*THE RNA'S OF INFLUENZA VIRUS**

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There are many indications that the nucleic acid of influenza virus is not a single molecule. In 1956, Burnet[1] proposed that several independent genetic linkage groups exist in the virus to explain the high recombination frequency observed in genetic experiments with various strains of influenza virus. The same conclusion was reached by Hirst[2] and Simpson[3] who found up to 50 per cent genetic recombination in marker rescue experiments with partially inactivated influenza virus. In contrast, Newcastle disease virus (NDV), a member of the parainfluenza group of myxoviruses, failed to show genetic recombination.[2] The possible existence of influenza virus RNA in several pieces is further suggested by the ready occurrence of multiplicity reactivation[4] and by the preservation of some virus-specific functions such as the ability to synthesize hemagglutinin and neuraminidase after chemical inactivation of viral infectivity.[5] Similarly, it was suggested[2] that the von Magnus phenomenon,[6] a unique characteristic of influenza virus, might indicate an uncoordinated replication of discrete segments of viral RNA. Loss of infectivity of von Magnus-type influenza virus by γ-ray irradiation follows a multiple-hit curve. In contrast, inactivation of standard virus follows a single-hit curve.[7] An infection caused by several "incomplete" von Magnus virus[6] particles could be the reason for the observed multiple-hit inactivation curve of von Magnus virus.

More recently the RNA from purified influenza virus was found to be heterogeneous in sucrose gradients.[8] The present report provides direct evidence that the RNA of standard virus, von Magnus-type incomplete virus, and the virus-specific RNA from infected cells can be resolved into several distinct components by polyacrylamide gel electrophoresis.

Materials and Methods.—Standard buffer contains: 0.1 *M* NaCl, 0.01 *M* Tris (pH 7.4), 0.001 *M* ethylenediaminetetraacetate (EDTA).

Virus: The PR8 and WSN strains of influenza virus and the L Kansas strain of NDV were used.

Virus growth: Standard influenza virus was grown for 32 hr as described[8] and von Magnus virus by inoculating 0.2–0.8 ml undiluted stock virus[8] after the first or second undiluted passage in 10-day-old embryonated eggs. After 15–20 hr at 37°C the allantoic fluid was harvested for virus purification.

Virus assay: The hemagglutinin titer and viral infectivity were determined as described,[8] except that virus dilutions for the infectivity assay were made in serum-free medium 199.

Virus purification was carried out at 0–4°C. After centrifugation at 12,000 *g* for 10 min, calf serum was added to the allantoic fluid to a concentration of 5%. The virus was then precipitated by the addition of an equal volume of a saturated, neutralized $(NH_4)_2SO_4$ solution. After stirring for 15 min the solution was centrifuged for 5 min at 30,000 *g*. The pellet was dissolved in about 5% of its original volume of standard buffer. After removal of an unidentified insoluble material by centrifugation for 10 min at 8000 *g*, the supernatant was incubated for 10 min at 5°C with 1 μg of RNase per ml. The solution was then placed over 2 layers of sucrose in standard buffer in a Spinco SW50 rotor tube: the top layer 2 ml of a light (1.1 g/ml) 20% sucrose solution, the bottom layer

0.3 ml of a heavy (1.32 g/ml) 65% sucrose solution in D_2O. After centrifugation for 40 min at 50,000 rpm, the virus was visible as an opaque band at the density interface between the light and the heavy sucrose solutions. After appropriate dilution, the virus was banded to equilibrium in a sucrose density gradient.[8] This procedure yields the following recoveries of purified virus with a density ranging from 1.20 gm/ml to 1.24 gm/ml: about 50% or more of both hemagglutinin and infectivity of standard virus and about 50% of the infectivity and 15–30% of the hemagglutinin of von Magnus virus.

Isolation of the RNA was done as described previously,[9] with the following modification. About 1% (w/v) dithiothreitol and 10% phenol were added to the virus solution in standard buffer, and then sodium dodecylsulfate (SDS) was added to a final concentration of 1%. After 5 min incubation and 3 phenol extractions at room temperature, the RNA was precipitated with alcohol, redissolved in 0.002 *M* EDTA (pH 7.5), and reprecipitated with alcohol.

Preparation of intracellular virus-specific RNA in cell culture: About 10^7 primary chick cells were seeded on a 10-cm plastic Petri dish.[8] After incubation for 1 hr at 41°C the cells were then infected with 3 ml of a 1:1 dilution of stock virus[8] (WSN) in Eagle's medium. After 45 min incubation, 3 ml of medium 199 containing 2% calf serum was added to the culture. (If these cells are further incubated at this stage, they are all dead after 12–16 hr.) The recovery of (mainly cellular) nucleic acids[8] from one such Petri dish of infected cells was about 2.5 A_{260} units.

RNA gel electrophoresis was carried out essentially as described by Bishop *et al.*,[10] with the following modifications. An ethylene diacrylate cross-linked polyacrylamide gel which can be dissolved by piperidine[11] was used exclusively. Acrylamide was purified as described previously.[12] Ethylene diacrylate was added to the purified acrylamide solution as obtained from Borden Chemical Company. A stock solution in H_2O containing 30 gm acrylamide and 2 gm ethylene diacrylate in 100 ml was used in all experiments. The gels were polymerized in Plexiglass tubes (0.7-cm i.d.) by the addition of $(NH_4)_2S_2O_8$ to a final concentration of 0.4 mg per ml. One hr after polymerization the gels were preelectrophoresed for 30 min at 50 v and used directly or stored in the refrigerator. RNA was dissolved in buffer containing 0.004 *M* Tris acetate pH 7.2, 0.002 *M* sodium acetate, 0.001 *M* EDTA, 0.3% SDS, and 15% glycerol for application to a polyacrylamide gel column. About 30–80 μliters of this solution, which is called electrophoresis sample buffer, was applied to a gel column.

Measurement of radioactivity: After electrophoresis the gel column was frozen and fractionated into 1-mm slices by stacked razor blades. If P^{32} was counted and excess H^3 was used as reference marker, each slice was placed in 1 ml 0.2 *M* piperidine, and if H^3 was counted, in 0.05 ml 1 *M* piperidine in a scintillation vial. After shaking for 15 min at 37°C, the polyacrylamide slices were dissolved. The radioactivity was measured in a Tri-Carb liquid scintillation counter after the addition of 10 ml Bray's scintillation fluid.

Results.—Electrophoretic patterns of the RNA of standard influenza virus which had an infectious unit/hemagglutinin (I/HA) ratio of approximately 10^7 are shown in Figure 1. Five components of P^{32}-RNA recovered from purified PR8 influenza virus (the same pattern was obtained for WSN virus) and the three H^3-cell RNA's are seen in Figure 1*A*. Further resolution of the P^{32}-RNA was obtained after prolonged electrophoresis under the same conditions as shown in Figure 1*B*. In order to correlate these electrophoretic patterns of influenza virus RNA with its previously described distribution in sucrose gradients,[8] an aliquot of the same P^{32}-RNA preparation was sedimented in a sucrose gradient. As shown in the insert of Figure 1*A*, the P^{32}-influenza RNA sediments as a broad peak showing the previously reported[8] heterogeneity. The apparent difference of the relative position of the bulk of the P^{32}-RNA to the position of the 18*S* H^3-cell RNA after fractionation by sedimentation or by gel

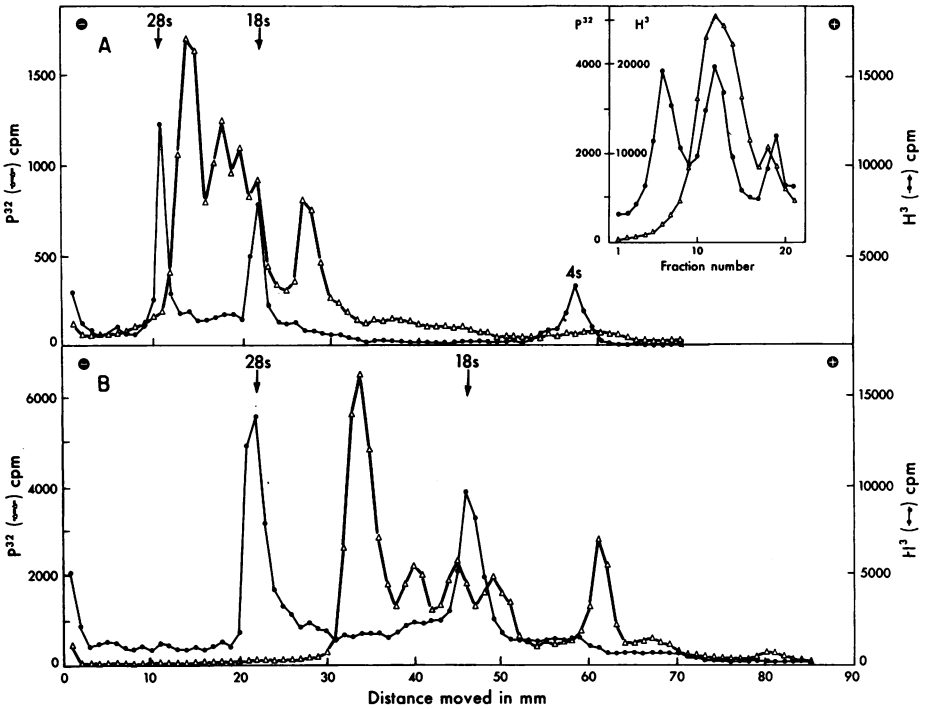

FIG. 1.—Electrophoretic patterns of PR8 influenza virus P^{32}-RNA (Δ — Δ) and H^3-cell RNA (● — ●) after polyacrylamide gel electrophoresis for 2.5 hr (*A*) and 4 hr (*B*) at room temperature at 70 v and 5 ma per tube. Ethylenediacrylate cross-linked gels (7-mm diameter, 75-mm length) were used at 2.7% acrylamide concentration. Subsequent to electrophoresis the gels were sliced, dissolved in piperidine, and counted in Bray's scintillation fluid (*Methods*). Sucrose gradient sedimentation of an aliquot of the same RNA that was used in the experiment illustrated in (*A*) is shown in the insert of (*A*) for comparison with the electrophoretic patterns. The RNA was fractionated by centrifugation in 5–20% (w/v) sucrose in standard buffer for 4 hr at 50,000 rpm in a Spinco SW50 rotor at 5°C.

electrophoresis is most likely due to the different ionic strengths used for gel electrophoresis (0.06 *M* salt) and sucrose gradient sedimentation (0.11 *M* salt). It was found that the sedimentation constant of influenza virus RNA is more reduced, corresponding to a more unfolded tertiary structure, at low ionic strength, than cell RNA.[8] Probably for this reason the bigger components of influenza RNA migrate more slowly and register as bigger RNA's in the gel than 18*S* cell RNA.

The RNA of incomplete von Magnus-type influenza virus: Assessment of purity of von Magnus virus is more difficult than that of standard virus, because the allantoic fluid from which it is purified is much more contaminated with cellular debris than allantoic fluid containing standard virus. A sucrose density gradient distribution of PR8 von Magnus virus is similar to that of PR8 standard virus.[8] The maxima of infectivity, hemagglutinin, radioactivity, and absorbancy at 260 mμ all coincide at a density of about 1.23 gm/ml, which is the density of standard virus. Hemagglutinin and radioactivity of von Magnus virus, how-

ever, show a more asymmetrical density distribution toward lower densities than standard virus. The electrophoretic pattern of the RNA of purified von Magnus virus (Fig. 2) indicates that the RNA's which are found in standard virus are also present in von Magnus virus, but the share of the RNA components with a lower mobility than 18S cell RNA is reduced. Details of these quantitative differences between the RNA patterns of standard virus and von Magnus virus were found to depend on the virus inoculum, the incubation time in the egg, and the virus strain used in a number of experiments. There are also new, smaller RNA components in von Magnus virus that are not seen in the electrophoretic pattern of standard virus RNA. Their relative amounts varied in a number of experiments and seemed to increase with decreasing I/HA ratio. Preliminary hybridization experiments of the slowest-sedimenting RNA's of von Magnus virus (corresponding to the RNA's with the highest electrophoretic mobility) suggest that at least part of these RNA's are virus-specific. In addition, von Magnus virus preparations of the second and third undiluted passage differ from standard virus in their P^{32}-RNA content as measured by the ratio of P^{32} in RNA to total P^{32} in virus. An average of 3 ± 1.5 per cent (average of 6) of the total P^{32} present in von Magnus virus was found in the RNA compared to 11 ± 2 per cent (average of 3) in standard virus. This result agrees with previous findings of Ada and Perry.[13]

Occasionally in the electrophoretic pattern of the nucleic acid of some preparations of "purified" von Magnus virus a very sharp P^{32} peak appeared near the top of the gel column (not in Fig. 2). The material in this P^{32} peak is resistant to RNase but is completely digested by DNase as tested by electrophoresis of the digest. Since this peak was never found in the RNA pattern of standard virus and appears only occasionally in the pattern of von Magnus virus nucleic acid, it is probably a distinct cellular DNA component of small size like mitochondrial DNA from damaged mitochondria. This is suggested by an

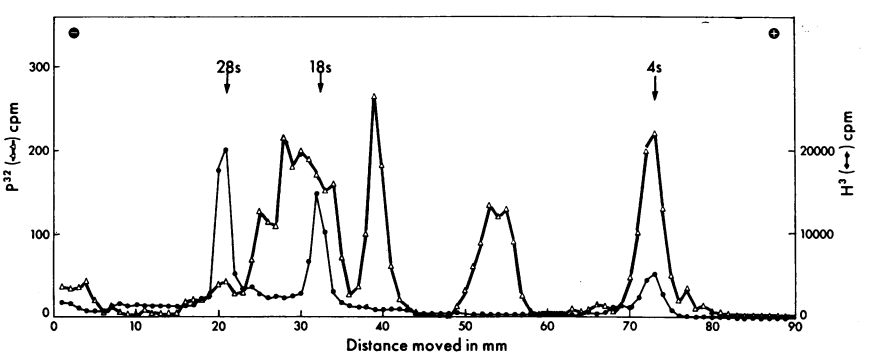

FIG. 2.—Gel electrophoresis of P^{32}-RNA (Δ — Δ) of purified von Magnus-type influenza virus after the third undiluted passage, together with H^3-cell RNA (● — ●). The virus was grown by innoculating 0.3 ml undiluted PR8 influenza virus after the second undiluted passage with 1 mc $H_3P^{32}O_4$ in embryonated eggs. After 20 hr of incubation the allantoic fluid was harvested for virus purification. Purified virus had an infectivity/hemagglutinin ratio of 6×10^3. The RNA was fractionated on a 2.7% gel column for 2.5 hr at 70 v as described for Fig. 1.

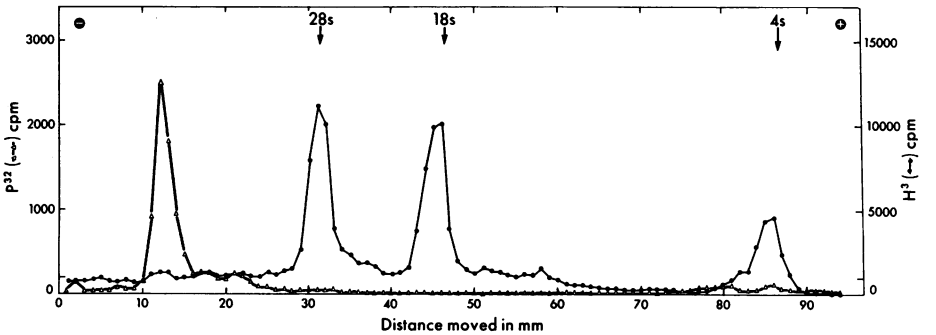

FIG. 3.—Electrophoretic pattern of P^{32}-NDV RNA (Δ — Δ) isolated from purified NDV (density 1.20–1.24 gm/ml) after the third undiluted 18-hr passage in embryonated eggs together with H^3-cell RNA (● — ●). All P^{32} of this preparation was rendered soluble in 5% trichloroacetic acid after digestion for 30 min at 36° in 0.2 *M* NaCl with 10 μg RNase per ml. Electrophoresis was carried out on a 2.6% gel column for 3 hr as described for Fig. 1.

experiment in which the described P^{32} component was co-electrophoresed with C^{14}-mitochondrial DNA from a human cell line[14] after RNase treatment. Both P^{32}- and C^{14}-DNA formed very sharp peaks at 17 and 16 mm, respectively, in a 2.7 per cent gel after electrophoresis for six hours at 60 volts, indicating that the two DNA's have very similar structures.

In contrast to the apparent changes of the RNA pattern of influenza virus, the RNA of NDV remains a homogenous 57*S*[9] component after three undiluted passages of the virus in embryonated eggs. As shown in Figure 3, almost all the P^{32}-RNA extracted from purified NDV migrates as a single component much more slowly than the 28*S* cell RNA. This result is compatible with previous observations that NDV does not show a von Magnus phenomenon.[15]

Intracellular virus-specific RNA: In order to test whether the heterogeneity of influenza virus RNA is original or a secondary product of a late intracellular or an extracellular degradation, intracellular virus specific RNA synthesis[8] was examined. The earliest time at which RNA is synthesized in the presence of actinomycin D was picked because it is the least likely to allow RNA degradation to occur. As shown in Figure 4, there is a maximum rate of single- and

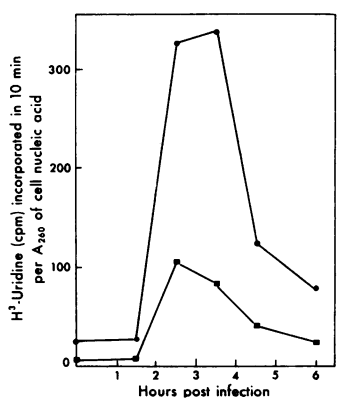

FIG. 4.—Rate of H^3-RNA synthesis in WSN influenza virus-infected chick embryo fibroblasts in the presence of actinomycin D at various times after infection.

Freshly seeded cells were infected with WSN virus at a multiplicity of 10–100 (*Methods*). At 0.5 before and 1, 2, 3, 4, and 5.5 hr after infection, 25 μg actinomycin D was added to a culture and 30 min later 100 μc H^3-uridine (25 c/mM). After incubation for 10 min at 41°C, the total nucleic acids were extracted from the cells.[8] The total H^3-RNA (● — ●) and the RNase-resistant H^3-RNA (■ — ■) per 1 A_{260} of extracted nucleic acid (consisting mainly of cellular DNA and RNA) was then determined.

double-stranded RNA synthesis in influenza virus-infected chick cells between 2.5 and 3.5 hours post infection. The electrophoretic pattern of this RNA and P^{32}-RNA from standard virus (WSN) as shown in Figure 5 indicates that a peak of intracellular H^3-RNA coincides approximately with each component of the viral P^{32}-RNA. In addition there is H^3-RNA with a lower mobility than the P^{32} RNA's from mature virus. This H^3-RNA most likely is double-stranded or partially double-stranded influenza virus RNA[8] because heat denaturation converts almost all of it to RNA with the electrophoretic mobility of viral RNA (Fig. 5*B*). Since an infected cell contains mostly free single-stranded viral RNA and only about 15–25 per cent double-stranded virus-specific RNA and less than 5 per cent single-stranded RNA complementary to viral RNA,[8] it is probable that most of the H^3-RNA components present at an early time during the eclipse period are the same as those obtained from mature virus. The possi-

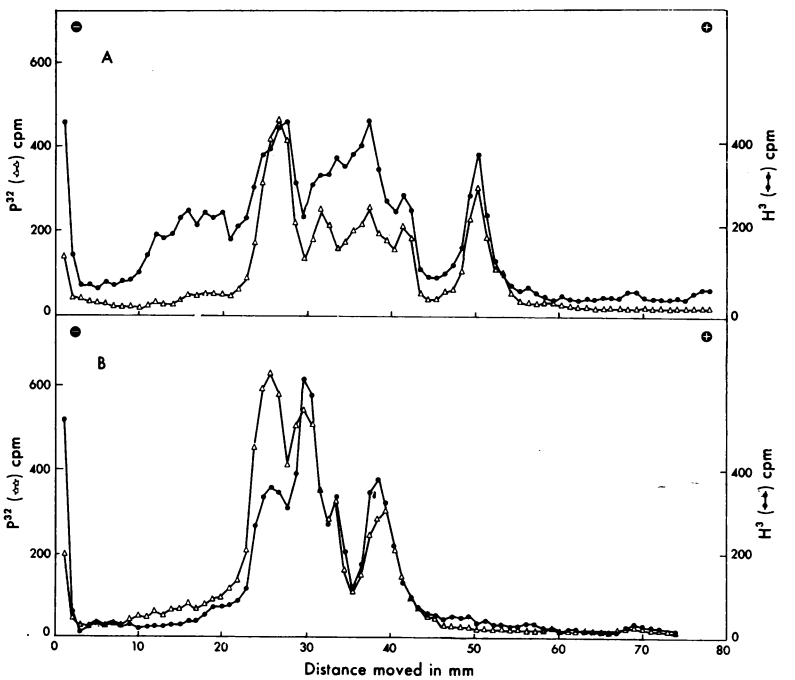

FIG. 5.—Coelectrophoresis of intracellular WSN influenza virus-specific H^3-RNA (● — ●) and P^{32}-RNA (Δ — Δ) isolated from mature WSN influenza virus before (*A*) and after (*B*) heat denaturation. The RNA synthesized in virus-infected cells was labeled with H^3-uridine in the presence of actinomycin D at 2.5 hr post infection for 1 hr and isolated as described in Fig. 4.

(*A*) One aliquot containing about 1.8 A_{260} and 15,000 cpm H^3 was dissolved in electrophoresis sample buffer and electrophoresed with P^{32}-RNA from purified WSN influenza virus on a 2.9% gel column for 4.5 hr as described for Fig. 1.

(*B*) Another equal aliquot of the described nucleic acid preparation of infected cells was dissolved in 300 μliter 0.002 *M* EDTA (pH 7.5) together with P^{32}-RNA from WSN influenza virus. The solution was boiled in a sealed ampule for 3 min and then quickly cooled in melting ice. After alcohol precipitation the RNA was electrophoresed on a 2.7% gel column for 3.5 hr as described for Fig. 1.

bility that identical RNA-breakdown products are obtained from an infected cell as well as from infectious virus seems very unlikely, in particular since the unlabeled cell RNA's are intact in sucrose gradients. It should be noted that the electrophoretic resolution of labeled viral RNA's of the cell is less distinct and reproducible than that of the virus because of impairment by the large excess of unlabeled cellular DNA and RNA.

Since it is likely that there are several single-stranded RNA species in influenza virus as well as in the virus-infected cell, one might expect that there are also several corresponding double-stranded or partially double-stranded RNA's in the infected cell. To test this, all partially double-stranded RNA was converted to completely double-stranded, RNase-resistant RNA prior to fractionation by digestion with a low concentration of RNase. The digest was analyzed by gel electrophoresis in the presence of P^{32}-cell RNA. At least four RNase-resistant H^3-RNA components were resolved as illustrated in Figure 6. Their electrophoretic mobilities are lower than that of 18*S* cell RNA. About 90 per cent of the original intracellular, virus-specific H^3-RNA migrated slightly faster than 4*S* cell RNA after RNase digestion. This indicates that the employed RNase concentration is sufficient to digest all single-stranded RNA to a size smaller than 4*S* cell RNA, and it has been previously shown to minimize any single-chain scissions in double-stranded RNA.[16] It therefore seems likely that the single-stranded RNA components of influenza virus have double-stranded counterparts in the infected cell.

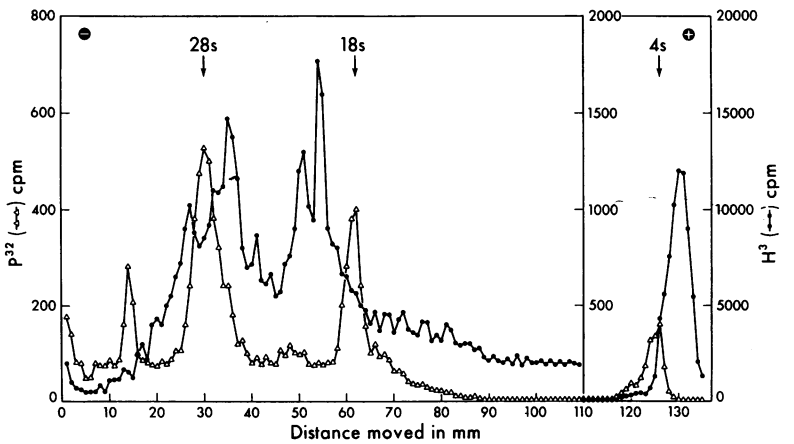

FIG. 6.—Electrophoresis of RNase-resistant intracellular H^3-RNA (● — ●) extracted from WSN influenza virus-infected cells together with P^{32}-cell RNA (△ — △). Intracellular virus-specific H^3-RNA was prepared as described for Fig. 5. About 3.8 A_{260} nucleic acid extract from infected cells, containing 30,000 cpm H^3, was dissolved in 200 μliters buffer, 0.2 *M* NaCl, 0.01 *M* Tris (pH 7.4), and 0.002 *M* $MgCl_2$ and incubated with 0.2 μg RNase and 5 μg DNase for 20 min at room temperature. After the addition of EDTA to a final concentration of 0.01 *M* and SDS and mercaptoethanol to 1% (w/v), the solution was three times extracted with phenol and the RNA recovered by alcohol precipitation. (Enough cellular nucleic acids are still alcohol-precipitable to serve as carrier nucleic acid.) The H^3-RNA was then dissolved in electrophoresis sample buffer, mixed with P^{32}-cell RNA, and analyzed by gel electrophoresis on a 2.7% gel column for 4 hr as described for Fig. 1.

Discussion and Summary.—The described experiments suggest that influenza virus contains probably five physically distinct RNA components. Virus-specific RNA components which are probably the same are also detectable at an early time during the eclipse period in the infected cell. The RNase-resistant RNA of the influenza virus-infected cell can also be resolved into at least four distinct components. Thus the RNA of mature influenza virus and the single- and the double-stranded virus-specific RNA in the infected cell all show a specific and very similar heterogeneity. Since none of these RNA preparations have been found to be infectious there is no proof that they represent the intact RNA of the virus, but their apparent heterogeneity is consistent with a number of properties of influenza virus discussed in the introduction. The behavior of influenza virus RNA suggests a fundamental difference between the RNA structures of the two types of myxoviruses, the influenza and parainfluenza viruses. The same methods of extraction of the RNA yield a large, single RNA molecule if applied to NDV, a typical parainfluenza virus, but several small RNA components if applied to influenza virus. The heterogeneity and small size of influenza RNA (not $>18S_{w,20}$, which is smaller than any known viral RNA) as well as the relative complexity of the virus further suggest that influenza RNA may consist of several pieces since one large RNA could not be extracted.[8, 17]

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