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*IN VITRO SYNTHESIS OF VIRAL RNA BY THE POLIOVIRUS RNA POLYMERASE**

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Infection of cultured mammalian cells by either Mengovirus or poliovirus causes the appearance of a new cytoplasmic RNA polymerase.[1, 2] The enzymes induced by the two viruses are quite similar. Both show a requirement for magnesium ions, are inhibited by manganese ions, require all four nucleoside triphosphates for maximal activity, and catalyze the incorporation of each of the nucleoside monophosphates into RNA. The two polymerases are found in particulate cytoplasmic

structures and neither is sensitive to actinomycin, a compound which inhibits all normal RNA synthesis.

A new cytoplasmic, virus-specific polymerase activity was originally sought because autoradiographic experiments had shown viral RNA synthesis to be an actinomycin-insensitive, cytoplasmic process.[3] Since the polymerase activity which was found appeared in the cytoplasm of infected cells with approximately the kinetics of viral RNA synthesis and was insensitive to actinomycin,[2] it was presumed to mediate viral RNA synthesis in the cytoplasm without the involvement of DNA. The present communication describes experiments which demonstrate that the bulk of the product of the *in vitro* reaction is poliovirus RNA and that a small proportion is double-stranded RNA. Experiments on the structural localization of the polymerase within the cytoplasm are also presented, and the mechanism of the reaction is discussed.

Localization of the Viral RNA Polymerase.—Recent evidence has focused attention on a large particulate structure in the cytoplasm of poliovirus-infected HeLa cells as the center of most virus-directed RNA and protein synthesis.[4-6] Horne and Nagington[7] had previously observed, in the electron microscope, membranous cytoplasmic structures in poliovirus-infected cells. In the following experiment an attempt has been made to ascertain if the viral polymerase is localized in a similar structure.

A cytoplasmic extract was prepared, as previously described,[8] from HeLa cells infected for 3.75 hr with poliovirus. Equal aliquots were submitted to varying degrees of centrifugation and the pellets resuspended and assayed for total polymerase activity. Since all of the activity is sedimented by a 9,000,000 *g*-min centrifugation (60 min at 150,000 × *g*), this was taken as 100%, and the incorporation by the other fractions was expressed as per cent of maximum.

Most of the viral RNA polymerase is associated with structures sedimenting between 50,000 *g*-min and 250,000 *g*-min (Table 1). Only 20% sediments at 50,000 *g*-min which is known to bring down mitochondria. Another 20% is associated with structures which will only sediment in the ultracentrifuge.

A related experiment was performed by Becker *et al.*,[4] who assayed the localization of acid-insoluble radioactivity after actinomycin-treated, poliovirus-infected cells were exposed for 5 min to uridine-H^3. Their data is given in the lower row of Table 1. The partitioning of polymerase activity and the partitioning of a 5-min pulse of uridine are almost identical, indicating that the product of the polymerase reaction retains its association with the large virus-specific particulates. This experiment also supports the thesis that the viral polymerase is responsible for viral RNA synthesis since the *in vivo* uridine incorporation is known to be largely into viral precursor RNA.[9]

TABLE 1

LOCALIZATION OF THE VIRAL RNA POLYMERASE

	—Centrifugation Force in 1,000 *g*-min—			
	50	250	600	9,000
Per cent of viral RNA polymerase sedimented	24	68	79	100
Per cent of *in vivo* uridine label sedimented	29	57	79	

The sedimented material was resuspended in 0.25 *M* sucrose plus 1 mM $MgCl_2$ and assayed for total polymerase activity as described.[2] The precursor was GTP-C^{14} (15 $\mu c/\mu M$; Schwarz BioResearch), and preparations were incubated for 15 min. Results of 5-min uridine label to poliovirus-infected actinomycin-treated cells taken from Becker *et al.*[4]

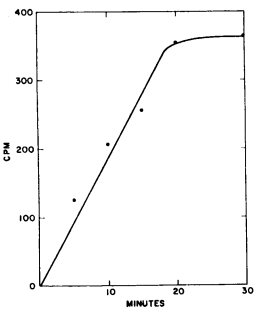

FIG. 1.—Kinetics of GMP^{32} incorporation by 600,000 g-min sediment. Cells (4×10^8) were infected for 3.75 hr with poliovirus and cytoplasmic extract prepared.[8] This was centrifuged for 600,000 g-min and the precipitate resuspended in 0.25 M sucrose, 1 mM $MgCl_2$ at about 5 mg of protein per ml. This was diluted 1:1 at 0°C with a prepared incubation mixture to give a final concentration per ml of 6 μmoles sodium phosphoenolpyruvate (Calbiochem), 40 μg phosphoenolpyruvate kinase (Sigma), 15 μmoles $Mg(Ac)_2$ 2.5 μg actinomycin D, 125 mμmoles each of ATP, UTP, and CTP, and 30 mμmoles α-P^{32} GTP (5×10^4 cpm/mμmole; kindly provided by Dr. T. August of the Albert Einstein Medical College, Bronx, New York). At various times after the start of incubation at 35°C, 0.03-ml samples were pipetted into 0.5 ml 4°-saturated sodium pyrophosphate (about 0.15 M) and 2 ml of 10% trichloracetic acid (TCA) rapidly added. Samples were collected on Millipore filters and counted.

The kinetics of incorporation of GMP^{32} into an acid-insoluble product by the 600,000 g-min sediment are depicted in Figure 1. There is some variation among different preparations, but incorporation is generally linear for 15 min and is ended by 30 min. Linearity for 15 min was shown by all preparations described in Table 1. Equivalent preparations from uninfected cells incorporate no measurable amount of GMP.

The preparation of cytoplasmic fractions in the above experiments involved suspension of the infected cells in a small volume of hypotonic medium and disruption of the cells with a Dounce homogenizer.[8] This method is more gentle than those used previously to isolate polymerase-containing structures,[1] and has been employed in all succeeding experiments. It achieves a partial purification of the polymerase since the sediment from a 600,000 g-min centrifugation of a cytoplasmic extract contains 80% of the total polymerase activity of the cell, but only about 25% of the cytoplasmic ribosomes and little soluble protein.

Sedimentation Analysis of the Product of the Viral RNA Polymerase.—The detergent sodium dodecyl sulfate (SDS) is known to free ribosomal RNA from ribosomal protein[10] and has been used at 0.5% to free viral RNA from the particulates of the cell.[5, 11] It does not degrade whole virus particles[12] and offers the advantage of protection against nuclease activity. SDS was therefore employed here to free the polymerase products and allow their characterization by sedimentation analysis in sucrose gradients containing 0.5% SDS.

When an enzyme preparation is incubated for 15 min with GTP^{32} and then treated with 0.5% SDS, the released material sediments in a sucrose gradient with the profile shown in Figure 2. By reference to the ribosomal RNA peaks of optical density, the sedimentation coefficient of the main, heavy peak is 35S while that of the lighter, minor peak is 16S. There is some labeled RNA sedimenting between 35S and 16S. The small amount of material lighter than 10S is mainly GTP^{32} which is trapped during precipitation of the large amount of protein and lipid found at the top of the gradient. The insert in Figure 2 shows the sedimentation pattern of a similar extract from actinomycin-treated poliovirus-infected cells which had been labeled *in vivo* with uridine-C^{14}. The two profiles are quite similar, indicating that the *in vitro* reaction closely resembles that occurring in the cell. Moreover, it is known that viral RNA, extracted from purified virus, sediments at 35S,[13] so the major peak of the product of viral RNA polymerase is in all likelihood viral RNA. The nature of the 16S material is described below.

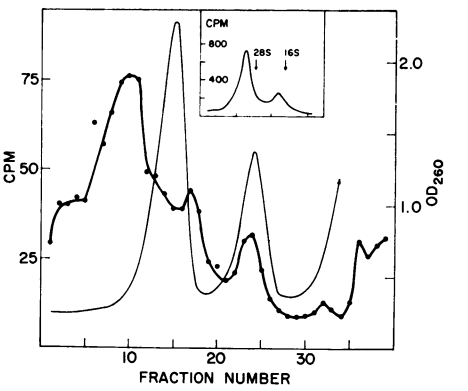

FIG. 2.—Sedimentation analysis of the polymerase product. Procedures as in Fig. 1, except that after 15 min of incubation, the reaction mixture was cooled and SDS added to 0.5%. It was then layered on a 15–30% sucrose gradient (0.1 *M* NaCl, 1 mM Tris-HCl, pH 7.4, 0.5% SDS) and centrifuged for 15 hr at 23,000 rpm in the SW25 rotor of the Spinco Model L ultracentrifuge. Fractions were collected, and the OD_{260} was recorded automatically.[5] The acid-precipitable radioactivity was determined for each fraction. The GTP^{32} used in this and all later experiments was purchased from International Chemical and Nuclear Corp., City of Industry, Calif., and had a specific activity of about 3×10^5 cpm/mμmole. Insert is taken from Baltimore *et al.*[11] and represents a sucrose gradient of the SDS-treated, 600,000 *g*-min pellet from infected cells which were labeled with uridine-C^{14}.

Sucrose gradient analyses have been performed on the material labeled during 5, 10, 15, 20, and 30 min of *in vitro* incubation. The amounts of material at 35S and 16S increase in a roughly parallel manner to the total incorporation into acid-insoluble material. Incubation for a shorter time (2–5 min) results in a diffuse pattern with no sharp 16S peak, but with labeled RNA distributed from 35 to 16S.

Identification of the 35S Material as Viral RNA by a Hydrodynamic Criterion.—It has been shown with 3 different types of viral RNA that configurational changes induced by different salt concentrations are greater than the changes observed with ribosomal RNA under the same conditions.[14–17] Of special relevance is the demonstration that the heavy component of ribosomal RNA from Krebs ascites cells sediments less rapidly than encephalomyocarditis virus RNA under high salt conditions but more rapidly under low salt conditions. On the assumption that poliovirus RNA would show similar effects, the following experiment was designed to use the differential sensitivity to salt conditions as a criterion for identification of the majority product of the polymerase reaction.

It was first demonstrated that poliovirus RNA is more drastically affected in its sedimentation behavior by ionic strength than is ribosomal RNA. When RNA extracted from poliovirus was sedimented through the usual 15–30% sucrose gradient (0.1 *M* NaCl, 5mM Tris-HCl, pH 7.4, and 0.5% SDS), the viral RNA sedimented more rapidly than the 28S component of ribosomal RNA. When the gradient was prepared in 1 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 0.5% SDS,

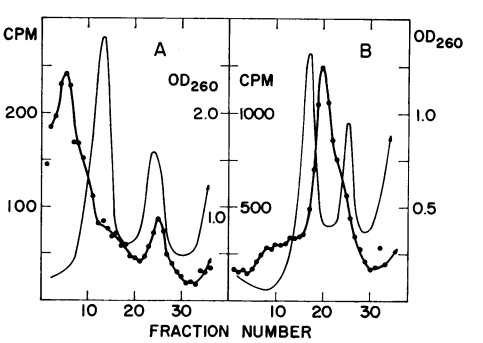

FIG. 3.—Sedimentation of the polymerase product under different ionic conditions.

(*A*) 15–30% sucrose gradient made in 0.1 *M* NaCl, 5 mM Tris-HCl, pH 7.4, and 0.5% SDS.

(*B*) 15–30% sucrose gradient made in 1 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 0.5% SDS.

Other procedures as in Fig. 2.

the viral RNA was located nearer the top of the tube than the heavy ribosomal component. This differential behavior of poliovirus RNA was used in the next experiment as a criterion for the identification of the polymerase product.

An enzyme preparation was incubated with GTP^{32} for 20 min at 35°C, and treated with 0.5% SDS. A portion of this material was layered on the usual 15–30% sucrose gradient. After 15 hr of sedimentation at 23,000 rpm the count profile and optical density tracing were similar to those shown in Figure 2; the major peak of radioactivity was about 7 fractions on the heavy side of the peak of 28S ribosomal RNA (Fig. 3*A*). A second portion of the SDS-treated incubation mixture was layered on a 15–30% sucrose gradient with 1 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 0.5% SDS, and was centrifuged for 18 hr at 25,000 rpm. The major peak of counts was now about 3 fractions to the light side of the heavy component of the ribosomal RNA (Fig. 3*B*). The fact that the polymerase product behaved identically to viral RNA strongly supports the thesis that the majority product of the reaction catalyzed by the viral RNA polymerase is viral RNA.

The Nature of the Material Sedimenting at 16S.—Montagnier and Sanders[18] have recently demonstrated that a double-stranded form of viral RNA exists in encephalomyocarditis virus-infected Krebs ascites cells. This material has a sedimentation coefficient close to that of the smaller component of ribosomal RNA and thus is about 16S. They showed that it is infectious, resists degradation by ribonuclease, bands in cesium sulfate at a lower density than viral RNA, and has a sharp melting point. In actinomycin-treated, poliovirus-infected HeLa cells a similar 16S RNA is synthesized[9] which has been shown to be ribonuclease-resistant and to have a lower density in cesium sulfate than viral RNA.[11] These two studies strongly suggest that the 16S material which is labeled *in vitro* is double-stranded. To test this possibility the density and ribonuclease sensitivity of the 16S RNA was determined.

Figure 4 shows the optical density and count profiles of the 35S and 16S regions of the sucrose gradient after equilibration in a cesium sulfate gradient. It is clear that the 35S material is entirely of the density of single-stranded RNA while the 16S RNA shows a second major band of lighter density. The broadening of the lighter RNA peak (cf. Fig. 2 in ref. 11) is not understood and may indicate that the product of *in vitro* synthesis has an, as yet unresolved, heterogeneity.

The test of sensitivity of the two classes of RNA to ribonuclease is shown in Figure 5. Almost 90% of the 35S RNA was digested while at most 20% of the 16S RNA was hydrolyzed. The 10% residuum in the 35S RNA probably represents the "core" of RNA which is not rendered acid-soluble by ribonuclease.

Thus, the 16S RNA from the *in vitro* reaction mixture, just as the 16S material from infected cells, has a lighter density than 35S RNA and is not digestible by ribonuclease—two properties expected of double-stranded RNA.

Conclusions.—The viral RNA polymerase has been shown to be localized in large, cytoplasmic structures. Its product has been identified as viral RNA by its sedimentation behavior. Some of the RNA made by the polymerase is found in a double-stranded form. From these results it is clear that the cytoplasmic RNA

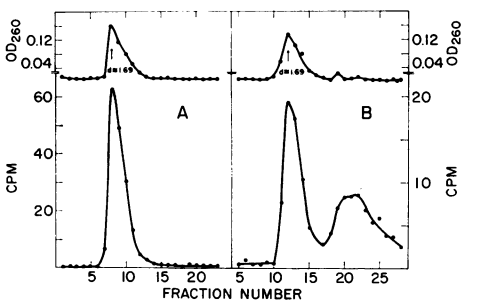

FIG. 4.—Cesium sulfate equilibrium sedimentation of the 35S and 16S components of the polymerase product. An SDS-treated enzyme preparation which had been incubated for 30 min with GTP^{32} was layered on a 15–30% sucrose gradient. The 35S and 16S regions were separately pooled and the RNA from each precipitated with ethanol. The RNA was redissolved in 4 × SSC (SSC is 0.15 *M* NaCl, 0.015 *M* Na citrate, pH 7.5) plus 0.5% SDS and part saved for digestion with ribonuclease. The remaining RNA was taken to a density of 1.60 with a solution of concentrated cesium sulfate and centrifuged at 33,000 rpm in the SW 30 head of the Spinco Model E ultracentrifuge for 65 hr. The gradients were collected in about 30 fractions, of which part was used to measure optical density, part for determining density by refractometry, and the rest for determination of radioactivity. (*A*) 35S RNA; (*B*) 16S RNA. Upper curves depict OD_{260} profiles.

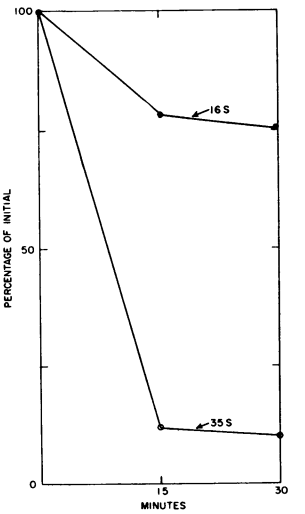

FIG. 5.—Ribonuclease sensitivity of the two components of the polymerase product. The pooled 16S and 35S fractions described in Fig. 4 were reprecipitated, dissolved in SSC, and treated with 10 μg/ml ribonuclease at 25°C. Both samples were adjusted to the same optical density with HeLa RNA before ribonuclease was added. Samples were taken before addition of ribonuclease and at 15 and 30 min of incubation. These were precipitated with 10% trichloracetic acid (plus 0.5 mg carrier yeast RNA), and their acid-insoluble radioactivity was determined. The insoluble radioactivity was expressed as per cent of initial.

polymerase appearing after poliovirus infection of HeLa cells is the enzyme responsible for the synthesis of viral RNA.

Weissman *et al.*[19] have recently demonstrated that the RNA polymerase induced by an RNA bacteriophage[20] catalyzes the synthesis of double-stranded RNA. Their result, in conjunction with the finding that the poliovirus-induced RNA polymerase produces some double-stranded RNA, suggests that viral RNA polymerases utilize RNA as a template for the synthesis of more RNA. Double-stranded RNA can be thought of either as a template for the synthesis of single strands of RNA or as an intermediate which separates into single strands after its synthesis. A choice between these alternatives is not possible at present.

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† Guest investigator from The Rockefeller Institute.

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COMPACTIFICATIONS OF HOMOGENEOUS SPACES AND CONTRACTIONS OF LIE GROUPS

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1. *Introduction.*—Let H be a closed subgroup of a connected Lie group G. In case G/H is a noncompact irreducible Riemannian symmetric space Satake[1] and Furstenberg[2] have given equivariant imbeddings of G/H into compact spaces on which G also acts. Satake's compactification, designed to be used in the theory of automorphic functions, is finite dimensional, while Furstenberg's is infinite dimensional and adapted to the theory of harmonic functions on G/H. C. Moore[3] has described the relation between the two methods.

In this note we indicate another method which applies to a much wider class of homogeneous spaces and in the symmetric space case leads to simpler computations. Our method (imbedding G/H in a Grassman manifold) is not particularly new[4] but does not seem to have been exploited to its full potentialities. The main idea proceeds as follows. Let V be a real vector space; suppose that there is a linear representation $\rho: G \to A(V)$ of G by linear transformations of V, and that there is a subspace V' of V invariant under $\rho(H)$ such that the connected component of the identity of H is equal to the connected component of the identity of

$$\{g \in G: \rho(g)(V') \subset V'\}. \tag{1.1}$$