Virus-Specific Double-Stranded RNA in Poliovirus-Infected Cells

Abstract. *Poliovirus-infected HeLa cells contain a small amount of an RNA that sediments slightly faster than 16S. This RNA is less dense than viral RNA and resists digestion by ribonuclease. It is therefore, considered to be a double-stranded form of poliovirus RNA. A significant amount of this material is found in association with a membranous virus-specific particulate.*

The recent demonstration of the natural occurrence of double-stranded RNA in reovirus and wound tumor virus (*1*, *2*) has focused attention on a general question about RNA viruses; namely, is double-stranded RNA implicated in the synthesis of the RNA of single-stranded viruses? The fact that the single-stranded DNA of bacteriophage ϕX174 becomes double-stranded during replication (*3*) is an additional reason to look for double-stranded structures during the multiplication of the single-stranded RNA viruses. Montagnier and Sanders (*4*) have provided conclusive evidence that a double-stranded infectious RNA exists in cells infected with encephalomyocarditis virus (a single-stranded RNA virus). They showed that this material is less dense and less sensitive to ribonuclease than a single-stranded RNA and that it has a sharp melting temperature. Their results suggest that single-stranded RNA is replicated by way of a duplex molecule. This report offers evidence that there is similar material in HeLa cells infected with poliovirus. Moreover, at least some of the double-stranded material can be found in virus-specific membranous (desoxycholate-senstive) structures that have been identified in the cytoplasm of poliovirus-infected HeLa cells as the probable site of viral RNA synthesis (*5*, *6*).

The growth and infection of suspension cultures of HeLa cells with poliovirus has been described (*7*). Cytoplasmic extracts of infected cells were prepared in a hypotonic buffer (0.0015*M* $MgCl_2$, 0.01*M* NaCl, 0.01*M* tris, *p*H 7.4) (*8*) and cytoplasmic virus-specific structures were isolated by centrifugation for 30 minutes at 20,000*g* in 1.5-ml lusteroid tubes (*5*, *6*, *9*). Treatment of this pellet with 0.5 percent sodium dodecyl sulfate (recrystalized once) according to the procedure of Gilbert (*10*) released RNA. This procedure is equivalent to phenol extraction except that whole virus is not disrupted (*11*) although the RNA from ribosomes and virus-specific structures other than whole virus is released.

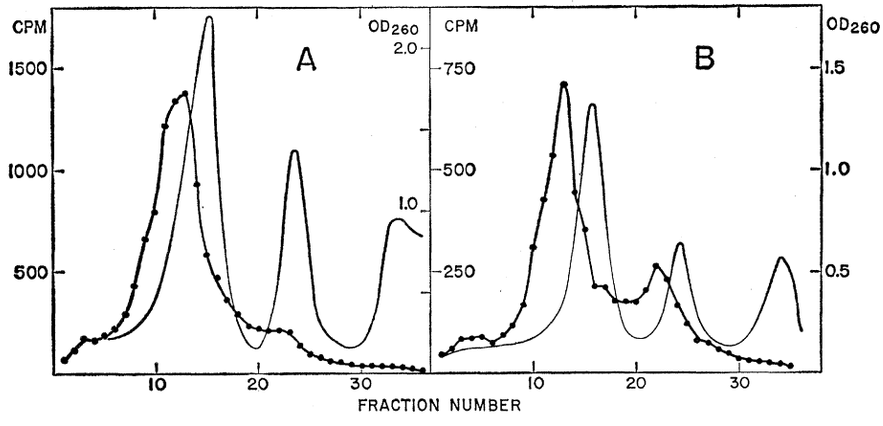

Fig. 1. Sedimentation analysis of cytoplasmic extract treated with sodium dodecyl sulfate from poliovirus-infected HeLa cells. HeLa cells were harvested by centrifugation from 500 ml of culture (4×10^5 cells per milliliter), washed once with serum-free medium, and resuspended (4×10^6 cells per milliliter) in warm serum-free medium containing actinomycin D (5 μg/ml) with about 50 plaque-forming units of poliovirus per cell. The culture was divided and 10 μc of uridine-2-C^{14} (30 $\mu c/\mu M$, New England Nuclear Corp.) was added 2.5 hours after infection to (*A*) and 4.0 hours after infection to (*B*). Each culture was further incubated for 1.5 hours and harvested by centrifugation. The cells were disrupted by homogenization in hypotonic buffer (*9*) and the nuclei were removed by centrifugation (800*g*, 10 minutes). The large cytoplasmic particulates were prepared by centrifugation of the cytoplasm at 30,000*g* for 20 minutes and resuspended in 0.1*M* NaCl, 5m*M* tris-HCl, *p*H 7.4, and 0.5 percent sodium dodecyl sulfate. This extract was layered onto a 30 to 15 percent linear sucrose gradient made with the same solution and fractionated by sedimentation (14 hours at 24,000 rev/min in the SW 25 rotor of the Spinco model L ultracentrifuge). Solid line corresponds to the optical density at 260 mμ, and the two major peaks are 28*S* and 16*S*. Most of the radioactive material (dotted line) sediments faster than the fastest material in the optical density peak and by comparison (*17*) is 35*S*.

Radioactive RNA from the cytoplasm of infected actinomycin-treated cultures was first analyzed by zone sedimentation in sucrose gradients containing sodium dodecyl sulfate (*10*). Fractions from the sucrose gradients (about 0.4 ml) were adjusted to a density of 1.60 (final volume 3 to 4 ml) with a concentrated cesium sulfate solution and the tubes were centrifuged for 65 hours at 33,000 rev/min (*12*). Fractions of about 0.1 ml were collected (*8*) and analyzed for radioactivity, refractive index, and absorbancy at 260 mμ. The Cs_2SO_4 (*12*) was dissolved at a density of about 2.0 in water and passed through activated charcoal twice. The optical density at 260 mμ of the concentrated solution was about 0.150.

It has previously been observed that radioactive RNA obtained by extraction with phenol and sodium dodecyl sulfate from cells infected with poliovirus and treated with actinomycin (the total virus-specific RNA) had two peaks of radioactivity when examined by sucrose-gradient sedimentation analysis (*13*, *14*). The major peak sedimented at 35*S* in 0.05*M* NaCl and 0.0001*M* $MgCl_2$, and was shown to be identical to the RNA that enters virus particles. The second peak had a sedimentation coefficient slightly over 16*S* and, relative to the 35*S*, was greater in amount late in infection. For purposes of discussion this material will be referred to as 16*S*. When Montagnier and Sanders (*4*) reported the discovery of a two-stranded infectious RNA with an *S* value of slightly more than 16*S*, which accumulated during infection of Krebs ascites cells with encephalomyocarditis virus—an RNA virus with about the same size RNA as polio—it became obvious that the 16*S* material from poliovirus-infected cells needed reexamination.

Accordingly, a culture of infected actinomycin-treated cells was divided, and one-half was labeled with C^{14}–uridine from 2.5 to 4 hours of infection and the other half from 4 to 5.5 hours of infection. The RNA released by

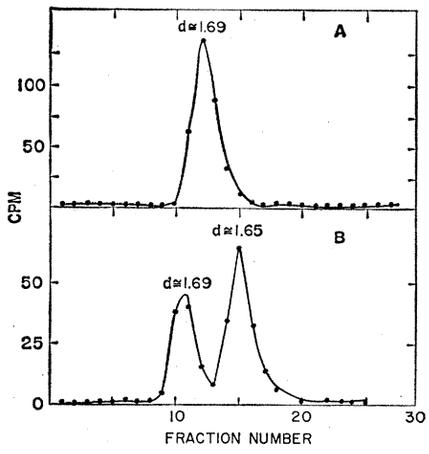

Fig. 2. Cesium sulfate equilibrium sedimentation of the 16*S* and 35*S* poliovirus-specific RNA. The peak tubes from the gradient shown in Fig. 1*B* were taken to a density of 1.60 with concentrated cesium sulfate in a volume of about 3 ml. The suspensions were centrifuged at 33,000 rev/min for 65 hours (*12*) and the radioactivity in the acid precipitable fraction was determined. (*A*), 35*S*; (*B*), 16*S*.

treatment of the virus-specific cytoplasmic structures with sodium dodecyl sulfate was fractionated by sedimentation through a sucrose gradient (Fig. 1). Although radioactive 35*S* and 16*S* RNA is present in both samples, there is more 16*S* RNA than 35*S* RNA

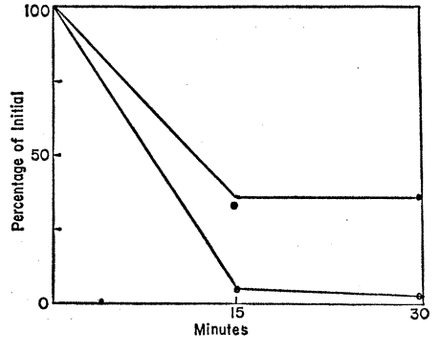

Fig. 3. Ribonuclease sensitivity of the 16*S* and 35*S* poliovirus-specific RNA's. Tubes on either side of the peaks shown in Fig. 1*B* were pooled, shaken once with an equal volume of phenol at room temperature and, after discarding the phenol, were precipitated by adding 2.5 OD_{260} units of ribosomal RNA from HeLa cells, NaCl to 0.25*M*, and 2 volumes of ethanol. After 1 hour at −20°C, the RNA was sedimented for 30 minutes at 50,000*g* in the S-50 rotor of the Spinco model L ultracentrifuge and dissolved in 0.15*M* NaCl, 0.015*M* sodium citrate, *p*H 7.5. Portions were incubated with 10 μg of ribonuclease for various times and the radioactivity in the acid-precipitable fraction was determined. Results are expressed as percentage of initial radioactivity.

in the sample from cells labeled later in infection. The 35*S* and 16*S* RNA taken from the sucrose gradients were then compared with respect to density by equilibrium sedimentation in a Cs_2SO_4 gradient (Fig. 2). Only a single band of radioactivity with a density of about 1.69 was found in the RNA from the 35*S* region, while the 16*S* material gave two bands of approximately equal amounts at density 1.65 and 1.69. Although not shown in Fig. 2, a peak of optical density at 260 mμ, which derived from the host cell ribosomal RNA in the preparations, was always found to coincide with or be very slightly more dense than the 35*S* RNA. These findings duplicate those of Montagnier and Sanders which showed RNA from purified encephalomyocarditis virus to be more dense than the two-stranded RNA, although the absolute densities they reported were somewhat lower than those found for the poliovirus material.

The relative sensitivity to digestion by pancreatic ribonuclease of 35*S* and 16*S* RNA from poliovirus-infected cells was compared next (Fig. 3). It was found that a substantial fraction (from 30 to 75 percent in different experiments) of the 16*S* material which had been labeled late in infection was resistant to high concentrations of ribonuclease, while more than 95 percent of the 35*S* RNA was degraded to acid soluble products. Resistance to pancreatic ribonuclease digestion has been shown to be characteristic of the double-stranded RNA from reovirus (*1*) as well as the double-stranded form of infectious encephalomyocarditis virus RNA (*4*). The part of the 16*S* material which is sensitive to ribonuclease is probably the "tail" from the large 35*S* peak, which is evident in Fig. 1.

The results of Montagnier and Sanders (*4*) and those presented here make clear the existence of a double-stranded form of viral RNA in cells infected with either encephalomyocarditis virus or poliovirus. There is, however, no evidence concerning the role of this type of structure in virus replication. The fact that the double-stranded material can be isolated in the virus-specific particulates, where the viral RNA polymerase and the virus-specific RNA pulse-labeled for 5 minutes are also found (*5*, *15*), strongly suggests a role for the 16*S* material in RNA replication.

For example, the double-stranded structure may be composed of a viral RNA molecule and its base-paired complement, and then serve as a template for the synthesis of viral RNA. If this is the case it is a restricted template in the sense that many more viral RNA molecules seem to be formed than complementary molecules. This conclusion is based on the observations that the 35*S* RNA, which comprises at least 90 percent of the RNA formed in the infected cell, has a base ratio showing an imbalance of adenylic and uridylic acids which would not be true if the complement were made as whole 35*S* molecules in an amount equal to that of viral RNA (*13*, *14*). Moreover, most of the RNA formed in the infected cell enters whole virus particles as single-stranded 35*S* material (*14*).

From these observations as well as the fact that the proportion of double-stranded 16*S* compared to 35*S* RNA is small both in total RNA extracted from infected cells (*14*) and in the particulates as isolated in our experiments (about 1 to 2 percent at 2.5 to 4 hours and 10 percent at 4 to 5.5 hours of which about one-half bands at the double-stranded density), it seems certain that relatively little complementary RNA is formed in the infected cell or alternatively that it is destroyed almost as soon as it is formed. It is interesting in this connection that recent evidence clearly indicates that the RNA polymerase of normal cells copies only one of the two DNA strands in the manufacture of messenger RNA (*16*). Thus, there is a precedent for copying only one of the two strands of a duplex nucleic acid molecule.

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Electrophoretic and Immunological Studies of Squid Axoplasm Proteins

Abstract. *By disc electrophoresis of the axoplasm of* Dosidicus gigas, *14 protein bands have been resolved. Antibodies to the intra-axonal proteins and to squid blood proteins were produced in rabbits. By Ouchterlony's technique, six antigenic components can be demonstrated in axoplasm; the combined use of disc electrophoresis and immune diffusion in agar resolves seven antigenic components in axoplasm; none of these components is detectable in squid blood.*

The giant axons of the squid permit the isolation of macroscopic quantities of cytoplasm of peripheral nerve. Studies on certain proteins in the axoplasm from *Loligo pealii* obtained at Woods Hole, Massachusetts (*1*), and from *Dosidicus gigas* caught near Valparaiso, Chile (*2*), were reported previously from this laboratory. *Dosidicus* offers the technical advantage of being larger; its average giant axon is 1.2 mm wide and 20 cm long; moreover, the axon can be freed from the sheath of connective tissue and thus obtained within only a thin layer of Schwann cells (*3*). By extrusion of the axoplasm from such a clean preparation it is possible to isolate intracellular material which we believe is practically free from nonneuronal contaminants. In addition to characterizing the structure and function of these intracellular proteins, studies were made to determine the number of protein species present in the axoplasm and to determine which of these proteins are specific to the neurons.

Davison and Taylor (*2*), by moving-boundary electrophoresis, showed that the axoplasm of *D. gigas* contains at least two different components: the major and fastest migrating fraction at neutral *p*H probably includes the proteins which make up the neurofilaments; a minor and slower-moving boundary appeared inhomogeneous. However, they were not able to define further the number of protein species present. We have made subsequent studies of the intra-axonal proteins from *D. gigas* by means of a variety of electrophoretic techniques, using paper, cellulose acetate, glass paper, agar gels, and polyacrylamide gels as support media. Electrophoresis on the latter medium has resolved at least 14 components.

In the experiment reported here, electrophoresis on polyacrylamide gels was performed as described by Ornstein and Davies (*4*). A 7.5-percent acrylamide gel at *p*H 9.5 was used as an anticonvection and sieving medium. The only departure from the original method described (*4*) is that the sample was held down by a piece of dialysis membrane (cellophane) placed between the spacer gel and the cathode buffer reservoir. This modification was introduced to avoid further dilution of the protein solutions with the acrylamide solution and also to cope with difficulties in polymerizing the acrylamide solution when it was mixed with the sample. Figure 1 shows a stained gel after electrophoresis of *Dosidicus* axoplasm.

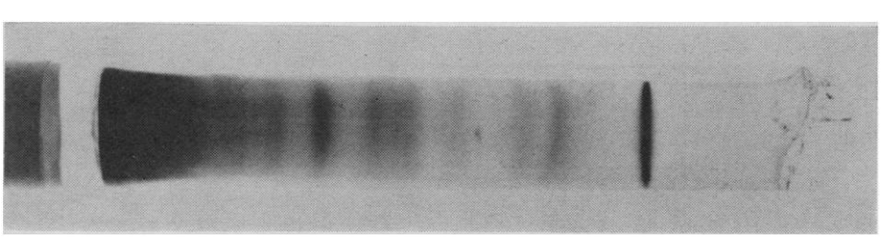

Fig. 1. Disc electrophoresis of squid axoplasm proteins. Cathode at the left, migration toward the right. Stained with amido black.

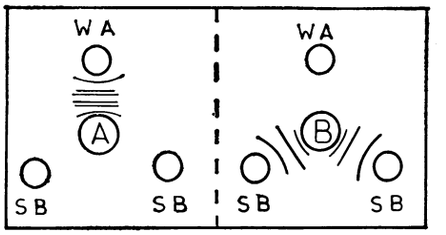

Fig. 2. Two-dimensional immune diffusion. Outer wells: *WA*, whole axoplasm; *SB*, squid blood. Center wells: *A*, axoplasm antiserum; *B*, squid blood antiserum.

To study the specificity of the intra-axonal proteins and to distinguish any which are common to axoplasm and to *Dosidicus* blood, some rabbits were immunized with proteins from whole axoplasm and other rabbits with squid blood. The lack of cross-reactivity between the antigenic proteins in axoplasm and those in squid blood, as studied by Ouchterlony's method, is illustrated

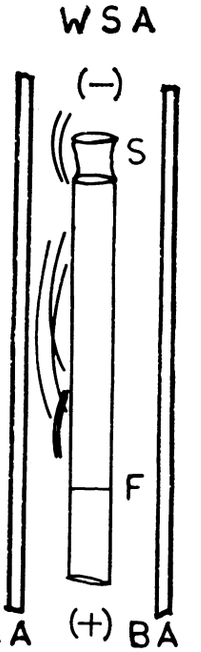

Fig. 3. Combined use of disc electrophoresis and immune diffusion in agar gel. *WSA*, Whole squid axoplasm; *S*, spacer gel; *F*, migration front; *AA*, axoplasm antiserum trough; *BA*, squid blood antiserum trough.