ISOLATION OF HIGH-MOLECULAR-WEIGHT, P^{32}-LABELED INFLUENZA VIRUS RIBONUCLEIC ACID*

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Several investigators[1-7] have reported the isolation of ribonucleic acid (RNA) from influenza virus and influenza virus-infected tissues by methods which could result in the isolation of intact viral RNA. But the only indication of the size of the RNA molecules in any of these preparations, a sedimentation coefficient of 11*S*, was given by Sokol, Schramek, and Šponar.[5] Schäfer[8] has quoted a value of 17*S* for RNA isolated from fowl plague virus, a virus similar to influenza, and recently, RNA's having considerably larger sedimentation coefficients have been isolated from other larger, lipid-containing viruses.[9, 10]

A particle weight of between 200 and 400 $\times$ 10^6 may be calculated from the various estimates of influenza virus dimensions, density, and hydration.[11] Depending on the influenza strain and the analytical methods employed, RNA has been estimated to represent from 0.7 to 1.2 per cent of the dry weight of the virus in preparations having high infectivity.[12-17] Thus, if the RNA of influenza virus

particles exists as one single-stranded molecule, isolated intact RNA could be expected to sediment about as rapidly as, or more rapidly than, tobacco mosaic virus (TMV) RNA, which possesses a sedimentation coefficient of 33*S*. In this communication several properties of RNA isolated from a mixture of unlabeled TMV and P^{32}-labeled influenza virus and of RNA isolated from influenza virus host tissue are examined.

Materials and Methods.—The PR-8 (Squibb) strain of influenza A, obtained from Dr. E. H. Lennette of the Viral and Rickettsial Disease Laboratory, Berkeley, was passaged in embryonated eggs incubated at 37°C. To increase the efficiency of P^{32} incorporation, 0.2 ml of a suspension of calcium phosphate in $CaCl_2$ solution was injected into the allantoic cavities of 10-day-old eggs to precipitate a portion of the unlabeled inorganic phosphate present in allantoic fluid (A.F.). The suspension was prepared by slowly adding 2 ml of 0.02 *M* $CaCl_2$ to 2 ml of 0.02 *M* potassium phosphate, pH 7.0. The precipitate was collected by centrifugation, was washed with 2 ml of 0.2 *M* $CaCl_2$, and was suspended in 6 ml of 0.2 *M* $CaCl_2$.

Six hr after $CaCl_2$ injection, the eggs were inoculated with 0.1 ml of a 1:1000 dilution of A.F. from infected eggs, and at 7 hr 1 mc of neutralized P^{32} sodium phosphate was injected per egg. At 46 hr the eggs were refrigerated (4°C) for 4–6 hr, after which the A.F. was harvested and held at 0–4°C.

Hemagglutination and infectivity: Hemagglutination (HA) titers of virus solutions were determined using either twofold or fractional serial dilutions with 0.1 *M* potassium phosphate, pH 7.0, in the wells of Perspex hemagglutination trays. An equal volume of 1% (v/v) chicken erythrocytes was added to each well, and the HA titer was read as the reciprocal of the highest dilution for which hemagglutination was complete after 1 hr at room temperature. An HA unit is defined as the amount of virus in 1 ml of a solution having an HA titer of one.

The 50% egg infectious dose (EID_{50}) of virus preparations was determined by the method of Reed and Muench[18] using ten eggs at each dilution.[19]

Isolation of virus: A suspension of Macaloid,[20] an anionic clay, was prepared as described elsewhere.[21] The isolated A.F. was centrifuged at 1000 × *g* for 20 min. Macaloid suspension [4% (w/w)] was added to the supernatant to a final concentration of 0.01%. The mixture was stirred at 4°C for 5 min and was centrifuged at 1000 × *g* as before. The clear supernatant contained about one third of the ribonuclease activity of untreated A.F., but the HA titer was not reduced.

Five ml of a 65% sucrose solution in 0.1 *M* potassium phosphate, 0.005 *M* mercaptoethanol, pH 7.0, and, above it, 5 ml of 10% sucrose in the same buffer were placed in the bottom of Spinco SW25 rotor tubes. Twenty-three ml of the supernatant fluid obtained from the Macaloid treatment were layered over the 10% sucrose, and the tubes were centrifuged at 25,000 rpm for 3.5 hr. This caused all of the HA activity to sediment, forming an opalescent band at the interface between the 10% and 65% sucrose layers. A turbid virus solution (3–5 ml) was recovered from each tube with a syringe and "J"-tipped needle.

Agarose column chromatography: A column of 250–400-μ diameter 4% agarose (Bausch and Lomb Seakem) beads, prepared according to Hjertén,[22] was equilibrated with column buffer [0.02 *M* sodium phosphate, 0.002 *M* KCl, 0.0005 *M* $MgCl_2$, 0.0005 *M* $CaCl_2$, 0.02% Tween 80 (Atlas Chemical), pH 7.0]. Concentrated virus from the previous step was applied and was eluted with column buffer at a linear fluid velocity, in the column, of 4 cm/hr. Aliquots of column effluent were assayed for HA activity, radioactivity, and sucrose. Sucrose was determined by colorimetric assay after heating with acid-chromate cleaning solution.

Isolation of viral nucleic acid: Fractions represented by the first peak of radioactivity eluted from the agarose column (Fig. 1) were pooled. Appropriate amounts of unlabeled TMV and mercaptoethanol, to a final concentration of 1%, were added to the pooled material. Then, in rapid succession, Macaloid was added to a final concentration of 0.1%; sodium dodecyl sulfate (SDS), as a 20% solution, was added to a final concentration of 1%; and the mixture was warmed to 30°C in a hot-water bath. One-hundredth vol of 0.5 *M* ethylenediaminetetraacetic acid (EDTA), pH 8.1, was added, and, after 1 min, the mixture was cooled to 4°C. The solution was extracted three times with phenol (equilibrated with 0.05 *M* Tris·HCl) and three times with ether as described elsewhere.[20, 21] The ether was removed by bubbling nitrogen through the solu-

tion, and low-molecular-weight, P^{32}-labeled compounds were removed by one of two procedures, either (1) chromatography on a column of Sephadex G25 equilibrated with 0.2 *M* potassium acetate, pH 5.5, followed by precipitation with 2 vol of 95% ethanol, or (2) dialysis against 0.2 *M* potassium acetate, pH 5.5, for 12 hr, and then against 0.025 *M* Tris·HCl, 0.05 *M* KCl, 0.001 *M* EDTA, pH 7.2, for 6 hr followed by precipitation by the addition of 0.1 vol of acetate buffer (2 *M* potassium acetate, 0.3 *M* acetic acid) and 2 vol of ethanol. Concentrated RNA solutions were prepared by dissolving the ethanol precipitate in RNA buffer (0.05 *M* Tris·HCl, 0.1 *M* KCl, 0.002 *M* EDTA, 5 μg/ml SDS, pH 7.2).

Isolation of nucleic acid from chorioallantoic membranes: After the A.F. had been removed from eggs as described above, the eggs were cut into halves around the smaller circumference, and the embryos were removed. The chorioallantoic membranes were separated from each shell half and were washed three times with 0.05 *M* sodium phosphate, pH 7.0, and twice with 0.05 *M* Tris·HCl, pH 7.2. About 2 ml of settled membranes were obtained per egg. Sodium dodecyl sulfate and Macaloid were added to final concentrations of 2% and 0.4%, respectively. The solution was frozen in liquid N_2 and thawed in a 40°C water bath three times and was diluted with 2 vol of water. The mixture was extracted with phenol and ether and was further treated as described above for viral RNA.

Velocity sucrose gradient centrifugation: An SW25 rotor of a Spinco model L centrifuge was used to separate RNA components on the basis of sedimentation coefficient. Eleven to 36% exponential sucrose gradients were formed with a constant volume mixer.[23] Three-tenths to 1 ml of RNA solution were applied, and the zones were separated in a period of 27–32 hr at 25,000 rpm and about 4°C. The contents of the tubes were analyzed directly for absorbancy at 260 mμ with a spectrophotometer, flow cell, and recorder, and the radioactivity of aliquots was measured with a gas-flow counter.

Nuclease digestion: Susceptibility of preparations to ribonuclease (RNase) was tested by incubating 50–200-μl aliquots with 2 ml of 2.2 μg/ml pancreatic RNase in 0.05 *M* Tris·HCl, 0.2 *M* NaCl, 0.002 *M* EDTA, pH 7.2, at 50°C. Differential susceptibility to deoxyribonuclease (DNase) was tested by incubating duplicate aliquots, one with 2 ml of 1.1 μg/ml RNase and the other with 2 ml of 1.1 μg/ml RNase plus 5.5 μg/ml pancreatic DNase. The buffer was 0.1 *M* Tris·HCl, 0.01 *M* $MgCl_2$, pH 7.2,[24] and the temperature 40°C.

RNA base ratios: The precipitates formed after the addition of carrier RNA and TCA to sucrose gradient fractions were washed with ethanol and ether. The dried RNA was hydrolyzed in 50–100 μl of 0.04 *M* KOH for 18 hr at 37°C. The hydrolysate was adjusted to pH 3–4 with $HClO_4$, and $KClO_4$ was removed by centrifugation. The nucleotides were separated by ionophoresis on Whatman 3MM paper in 0.05 *M* ammonium formate, pH 3.5.[25] The four UV-quenching spots and ten other areas of the paper along the direction of ionophoresis were cut out and counted in vials with toluene-base scintillation fluid.

Results and Discussion.—Virus purification: Because of the observation of Knight,[26] purification of the virus was carried out in phosphate-containing buffers. Sedimentation through a layer of 10 per cent sucrose and onto the surface of a 65 per cent layer separated HA activity from most of the other components of A.F. but did not cause as much loss of HA units as does sedimentation onto tube surfaces. The sucrose and a large amount of P^{32}-labeled, low-molecular-weight materials

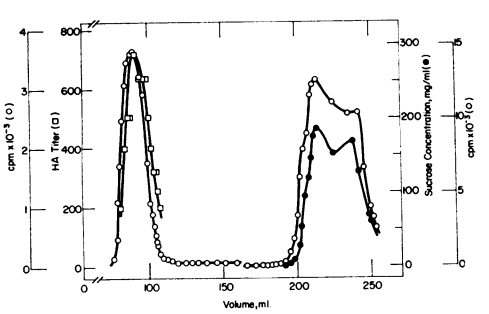

FIG. 1.—Elution of influenza virus from an agarose column. The 2.5-cm diameter column had a bed volume of 220 ml and was prepared and operated as described under *Materials and Methods*. Twenty ml of virus solution, concentrated by centrifugation onto a dense sucrose layer, were applied, and 20-μl aliquots from column effluent fractions were analyzed for HA (□—□), P^{32} radioactivity (○—○), and sucrose (●—●). Note that the scales for the two peaks of radioactivity are different.

were removed by chromatography on a column of agarose beads (Fig. 1). Beads of 4 per cent agarose have sufficient strength to allow a good flow rate to be maintained even in large columns and yet are permeable enough to provide partial separation of influenza virus from ribosomes,[27] the largest particle likely to be a major source of nonviral nucleic acid. When the column was operated in phosphate buffer alone, a large proportion of the virus was irreversibly lost by adsorption. However, when Tween 80, $CaCl_2$, and $MgCl_2$ were added to phosphate buffer, the loss of virus was greatly reduced in agreement with the results of Bengtsson and Philipson.[28] The use of glass or cellulose paper on the top of the column, to prevent disturbance of the bed, was avoided since this also caused loss of virus through adsorption. Fifty to 80 per cent of the HA units present in A.F. were recovered after column chromatography. When unlabeled, purified virus was mixed with P^{32}-labeled A.F. from uninfected eggs, no detectable radioactivity was found associated with the peak of HA activity eluted from the agarose column.

Properties of P^{32}-labeled virus: Since the peak of HA activity migrated as rapidly as or slightly slower than the peak of radioactivity (Fig. 1), ribosomes were probably not important contaminants of virus preparations. The concentration of inorganic phosphate in A.F., as determined by the method of Chen *et al.*,[29] varied from 3 to 4 mM, and the amount of $CaCl_2$ injected was calculated to reduce this to about 1 mM. The observed specific activity of purified virus was increased 2.5–3-fold, to between 70 and 200 cpm/HA unit, by the injection of $CaCl_2$. This did not reduce the yield of virus in HA units nor qualitatively change the appearance of influenza virus RNA sedimentation patterns.

As a measure of homogeneity, column-purified virus preparations were subjected to centrifugation on a gradient of concentrated sucrose. Since the distribution of radioactivity was similar at 46 hr (Fig. 2) and at 23 hr of centrifugation, the P^{32}-labeled material close to the meniscus probably has a lower density rather than a necessarily smaller sedimentation rate. It could represent either defective viral components or virus degradation products. The average density of the peak of HA activity was 1.20 gm/ml, a value slightly higher than the 1.19 gm/ml found by Lauffer and Taylor.[30]

For column-purified virus the ratio of EID_{50} to HA titer was found to be about 10^6, a value which is within a factor of 2 of those found by others.[13, 31]

General properties of viral and membrane nucleic acids: Dialysis against acetate buffer or Sephadex G-25 chromatography (as described in *Materials and Methods*) removed about 60 per cent of the total radioactivity present in solutions of nucleic acid isolated from column-purified virus plus TMV. If these

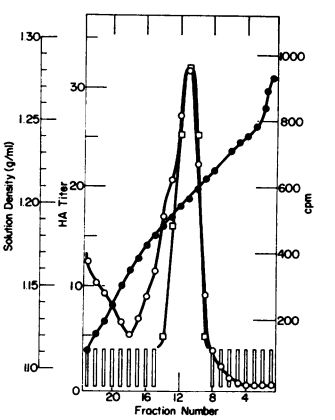

FIG. 2.—Distribution of P^{32} (○—○) and HA (□—□) after isopycnic density gradient centrifugation of column-purified influenza virus. A virus sample was layered on a 4.8-ml, linear, 30–75% (w/v) concentration gradient of sucrose in 0.1 *M* potassium phosphate buffer, pH 7.0. After centrifugation at 28,000 rpm and 4°C for 46 hr in an SW39 rotor, fractions were collected. Aliquots were appropriately diluted for HA and isotope measurements, and density (●—●) was estimated refractometrically by comparison with sucrose-phosphate buffer solutions of known density. The height of the vertical bars represents the range of HA values possible for those gradient fractions for which HA was negative at the lowest dilution tested.

steps were omitted, up to 75 per cent of the ultraviolet-absorbing material precipitated by ethanol failed to dissolve again in either RNA buffer or water. All of the radioactivity in dialyzed or Sephadex-treated nucleic acid preparations from influenza virus or from chorioallantoic membranes was precipitable by carrier RNA plus 7 per cent TCA. Incubation of viral nucleic acid with 2 μg/ml pancreatic RNase at 50°C reduced the TCA-insoluble counts to 3 per cent of the original number after 5 min and to 1 per cent of the original number after 2 hr. The corresponding figures for P^{32}-labeled nucleic acid from the membranes of either infected or uninfected eggs are 15 per cent after 5 min and 6 per cent after 2 hr. Incubations of these preparations over period of 2 hr with 5 μg/ml pancreatic DNase + 1 μg/ml RNase at 40°C did not cause an increase in the rate or extent of loss of TCA-insoluble counts compared with the rate and extent observed in parallel incubations with 1 μg/ml RNase alone.

RNA sedimentation: Figures 3 and 4 show the results of sucrose gradient cen-

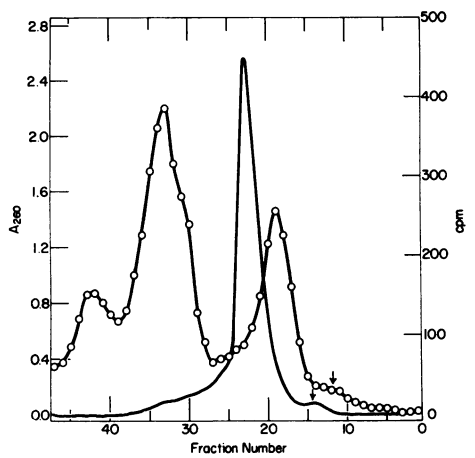

FIG. 3.—Distribution of counts (O—O) and absorbancy at 260 mμ (——) after a 30-hr velocity sedimentation of a mixture of P^{32}-labeled influenza virus RNA and unlabeled TMV-RNA on an exponential sucrose gradient. Absorbancy was recorded automatically as described in *Materials and Methods*, and 0.2-ml aliquots were taken for isotope measurements. Even though RNA preparations were completely TCA-precipitable, if aliquots of 0.1 ml or over were to be counted, they were processed by TCA precipitation and Millipore filtration. This was done to avoid loss of counts through absorption of beta-particles by sucrose from the gradients. Sedimentation in this and the two succeeding figures is from left to right. *Arrows* indicate peaks due to aggregated RNA.

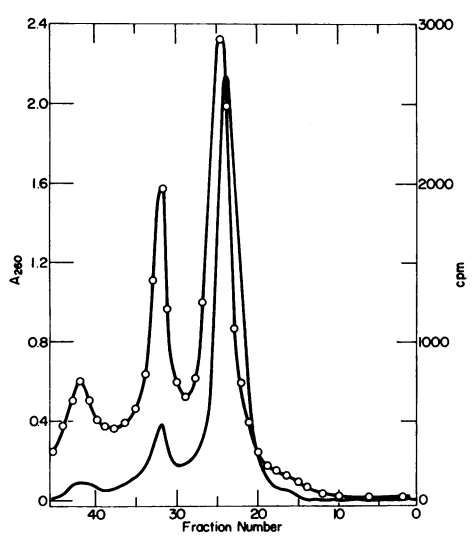

FIG. 4.—Distribution of counts (O—O) and absorbancy at 260 mμ (——) after a 28-hr velocity sedimentation of a mixture of P^{32}-labeled chorioallantoic membrane RNA and unlabeled TMV-RNA on an exponential sucrose gradient. Twenty-μl aliquots of collected fractions were plated directly on planchets for isotope measurements.

trifugation of P^{32}-labeled influenza virus RNA and of P^{32}-labeled chorioallantoic membrane RNA, respectively. In Figure 3 TMV-RNA apparently contributes all of the detectable absorbancy at 260 mμ. The sedimentation coefficients of the P^{32}-labeled RNA components were estimated by comparison of their positions to the position of the TMV-RNA peak ($S_{20,w}$ = 32.5 in RNA buffer), assuming distance from the meniscus to be proportional to sedimentation coefficient. On this basis the three principal P^{32}-labeled components of influenza virus RNA were assigned sedimentation coefficients of 7*S*, 19*S*, and 38*S*. The three principal components of membrane nucleic acid were assigned sedimentation coefficients of 5*S*, 20*S*, and 31*S*.

To investigate the nature of the partially nuclease-resistant material in membrane RNA, nucleic acid from infected membranes was incubated with DNase + RNase (under conditions described in *Materials and Methods*) for 8 min and was centrifuged on an exponential sucrose gradient in the manner used for the experiment shown in Figure 4. A broad peak of both absorbancy and radioactivity was observed in the 2–10*S* region, and no detectable radioactivity sedimented more rapidly than 12*S*. The results of this experiment and the nuclease digestion data reported above support the idea that the P^{32} label in both membrane and viral nucleic acid preparations has been wholly, within the limits of detection, incorporated into single-stranded RNA. The lower susceptibility of membrane RNA to RNase digestion could be due to its higher purine content.[32] From Table 1 it may be seen that all three influenza virus RNA components are characterized by a high uridylic acid content, but all membrane RNA components are characterized by a high guanylic acid content.

In Figure 3 small peaks of UV-absorbing and radioactive materials, marked by arrows, may be seen sedimenting more rapidly than the principal components. To test whether these small peaks, as well as the other components, represent covalently integral RNA or simply aggregates held together by secondary forces, a TMV-RNA-influenza RNA mixture was diluted to 40 μg/ml in 0.025 *M* Tris·HCl, 0.05 *M* KCl, 0.001 *M* EDTA, pH 7.2, and was heated to 80°C for 80 sec. The solution was cooled to 4°C, and after 30 min, the RNA was precipitated by the addition of potassium acetate and 2 vol of ethanol. This heat treatment is sufficient to dissociate

TABLE 1

BASE COMPOSITIONS OF INFLUENZA VIRUS AND CHORIOALLANTOIC MEMBRANE RNA'S

Influenza virus RNA	Nucleotides per 100 Nucleotides			
	C	A	G	U
7*S* Av. 4 ± SE	24.1 ± 1.2	20.4 ± 1.1	28.9 ± 1.4	26.7 ± 1.6
19*S* Av. 5 ± SE	23.5 ± 0.8	23.1 ± 1.7	20.5 ± 0.9	33.0 ± 1.7
38*S* Av. 6 ± SE	22.6 ± 0.7	22.0 ± 0.5	19.8 ± 1.2	35.7 ± 0.9
RNA from uninfected chorioallantoic membrane				
5*S* Av. 2 ± range	26.8 ± 1.0	20.5 ± 0.6	30.0 ± 0.9	22.7 ± 0.7
20*S* Av. 2 ± range	25.2 ± 0.9	21.4 ± 0.3	30.8 ± 0.8	22.7 ± 0.3
31*S* Av. 2 ± range	27.7 ± 0.5	16.8 ± 0.6	36.0 ± 1.9	19.6 ± 0.9
RNA from infected chorioallantoic membrane				
5*S* Single determination	25.9	19.7	31.1	23.0
20*S* Single determination	27.3	19.9	30.8	22.0
31*S* Single determination	28.3	17.3	34.8	19.5

Base compositions were determined using P^{32}-labeled RNA as described in *Materials and Methods*. In every case more than 91% of the radioactivity detectable along the entire length of the ionophoresis strip was associated with the four UV radiation-quenching spots from the hydrolyzed carrier RNA. For the RNA from influenza virus and from uninfected membranes, each value represents the average of the designated number of determinations from four and from two different preparations of RNA, respectively.

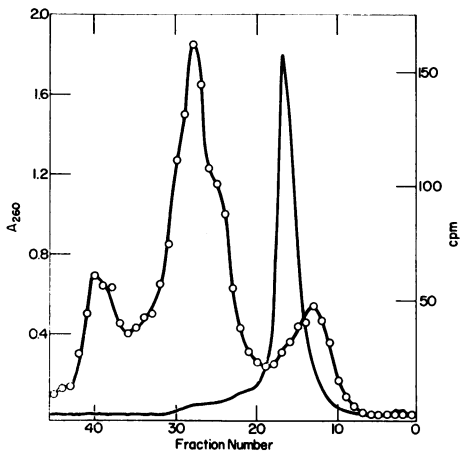

FIG. 5.—Distribution of counts (O—O) and absorbancy at 260 mμ (——) after a 31-hr velocity sedimentation of heated influenza virus RNA. A portion of the sample that was analyzed in Fig. 3 was heated to 80°C for 80 sec in pH 7.2 buffer containing EDTA. The RNA concentration was 40 μg/ml. The RNA was concentrated by ethanol precipitation and was applied to the gradient in the usual manner. One-tenth-ml aliquots of collected fractions were counted after TCA precipitation and Millipore filtration. The greater distance of sedimentation, as compared with Fig. 3, was due to a higher temperature of centrifugation.

the secondary force aggregates of fragments of *E. coli* ribosomal RNA molecules which were produced by introducing scissions into the ribose-phosphodiester backbone of intact molecules.[20] Figure 5 shows the sedimentation pattern of a heated influenza RNA-TMV-RNA mixture. The small, rapidly sedimenting peaks present before heating (*arrows*, Fig. 3) have disappeared, indicating that they were probably nonspecific aggregates. The amount of radioactivity in the 38*S* peak was reduced, while the size of the 19*S* and 7*S* peaks increased. If the final dialysis in the RNA preparation scheme (*Materials and Methods*) was extended by 12 hr, a pattern similar to that shown in Figure 5 was obtained, and heat treatment reduced the size of the 38*S* peak only slightly. The shape of the TMV-RNA sedimentation pattern was not altered by the heat treatment (Figs. 3 and 5) or by extended dialysis. Thus, by these tests, only part of the 38*S* influenza virus RNA is covalently intact, while 33*S* TMV-RNA is without detectable breaks in the ribose-phosphodiester chain.

Two phenomena associated with influenza virus are high rates of genetic recombination and high rates of cross-reactivation.[33] This has led some investigators to postulate a divided genome.[33, 34] A more or less random packaging of two or more RNA pieces could produce high recombination rates if two virus strains were replicating in the same cells. Since the molecular weight of single-stranded RNA varies approximately as the square of the sedimentation coefficient,[35, 36] and the molecular weight of TMV-RNA is about 2.1 million,[36] the molecular weight of 38*S* RNA may be estimated to be 2.8×10^6 and that of the 19*S* RNA to be 0.7×10^6. It is therefore possible that infectious particles of influenza virus could contain a single piece of 38*S* RNA. The production of complete virus particles after infection with 38*S* RNA would support this hypothesis, but we have not yet been able to demonstrate infectivity with our influenza virus RNA preparations. Since influenza virus infection produces particles varying in size and RNA content,[13, 37, 38] it is not unexpected that several sizes of RNA are also produced.

Summary.—Influenza virus labeled with P^{32} has been purified from allantoic fluid by centrifugation onto a dense sucrose solution layer followed by chromatography on an agarose column. The single-stranded RNA extracted from purified virus sedimented, on sucrose gradients, as three components at 7*S*, 19*S*, and 38*S*. A part of the 38*S* component retains its original sedimentation rate after being heated in dilute solution to 80°C for 80 sec, and these covalently intact molecules have an es-

timated molecular weight of 2.8×10^6. One such RNA molecule per particle could account for the amount of RNA found in purified virus preparations.

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