RNA template strands, and the growing new "plus" strands that, under certain conditions, gives rise to double-stranded and single-stranded viral RNA.

Preparations.—MS2 RNA and P^{32}-labeled MS2 RNA were prepared as described earlier.[5] *Escherichia coli* Hfr 3000, infected with MS2 phage, and uninfected *E. coli* B were grown in a vat fermenter in batches of 150 liters. The washed cells were stored at −12°.[6] Extracts were prepared by grinding in a mortar from 1 to 15 gm of frozen cells with the same weight of alumina at 4° for 15 min. Thirty μg of pancreatic DNase and 3–7 ml of a solution containing Tris-HCl buffer, pH 7.6 (10 mM), EDTA (1 mM), $MgCl_2$ (3 mM), and mercaptoethylamine (5 mM) were added per gram of cells. The mixture was centrifuged for 20 min at 0–4° and 16,000 rpm in the SS-34 rotor of a refrigerated Servall centrifuge and the supernatant was drawn off without disturbing the precipitate. The extracts usually contained 7–20 mg of protein/ml and their absorbance at 260 mμ (A_{260}) was 100–250/ml. RNA synthetase was assayed as described previously,[3] but Mn^{++} was omitted from the assay mixture in all experiments except that of Figure 1*b*, because there was only a small effect of this cation at the concentration used. Purification of RNA synthetase was carried out as reported,[6] except that extracts were prepared by grinding with alumina rather than by homogenization with glass beads, unless stated otherwise. C^{14}-labeled product of RNA synthetase was prepared by incubating the crude extract of infected *E. coli* (containing 0.5–2 mg of protein) or step 4 preparation (containing 0.1–0.2 mg protein) in 0.2 ml of the assay mixture.[3] C^{14}-UTP or C^{14}-GTP was used as labeled precursor. The product of DNA-dependent RNA nucleotidyl transferase (RNA polymerase) was prepared in the same way, using a crude extract from uninfected *E. coli* B. DNase was omitted during extraction and added after incubation only. The endogenous DNA of the extract served as template. RNase-resistant synthetase product was prepared as in earlier work.[6]

Phenol was distilled and shaken once with 0.5 *M* Tris-HCl buffer, pH 7.6, and once with water immediately prior to use. Sodium dodecylsulfate was recrystallized from ethanol.

Methods.—*Assay of RNase resistance:* RNase resistance was assayed by incubating the sample in 5 ml of SSC containing 50 μg of RNase A/ml for 30 min at 25°. The reaction was stopped with 0.5 ml of 60% trichloroacetic acid. Bovine serum albumin (100 μg) was added to the samples prior to acidification. In some experiments 100 μg of insoluble yeast RNA was used as a carrier instead of albumin; this gave rise to slightly higher blanks. The samples were filtered through Millipore HA filters (0.45 μ, 47 or 25 mm diameter, depending on the amount of precipitate) and washed with 6% trichloroacetic acid. If a sample contained radioactive nucleoside triphosphates, sodium pyrophosphate, pH 8, was added to a final concentration of 1.5% prior to acid precipitation. Radioactivities were determined as described previously.[1]

Phenol and sodium dodecylsulfate treatment: Samples (0.2 ml) containing the radioactive products were homogenized with 1.5 ml of phenol for 1 min at 25°, using a Vortex mixer. SSC (2.0 ml) was added and mixed. Following low-speed centrifugation, the phenol phase was drawn off as completely as possible with a Pasteur pipette. An additional 3 ml of SSC was added whereby the remaining phenol was brought into solution. In this solution RNase action is unimpaired. In some experiments the phenol extraction was carried out three times and phenol was finally removed by exhaustive ether extraction; essentially the same results were obtained. For treat-

TABLE 1

EFFECT OF PHENOL AND SODIUM DODECYLSULFATE ON RIBONUCLEASE SUSCEPTIBILITY OF FRESHLY PREPARED SYNTHETASE PRODUCT*

	C^{14}			P^{32}		
	No RNase (cpm)	RNase (cpm)	Resistance (%)	No RNase (cpm)	RNase (cpm)	Resistance (%)
(*A*) C^{14}-labeled product of RNA synthetase (P^{32}-MS2 RNA added as control)						
1. No treatment	2,064	131	6.3	1,767	44	2.5
Phenol extracted	2,319	1,538	66	1,766	28	1.6
2. Phenol extracted	1,645	1,114	68	1,680	13	0.8
100 μg MS2 RNA added and phenol extracted	—	1,095	67	—	9	0.5
Diluted 10-fold with SSC and phenol extracted	—	1,147	70	—	14	0.8
3. No treatment	404	51	13	111	0	<1
Sodium dodecylsulfate (0°)	468	132	28	—	0	<1
Sodium dodecylsulfate (37°)	479	276	56	—	0	<1
(*B*) C^{14}-labeled product of DNA-primed RNA nucleotidyl transferase (P^{32}-MS2 RNA added as control)						
4. No treatment	15,945	112	0.7	1,592	5	0.3
Phenol	18,093	114	0.6	1,532	6	0.4

* Expts. 1, 2, and 4: Each tube contained 0.2 ml of C^{14}-labeled product, prepared by incubating crude extracts of infected or normal *E. coli* for 15 min at 15° as described in *Preparations*. The specific activity of the C^{14}-UTP was 19,000 dpm/mμmole. To the chilled samples, 0.75 μg of P^{32}-labeled MS2 RNA and 0.025 ml of 0.25 *M* EDTA were added. Phenol treatment and RNase assay were then carried out as described in *Methods*. Blanks for each tube were prepared by adding the radioactive UTP after the incubation and carrying out all other procedures as above. The C^{14}-radioactivity of the blanks ranged from 10 to 87 cpm. Radioactivities were counted in the Packard liquid scintillation spectrometer (efficiency for C^{14}, 38%, for P^{32}, about 80%). Expt. 3: The incubations were as above; however, the specific activity of the C^{14}-UTP was 4300 dpm/mμmole. SDS treatment and removal of the detergent are described in *Methods*. Prior to RNase treatment 0.1 μg of P^{32}-MS2 RNA was added to the resulting samples in order to check the degree of completeness of the digestion. C^{14}-blanks ranged from 11 to 35 cpm and were subtracted from the values given.

ment with sodium dodecylsulfate the samples (0.3 ml) were incubated with 0.1 ml of a 10% solution for 10 min, at either 0° or 37°. The detergent was then removed by adding 0.1 ml of 3 *M* KCl and 0.75 ml SSC at 0° and centrifuging off the precipitate. In some experiments the RNA was then precipitated with ethanol three times. Alternatively, the sodium dodecylsulfate was removed by exclusion chromatography on Sephadex G-75. Neither the Sephadex chromatography nor the ethanol precipitations, if carried out at low temperature, altered the RNase susceptibility of the synthetase product.

Results.—Effect of phenol and sodium dodecylsulfate on the RNase susceptibility of synthetase product: As shown in Table 1 (expt. 1) only about 6 per cent of the product formed by a crude extract of infected *E. coli* at 15° was resistant to RNase when assayed directly. After extraction with phenol, the RNase resistance of the product rose to 66 per cent. In a large series of similar experiments, the values ranged from 5 to 10 per cent before, and from 50 to 60 per cent after phenol treatment. RNase-resistant synthetase product, obtained after phenol extraction, has been characterized as double-stranded RNA.[4, 6] Control experiments showed that purified C^{14}-labeled RNase-resistant synthetase product, dissolved in SSC, retained its resistance when treated with RNase in the presence of *E. coli* extract. Table 1 (expt. 3) shows that treatment with sodium dodecylsulfate, particularly if conducted at 37°, had a similar effect as the phenol treatment. On the other hand, phenol treatment did not confer RNase resistance to P^{32}-labeled MS2 RNA added to the extract containing C^{14}-labeled synthetase product (Table 1, expt. 1). It may be noted that addition of a large excess of unlabeled MS2 RNA or 10-fold dilution of the mixture prior to phenol extraction did not alter the effect of phenol

TABLE 2

SUSCEPTIBILITY TO RNASE OF FRESHLY PREPARED SYNTHETASE PRODUCT KEPT AT DIFFERENT TEMPERATURES BEFORE ASSAY*

Temperature (°C)	Radioactivity of Synthetase Product		
	No RNase (cpm)	RNase (cpm)	Resistance (%)
0	110	28	25
15	116	45	39
37	120	61	51
50	112	66	59

* The product was prepared with fresh step 4 enzyme by incubating 5 min at 15°. The specific activity of the C^{14}-UTP was 3600 dpm/mμmole. The samples (0.2 ml) were diluted 25-fold with SSC and kept for 15 min at the temperatures indicated before the RNase assay. Radioactivities were determined with the Tracerlab omniguard low-background system (efficiency for C^{14}, 11%). Blanks, 11 cpm (with RNase) and 2 cpm (without RNase), were subtracted from the values given.

on the synthetase product (Table 1, expt. 2). In contrast to the remarkable effect of phenol on the synthetase product was the finding that phenol treatment of the C^{14}-labeled product of the DNA-dependent RNA nucleotidyl transferase did not change its high susceptibility to RNase (Table 1, expt. 4).

By means of annealing experiments similar to those reported earlier,[1] it was shown that 60 per cent or more of the radioactive RNA synthesized by *crude* extracts of infected *E. coli* under synthetase assay conditions was viral RNA. Of this, 70–80 per cent consisted of "plus" strands. If conversion from sensitivity to resistance to RNase is due to an annealing process, whereby the growing, C^{14}-labeled "plus" strands of MS2 RNA anneal with predominantly unlabeled single "minus" strands to form an RNase-resistant duplex, it is clear that the new "plus" strands must be capable of annealing with great ease, for they do so under conditions in which *added* MS2 RNA itself does not anneal to a measurable extent. This means that the added MS2 RNA strands, even at high concentrations, compete poorly with the nascent "plus" strands for the available MS2 "minus" strands in the reaction mixture. Moreover, since treatment with phenol did not confer RNase resistance either to MS2 RNA or to the product of RNA nucleotidyl transferase, it is apparent that the conversion by phenol from RNase sensitivity to resistance is not a property of MS2 RNA or of newly synthesized RNA in general, but a specific feature of the "nascent" MS2 RNA produced by RNA synthetase.

The susceptibility of "nascent" RNA to RNase was compared to that of parental, P^{32}-labeled MS2 RNA by following the time course of digestion of each by RNase at low concentrations. No difference was found.

Effect of temperature and enzyme purification: The synthetase reaction can be stopped by diluting the samples with a 25-fold excess of SSC. In the experiment shown in Table 2, with *purified* (step 4) RNA synthetase, the RNase resistance of a diluted sample after further incubation for 15 min at 0° was 25 per cent. Incubation at higher temperatures increased the amount of RNase-resistant radioactivity. At 50° the resistance rose to 59 per cent. Thus, incubation of the product at elevated temperature had an effect similar to that of phenol or sodium dodecylsulfate treatment. As shown in Table 3, the RNase resistance of the product, assayed directly after incubation, was higher when the incubation was carried out at 37° than when conducted at 20°. Table 3 also shows that the product synthesized by crude extracts was less resistant to RNase than that formed by partially purified preparations (step 4) of RNA synthetase. The time course of synthesis of total radioactive RNA and of the fraction resistant to RNase (prior to phenol treatment) with crude extract at 15° is shown in Figure 1*a*. There was no increase

TABLE 3

SUSCEPTIBILITY TO RIBONUCLEASE OF PRODUCT OF SYNTHETASE REACTION ASSAYED DIRECTLY*

Enzyme	Incubation temperature (°C)	Specific activity (units/mg protein)	Radioactivity of Synthetase Product No RNase (cpm)	RNase (cpm)	Resistance (%)
Extract	20	0.035	246	15	6
	37	0.053	374	30	8
Step 4	20	1.02	763	86	11
	37	1.50	1133	217	19
Step 4 (after frozen storage)	20	0.75	132	36	27
	37	1.06	185	89	48

* Extract and step 4 enzyme were prepared and assayed as described in *Preparations* and *Methods*. The specific radioactivity of the C^{14}-UTP used for assaying the freshly isolated enzyme was 10,000 dpm/mμmole. Radioactivity was counted on a Nuclear-Chicago gas-flow planchet-counting system equipped with an end window (efficiency for C^{14}, 15%). Blanks ranged from 7 to 11 cpm. Step 4 enzyme was assayed again after frozen storage overnight using C^{14}-UTP with a specific radioactivity of 4300 dpm/mμmole and the Tracerlab low-background counting system. Blanks ranged from 0 to 10 cpm and were subtracted from the values given.

of the small amount of RNase-resistant material during the incubation. Figure 1*b* shows a similar experiment carried out with the step 4 enzyme, in which the fraction of RNase-resistant product increased from 24 to 49 per cent during the course of incubation.

Discussion.— The experiments reported in this paper show that, under certain conditions, the product of RNA synthetase is largely RNase-sensitive and therefore not in a hydrogen-bonded double helix. Following treatment with protein-denaturing agents (phenol, sodium dodecylsulfate), a large proportion of this material is rendered resistant to RNase with extraordinary ease, presumably by being converted into a double-helical form. The evidence so far available suggests that *in vivo* virus-specific double-stranded RNA (replicative form) provides the template for the formation of parental-type "plus" viral RNA strands. Synthesis proceeds by an asymmetric replication mechanism of a semiconservative nature, in which the new "plus" strand displaces the old one from the duplex.[6-9] It would appear that under *in vitro* conditions the enzyme holds the growing "plus" strand

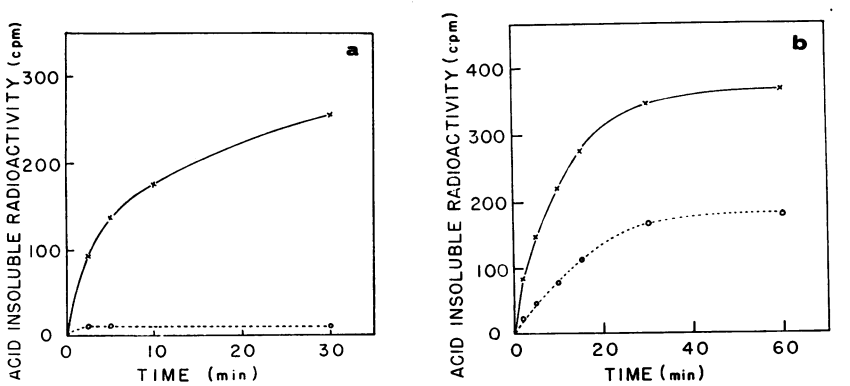

FIG. 1.—Formation of total and RNase-resistant acid-insoluble product by RNA synthetase as a function of time. Incubations were carried out (*a*) with crude extract at 5° and (*b*) with step 4 enzyme at 37°. Experimental details as described in *Methods* except that for (*b*) the extract was prepared by homogenization with glass beads, as originally described,[6] and the RNase treatment was with 100 μg of crystalline RNase/ml. —X—X—X—, total acid-insoluble radioactivity; - -O- -O- -C- -, RNase-resistant radioactivity.

in proximity—but without hydrogen bonding—to the "minus" strand of the template. This complex may, *in vitro*, be prevented from spontaneously giving rise to a duplex by attachment of proteins or ribosomes to the single strands. Removal of protein would allow hydrogen bonding to be established.

In pulse-labeling experiments *in vivo*, substantial amounts of RNase-resistant RNA are found even prior to phenol treatment.[10] While these results might be interpreted to mean that the formation of the RNase-sensitive complex, described above, could be a peculiarity of the *in vitro* reaction, they could also be explained by assuming that the complex has but a transient existence *in vivo*.

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[2] Abbreviations: RNA and DNA, ribonucleic acid and deoxyribonucleic acid; UTP, uridine 5'-triphosphate; RNase, pancreatic ribonuclease; DNase, pancreatic deoxyribonuclease; EDTA, ethylenediamine tetraacetic acid (the trisodium salt was used throughout); Tris, tris(hydroxymethyl)aminomethane; SSC, 0.15 *M* sodium chloride, 0.015 *M* sodium citrate, pH 7; dpm, disintegrations per minute; cpm, counts per minute; UV, ultraviolet; A_{260}, absorbance at 260 mμ, 1.0-cm light path; "plus" strands are defined as viral RNA strands of the parental type as opposed to "minus" strands which have the complementary base sequence.

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