*ISOLATION OF THE NUCLEIC ACID OF NEWCASTLE DISEASE VIRUS (NDV)**

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Newcastle disease virus (NDV) and other myxoviruses contain about one per cent RNA.[1-3] In the case of NDV an RNA content corresponding to 9000 nucleotides per average particle has been estimated by chemical analysis of purified virus.[4] Because of the difficulty in isolating high-molecular-weight RNA from myxoviruses it has been suggested that the RNA may exist in the infectious virus particle as multiple pieces in certain cases.[5, 6]

Recently, in this laboratory, the intact RNA was isolated from the mixture of Rous sarcoma virus (RSV) and its helper virus RAV which belong to the avian leukosis complex of viruses.[7] This result suggested that similar methods be used with a member of the myxovirus group.

This paper describes the purification of NDV and the isolation of a $57S_{20}$ RNA, one molecule of which probably accounts for the total RNA per virus particle. While this study was in progress a brief report appeared on the isolation of a 45–50*S* RNA from NDV.[8]

Materials and Methods.—The plaque-forming L-Kansas 48 strain of NDV was used in all experiments. Ten-day-old eggs were inoculated in the allantoic cavity with 1–3 $\times$ 10^2 PFU of virus. After incubation at 40°C for 40–44 hr, the eggs were chilled and the chorioallantoic fluid was harvested. To remove cell debris the fluid was centrifuged at 4000 rpm for 10 min in a Servall GSA rotor. The supernatant was then kept overnight at 4°C.

Virus purification: The virus was purified in three steps, all carried out in the cold (0–4°C). The following procedure was used to purify 800–1800 ml of allantoic fluid.

First step: Centrifugation to a density interface: Twenty-eight ml of allantoic fluid was layered on 5 ml of a sucrose-RbCl solution and centrifuged in the Spinco 30 rotor for 1 hr at 30,000 rpm. The sucrose-RbCl solution contained 60% sucrose, 15% RbCl, 0.01 *M* Tris hydroxymethylaminomethane·HCl (Tris·HCl), pH 7.3, and 0.001 *M* versene (EDTA); its density was 1.34 gm per ml. After centrifugation a visible band of virus was seen at about density 1.22 gm per ml in the steep density gradient between the sucrose-RbCl solution and the allantoic fluid. A second band of nonviral material appeared 3–5 mm above the virus zone at about density 1.04 gm per ml. The tube was then punctured at the bottom, the virus band collected, and the upper band of foreign material discarded. The virus solution was diluted with an equal volume of buffer, 0.01 *M* Tris·HCl, pH 7.3, 0.001 *M* EDTA and 1/4 vol of medium containing 4% calf serum.[9]

Second step: Ammonium sulfate precipitation: An equal volume of saturated ammonium sulfate, previously neutralized with Tris HCl buffer, was added dropwise to the virus solution. The precipitate was collected by centrifugation, and the supernatant containing about 1% of the starting infectivity was discarded.

Third step: Sucrose-D_2O gradient: The precipitate was redissolved in buffer containing 0.01 *M* Tris·HCl, pH 7.3, 0.1 *M* NaCl, and 0.001 *M* EDTA to make a final volume between 4 and 9 ml depending on the amount of virus present to ensure that the solution density was less than 1.07 gm per ml.

Up to 4.5 ml of the virus solution was then layered on top of an 18-ml linear gradient of sucrose in heavy water and then overlaid with mineral oil to fill the tube and centrifuged in a Spinco SW25 rotor at 25,000 rpm for 6 hr. The gradient was made, using 65% sucrose in D_2O (density 1.32 gm per ml) and an equal volume of that solution diluted 4-fold with H_2O. Both solutions and the final gradient contained 0.01 *M* Tris·HCl, pH 7.3, 0.05 *M* NaCl, and 0.001 *M* EDTA.

The virus band could be seen at about the middle of the tube. A second band of nonviral ma-

terial and varying in amount appeared between the virus band and the bottom of the tube at a density of approximately 1.276 gm per ml. For this reason the virus was collected by puncturing the tube in the wall between the two bands.

Growth of P^{32}-labeled virus: P^{32}-labeled virus was produced by inoculating 9-day-old eggs with P^{32} phosphate (1 mc per egg) and after 24 hr the eggs were infected with NDV. The allantoic fluid was collected and virus was purified as described above.

Assay of virus infectivity: The virus was assayed by a modification[10] of the original plaque technique[11] using complete medium containing 5% calf serum.[9]

Isolation of the RNA: The appropriate fractions containing virus collected from the final sucrose gradient step in virus purification were pooled and diluted 1:4 with buffer containing 0.01 *M* Tris·HCl, pH 8.5, 0.1 *M* NaCl, 0.001 *M* EDTA to lower the solution density. Mercaptoethanol and then sodium dodecyl sulfate (SDS) were added to make a 1% solution of each. The solution was then extracted 3 × with phenol previously equilibrated with buffer (0.01 *M* Tris·HCl, pH 8.5, 0.1 *M* NaCl, 0.001 *M* EDTA, and 0.05 *M* mercaptoethanol). The phenol remaining emulsified in the aqueous phase was removed by centrifugation at 15,000 rpm for 5 min. The nucleic acid was twice precipitated by the addition of $^1/_{10}$ vol of 16% sodium acetate pH 5.5 and 2 vol of ethanol in the cold.

P^{32}-RNA was isolated in the same way except that 300 mg TMV-RNA was added as carrier prior to the addition of SDS.

Results and Discussion.—The recovery of NDV infectivity during the purification described in *Materials and Methods* was in the range of 75–100 per cent after $(NH_4)_2SO_4$ precipitation and was 60–100 per cent over all three steps.

Zone centrifugation in a preformed sucrose-D_2O gradient, as employed in the final step of purification, achieves virus purification by separating components on the basis of sedimentation velocity and buoyant density. In the rather short time of centrifugation, small components, such as many serum and allantoic fluid proteins, remain on top of the gradient. Larger components such as NDV move rapidly down the gradient until they reach their buoyant density where they remain. Because of the density of NDV a sucrose solution in H_2O cannot be made with sufficient density for equilibrium centrifugation of the virus. Therefore, a sucrose solution in D_2O was employed for the density gradient step of purification. Salts such as CsCl were not used alone because they have been shown to inactivate infectious NDV[12] and other myxoviruses.[13]

Properties of purified virus: Results of the centrifugation of purified P^{32}-labeled NDV to density equilibrium in a gradient of sucrose D_2O is shown in Figure 1. The virus solution was layered over a preformed gradient of sucrose in D_2O (15–60%), and centrifuged for 20 hr at 36,000 rpm and 4°C in the Spinco SW39 rotor. Viral infectivity and radioactivity were found to coincide. The buoyant density of the virus in sucrose D_2O was 1.235 gm per ml (Fig. 1). This value agrees with that previously reported for NDV.[12]

Velocity sedimentation of NDV is shown in Figure 2. The virus solution containing purified P^{32}-labeled NDV was layered over a sucrose gradient (5–20% w/v) and centrifuged at 17,500 rpm for 40 min in the Spinco rotor SW39. The sharp portion of the peak of virus infectivity is seen to coincide with the sharp portion of the radioactivity peak. The small shoulder of radioactivity and infectivity on the leading edge of the virus peak may represent faster-sedimenting virus aggregates. The unusually broad sedimentation profile suggests a heterogeneous population of virus particles with respect to sedimentation velocity. Heterogeneity in the structure of the large lipid-containing RNA viruses has been implied previously by studies demonstrating heterogeneity in buoyant density of NDV[12] and RSV.[14] This

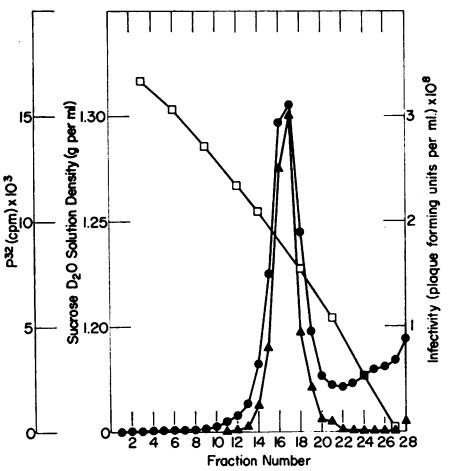

FIG. 1.—Distribution of infectivity (—▲—▲—) and P^{32} (—●—●—) after equilibrium density gradient (—□—□—) centrifugation of purified P^{32}-labeled NDV in sucrose-D_2O. The virus was layered over a 5.0-ml gradient of sucrose 15–60% (w/v) in D_2O containing 0.01 *M* Tris·HCl pH 7.3, 0.05 *M* NaCl, and 0.001 *M* EDTA and was centrifuged for 20 hr at 36,000 rpm in a Spinco model L SW39 rotor at 5°C. Fifty-μl aliquots of each fraction were diluted with 1 vol of water, placed directly in Bray's scintillation fluid,[24] and counted in a Tricarb liquid scintillation counter. Viral infectivity was determined for each fraction as described in *Methods*.

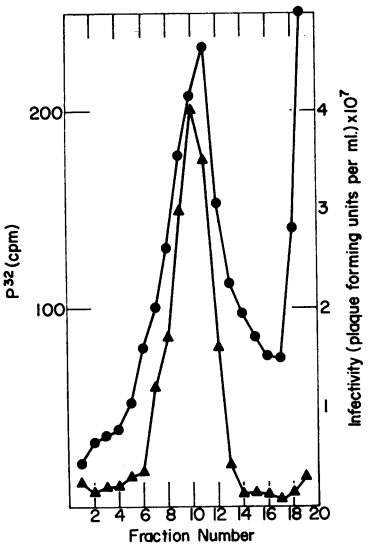

FIG. 2.—Distribution of infectivity (-▲-▲-) and P^{32} (-●-●-) after velocity sedimentation of purified P^{32}-labeled NDV. The virus was layered over a 5-ml gradient of sucrose (5–20% w/v) containing 0.01 *M* Tris·HCl pH 7.3, 0.1 *M* NaCl, and 0.001 *M* EDTA and centrifuged for 40 min at 17,500 rpm in a Spinco model L SW39 rotor at 5°C. Undiluted aliquots of each fraction were counted as described in Fig. 1. Viral infectivity was determined for each fraction as described in *Methods*.

phenomenon has been attributed to the imprecise manner of maturation of these viruses at the cell surface where individual virus particles may acquire different amounts of cell membrane in formation of the outer viral envelope.

The low-molecular-weight material on top of the gradient after velocity sedimentation of purified P^{32}-labeled virus contains about 40 per cent of the starting radioactivity. This observation suggests the possibility of breakdown of purified virus during storage resulting in low-molecular-weight P^{32}-labeled components. Another explanation would be the presence of contaminating low-molecular-weight P^{32}-labeled material such as inorganic phosphate associated with the virus and carried along during the initial purification. Such contamination would not be surprising in view of the fact that the P^{32}-labeled virus represents only 0.1 per cent or less of the total radioactivity in the chorioallantoic fluid before purification of the virus.

Properties of the viral nucleic acid: (a) *Preparation:* The nucleic acid from purified P^{32}-labeled NDV was isolated as described in *Materials and Methods* and fractionated by sucrose gradient centrifugation. The result shown in Figure 3 was obtained by layering 250 μl nucleic acid solution over a gradient of sucrose (5–20%) containing 0.1 *M* NaCl, 0.01 *M* Tris·HCl, pH 7.3, and 0.001 *M* EDTA and centrifuging for 2.5 hr at 5° at 36,000 rpm in a Spinco SW39 rotor. The carrier TMV-

RNA is represented by the A_{260} tracing. The amount of P^{32} NDV-RNA is too small to be detected optically but is represented by the radioactivity tracing. Two distinct P^{32}-labeled components are present and both are rendered acid-soluble by pancreatic ribonuclease indicating that they consist of RNA. The fast-sedimenting component of P^{32}-labeled RNA is thought to be intact viral RNA.

The amount of the slow component relative to the fast one and the base composition of the slow component vary from preparation to preparation. In addition, the slow component of RNA that was twice precipitated with alcohol before isolation by sucrose gradient centrifugation consists of 30 per cent TCA-soluble material. These observations indicate considerable heterogeneity of the slow component and it is not unlikely that it consists of degraded viral RNA. The possibility that RNA from other sources is also present has not been excluded.

The fact that the two viral RNA components are distinctly separated by centrifugation suggests that breakdown of intact viral RNA into small fragments occurs in the virus preparation prior to the isolation of the RNA, rather than during the isolation procedure itself. If the RNA was broken down during the isolation with phenol, the result would be random degradation of all RNA in the preparation and the appearance of a broad sedimentation band rather than two distinct peaks.

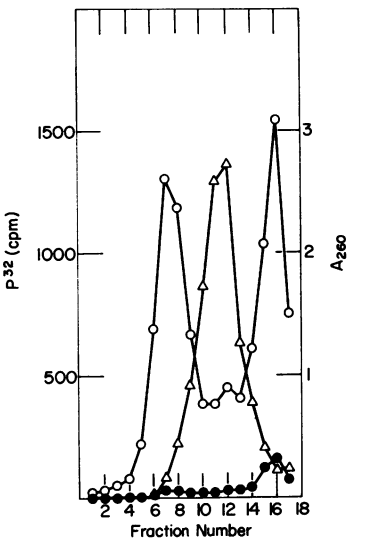

FIG. 3.—Distribution of P^{32} (—○—○—) and absorbancy at 260 mμ (—△—△—) after velocity sedimentation in a sucrose gradient of P^{32}-labeled NDV-RNA with a TMV-RNA marker. A 0.2-ml solution of P^{32} NDV-RNA and TMV-RNA was layered on top of a 5-ml linear sucrose gradient (5–20% w/v) containing 0.01 *M* Tris·HCl, pH 7.3, 0.1 *M* NaCl, and 0.001 *M* EDTA and centrifuged for 2.5 hr at 36,000 rpm in a Spinco model L SW39 rotor at 5°C. One aliquot of each fraction was counted after precipitation with TCA.[7] An aliquot of equal volume was incubated with pancreatic ribonuclease 1 μg/ml in solution with 0.2 *M* NaCl at 37°C for 1 hr prior to the determination of TCA-precipitable radioactivity (—●—●).

(b) *Base composition:* The base composition of the fast component of RNA shown in Table 1 is not compatible with complementary base pairing. The base composition of the slow RNA component was not reproducible in several experiments and was never identical with that of the intact viral RNA (Table 1). Since known ribonucleases hydrolyze RNA in such a way that the TCA or alcohol-insoluble fraction has a different average base composition from the starting material,[15–17] it is tenable that the slow-sedimenting RNA fraction is degraded viral RNA. Degradation of NDV-RNA could in part explain the considerable difference between the base composition of the present study and that previously reported.[18] The major reason for the difference in base compositions is probably related to the difference in methods used. In the previous study[18] the base composition of total P^{32}-labeled RNA of NDV-infected cells incubated with P^{32} in the presence of actinomycin D was determined. The values obtained for adenylic acid and cytidylic acid were higher in that study[18] than in the present one (intact viral RNA in Table 1) suggesting that P^{32} labeling of the terminal end of the cellular sRNA, which con-

TABLE 1

	Intact NDV-RNA (av. 7 ± SE)	Slowly sedimenting RNA component (a representative single expt.)
C	23.0 ± 0.4	26.3
A	23.8 ± 0.4	20.0
G	23.8 ± 0.5	33.4
U	29.4 ± 0.6	20.3

Base compositions were determined using P^{32}-labeled RNA. The viral RNA was fractionated by sucrose gradient centrifugation (Fig. 3), the appropriate fractions from the RNA peaks were pooled, carrier TMV-RNA was added, and the total RNA precipitated with ethanol. After alkaline hydrolysis the RNA hydrolysate was neutralized with $HClO_4$[23] and analyzed by high-voltage (40 v/cm) paper electrophoresis.[22] The paper strip was dried and cut into 0.5-cm sections. Each section was placed directly in scintillation fluid and counted in a Tricarb liquid scintillation counter. All radioactivity on the paper was confined to the four nucleotide spots visible with the UV lamp.

tinues in the presence of actinomycin D,[19, 20] contributed significantly to the P^{32}-labeled RNA fraction used in that study for base analysis.

(c) *Sedimentation velocity:* The sedimentation properties of intact NDV-RNA were studied in the Spinco model E analytical ultracentrifuge using UV optics.

In two representative experiments NDV-RNA was obtained by purification of 1350 ml (experiment A) and 1600 ml (experiment B) of allantoic fluid (see *Materials and Methods*) containing about 6×10^{11} PFU and 7×10^{11} PFU of NDV, respectively. The total RNA isolated as described in *Materials and Methods* was 114 μg in experiment A and 142 μg in experiment B. Each preparation of RNA was fractionated by sucrose gradient centrifugation as described in Figure 3. The fast-sedimenting component recovered from the sucrose gradient consisted of 20 μg (17% of the total RNA) in experiment A, and 30 μg (21% of the total) in experiment B. The slowly sedimenting component consisted of 95 μg (82%) and 112 μg (79%) in experiments A and B, respectively.

The fast component considered to be intact NDV-RNA was precipitated with alcohol and redissolved in 0.6 ml buffer containing 0.01 *M* Tris·HCl pH 7.4, 0.10 *M* NaCl, and 0.001 *M* EDTA for analysis in the analytical ultracentrifuge. Densitometer tracings from a sedimentation of intact NDV-RNA from experiment A are shown in Figure 4. About 80–90 per cent of the material sediments with a fairly sharp boundary. Of the remaining material very little trails and most appears to consist of an aggregate leading the sharp portion of the boundary. The sedimentation constant ($S_{20,w}$) calculated using the sharp portion of the boundary was 56.8 for the RNA from experiment A and 56.9 for the RNA from experiment B. TMV-RNA run under the conditions described in Figure 4 had the expected value of 32 $S_{20,w}$.

In 0.001 *M* EDTA the sedimentation constant of NDV-RNA was found to be 14 $S_{20,w}$ and in 0.2 *M* NaCl, 0.01 *M* Tris·HCl pH 7.4, and 0.001 *M* EDTA the sedimentation constant was 64 $S_{20,w}$. Such dependence of sedimentation velocity on salt concentration has been demonstrated for single-stranded RNA.[7, 22, 25]

If it is assumed that the viral RNA is single-stranded, as suggested by the base composition (Table 1), susceptibility to ribonuclease (Fig. 3), and dependence of sedimentation constant on salt concentration, an estimate of its molecular weight can be made with the empirical relation:

$$M = 1550\ (S_{20,w})^{2.1}$$

determined by Spirin[21] for TMV-RNA on 0.1 *M* NaCl, 0.01 *M* EDTA. Thus, a single-stranded RNA with $S_{20,w}$ of 57 in 0.1 *M* NaCl would have a molecular weight of 7.5×10^6. The value of this calculation is limited by the uncertainty of

whether Spirin's equation is applicable to all single-stranded RNA molecules. For example, in the case of the RNA from MS2 phage, the relationship of sedimentation constant and molecular weight has been shown to differ from that of TMV-RNA.[22] One RNA molecule of molecular weight 7.5×10^6 per virus particle would constitute more than two times the RNA content previously estimated by chemical analysis of purified virus.[4] This observation is consistent with the idea that purified preparations of virus contain many particles with degraded RNA. Such particles would significantly lower the average RNA content per particle.

Figure 5 shows a comparison of NDV-RNA with the RNA from RSV recently isolated in this laboratory,[7] the only known single-stranded RNA of comparable size. This comparison was of particular interest since the RSV-RNA, because of its scarcity, has not been analyzed in the analytical ultracentrifuge and it has been studied so far only with radioactive labels. The experiment was done by sedimenting simultaneously H^3-labeled RSV-RNA and P^{32}-labeled NDV-RNA with TMV-RNA on a sucrose gradient. The position of the H^3-labeled RSV-RNA clearly indicates that this RNA has a higher sedimentation constant than NDV-RNA in agreement with the previous estimate of 64*S* for RSV-RNA.[7]

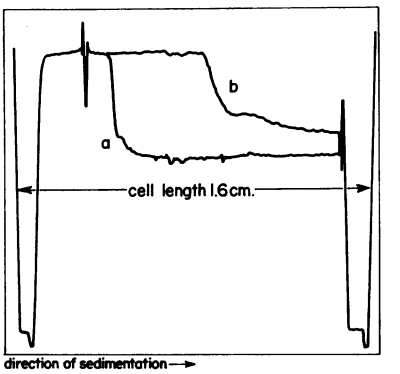

FIG. 4.—Ultraviolet patterns of NDV-RNA approximately 20 μg/ml in 0.01 *M* Tris·HCl pH 7.3, 0.10 *M* NaCl, and 0.001 *M* EDTA sedimenting at 44,770 rpm in the Spinco model E ultracentrifuge at 1.3°C. Photographs were taken every 4 min for calculation of the sedimentation constant. (*a*) A tracing of the first picture taken at full speed, and (*b*) the UV pattern 16 min later.

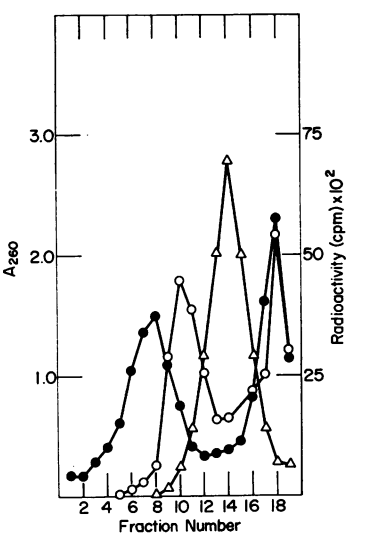

FIG. 5.—Distribution of H^3 (—●—●—), P^{32} (—○—○—), and absorbancy at 260 mμ (—△—△—) after velocity sedimentation in a sucrose gradient of H^3-labeled RSV-RNA, P^{32}-labeled NDV-RNA and TMV-RNA. The RNA's were centrifuged for 2.25 hr as described for Fig. 3.

Summary.—Newcastle disease virus (NDV) has been purified from chorioallantoic fluid with little loss of infectivity. Intact nucleic acid isolated using SDS and phenol extraction consists of single-stranded RNA. Two distinct RNA components are always recovered. One component is thought to be intact viral RNA with $S_{20,w} = 57$ in 0.10 *M* salt and an estimated molecular weight of 7.5×10^6. One such RNA molecule per virus particle would account for all of the RNA found in purified virus preparations. The other component which sediments at approximately 3*S* and is heterogeneous is thought to consist of degraded viral RNA.

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*ROLE OF THE RESIDUAL NUCLEOPROTEIN COMPLEX AND ACIDIC PROTEINS OF THE CELL NUCLEUS IN PROTEIN SYNTHESIS**

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It has been shown[1] that the residue from calf thymus nuclei obtained after thorough extraction of the nuclei with buffer or saline is capable of active amino acid incorporation *in vitro*. The incorporation of isotope label is DNA-dependent and appears to be different from the known ribosomal synthetic systems. Subsequently, a soluble DNA-RNA-protein complex has been isolated from the nuclear residue which manifests the same synthetic activity.[2] Incorporation of C^{14}-tryptophan into the residual complex and partial tryptic digestion of the labeled protein[2] show that the incorporation is in the nonhistone proteins (residual proteins) and that the isotopic label is in the polypeptide linkage. It has been suggested that