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Physical and Chemical Characterization of an Avian Reovirus

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The avian viral agent S1133 has previously been classified serologically as a member of the avian reovirus group. This viral agent grows in chicken embryo fibroblast cells, bands at a density of 1.37 g/ml in CsCl equilibrium density gradients, has a particle diameter of 75 nm, and has a morphology similar to that of human reovirus type 3. Its nucleic acid is comprised of double-stranded RNA and adenosine-rich oligonucleotides. The dsRNA is distributed among 10 segments with molecular weights of 2.7×10^6, 2.6×10^6, 2.4×10^6, 1.7×10^6, 1.5×10^6, 1.3×10^6, 1.2×10^6, 0.80×10^6, 0.74×10^6, and 0.68×10^6 for the largest (L_1) to the smallest (S_4) segment, respectively, as determined by polyacrylamide gel electrophoresis. These 10 segments migrate differently on polyacrylamide gels compared to those of human reovirus type 3. The capsid proteins of avian reovirus consist of eight species of polypeptides as determined by polyacrylamide gel electrophoresis. These are λ_1, λ_2, λ_3, μ_1, μ_2, σ_1, σ_2, and σ_3 with molecular weights of 140, 125, 115, 85, 72, 40, 36, and 32×10^3, respectively. Only polypeptide σ_2, which resides in the inner capsid or core, comigrated with the σ_2 polypeptide of type 3 reovirus. Antiserum against type 3 reovirus did not neutralize avian reovirus. Avian reovirus core particles were found to possess a transcriptase and a methylase activity.

A number of viral agents isolated from chickens have been classified as avian reoviruses, since they share some morphological and physiochemical features in common with mammalian reoviruses (4, 5, 9, 14). One group of 77 avian agents has been classified into five serotypes by means of neutralization tests (9), and it was later shown that prototypes from the five groups had common antigens (10). Some of the avian strains are antigenically related to the three serotypes of the human reoviruses (6). Several years ago it was shown that some of these avian agents contained both single-stranded and double-stranded (ds) RNA (11, 15), but only recently has there been a sufficiently detailed analysis to show that there is a similarity between the genomic and capsid structures of avian and human reoviruses (13).

We became interested in avian reovirus because of strong suggestive evidence that it might participate in a nonpermissive infection in L cells (12), and we wanted to study its genetics and the transcription of the viral genome under these circumstances. To this end we obtained from L. van der Heide a newly isolated avian agent that had been serologically classified as an avian reovirus (24). The present paper describes the purification and analysis of this virus and shows that the agent is remarkably similar structurally to type 3 human reovirus. Our results are in general agreement with those of Nick et al. (13) who used two different avian agents, where we have studied the same properties. The transcription of the avian viral genome in L cells will be described in an accompanying paper (23).

MATERIALS AND METHODS

Cells and virus. Primary and secondary cultures of chicken embryo fibroblasts (CEF) were prepared from 10-day-old embryos (25) and grown in minimum Eagle medium supplemented with 10% tryptose phosphate broth and 10% fetal calf serum. L cells were grown in a suspension culture in minimal Eagle medium supplemented with 5% fetal calf serum.

L. van der Heide kindly sent us a sample of the infectious agent he had recently isolated from chickens and designated S1133 (25). We will henceforth refer to this agent as avian reovirus. The R_2 strain of reovirus serotype 3 was also used and propagated in L cells (22).

The plaque assay for avian reovirus was performed with cultures of CEF. Secondary monolayer cultures of CEF were infected with 0.1-ml quantities of viral suspension, the virus was permitted to adsorb for 2 h at 20°C, and the cultures were then overlayed with 5 ml of a solution of minimal Eagle medium containing 1% Difco agar and 5% heat-inactivated fetal calf serum. After incubating the

plates for 3 days at 37°C, 3 ml of a similar overlay solution was added, and the plates were incubated for a further 2 to 3 days when the plaques were plainly visible. The cultures were then stained for 2 h with 2 ml of a 0.05% solution of neutral red in phosphate-buffered saline, and the plaques were scored.

Avian reovirus for purification was propagated in large monolayer cultures of CEF and harvested at approximately 20 h postinfection. The virus remained largely cell-associated at this time and was purified by just the same procedure described for R_2 virus and its various temperature-sensitive mutants (21, 22). To calculate the number of virus particles in a purified suspension, one unit of absorbancy at 260 nm (A_{260}) was taken to be equivalent to 2.1×10^{12} particles/ml (20). On the average purified avian reovirus contained 60 particles/PFU. To prepare labeled virus, 0.5 μg of actinomycin D per ml was added to a culture at the time of infection along with 1 μCi of [^{3}H]uridine per ml, 0.02 μCi of [^{14}C]uridine per ml, 5 μCi of ^{3}H-labeled amino acid mixture per ml, or 1 μCi of [^{35}S]methionine per ml in methionine-free medium.

Buffers, chemicals, and isotopes. SSC buffer contains 0.15 M NaCl plus 0.015 M sodium citrate (pH 7.4). Phosphate-buffered saline (pH 7.3) contains 0.13 M NaCl, 2.7×10^{-3} M KCl, 8.2×10^{-4} M Na_2HPO_4, 1.5×10^{-3} M KH_2PO_4, 9.1×10^{-4} M $CaCl_2$, and 5×10^{-4} M $MgCl_2$. STES buffer (0.3 M) contains 0.3 M NaCl, 1 mM EDTA, 50 mM Tris-chloride (pH 7.4), and 0.5% sodium dodecyl sulfate (SDS). Pancreatic RNase was obtained as the crystalline enzyme from Worthington Biochemicals Corp. Reovirus type 3 HAI antiserum (chicken) was obtained from Grand Island Biological Co. [5-^{3}H]uridine (25 Ci/mmol), [2-^{14}C]uridine (50 mCi/mol), [^{32}P]orthophosphate, L-[^{35}S]methionine (100 Ci/mmol), *S*-adenosyl [*methyl*-^{3}H]methionine (8.5 Ci/mmol), and L-^{3}H-labeled amino acid mixture in 0.1 N HCl solution were obtained from New England Nuclear.

Extraction of viral RNA and chromatography on Sephadex G-50. Purified virus in 1× SSC was made 0.5% with SDS and extracted three times with 90% phenol, and the phenol was removed from the aqueous layer by ether extraction. The resulting aqueous viral RNA solution was made to 0.3 M NaCl – 0.05 M sodium acetate (pH 5.3) – 0.5% SDS and layered onto a column of Sephadex G-50 (90 by 0.9 cm) preequilibrated in the same buffer. Fractions recovered from the column were pooled as described later. To concentrate each pool it was extracted three times with an equal volume of *n*-butanol to remove SDS diluted with 5 volumes of 5 mM triethylammonium bicarbonate, pH 8.0, and added to a short column (10 by 1 cm) of DE-11 cellulose (OH^- form). Fractions were eluted from the column with 1 M triethylammonium bicarbonate and evaporated to dryness under vacuum at 40°C.

Nucleotide analysis. Samples to be analyzed were desalted, dissolved in H_2O, adjusted to 0.3 M KOH, and hydrolyzed for 16 to 18 h at 37°C. Samples were neutralized with Dowex 50 (H^+ form) and analyzed for base content according to the procedure of Blattner and Erickson (2). In brief, nucleotides were adsorbed to and then eluted from a column (90 by 0.9 cm) of Dowex 50 (ammonium form) with 0.25 M ammonium formate, pH 4.1, at the rate of 1 ml/min. AMP was eluted before CMP under these conditions.

PAGE of dsRNA and fluorography. Polyacrylamide gel electrophoresis (PAGE) slab analysis of dsRNA has been described in detail (22). Fluorography was performed according to the method of Bonner and Laskey (3). In summary, after electrophoresis the gel was soaked in about 20 times its volume of Me_2SO for 30 min followed by a second 30-min immersion in fresh Me_2SO. The gel subsequently was immersed in 5 volumes of 22% (wt/vol) PPO (2,5-diphenyloxazole) in Me_2SO for 3 h and then washed with 3 liters of water for 1 h. Kodak X-ray film was placed in contact with the dried gel and exposed at −70°C. A total of 30,000 dpm of ^{3}H or 2,000 dpm of ^{14}C applied to the gel as dsRNA gave 10 easily detectable bands of dsRNA in a 24-h exposure.

PAGE analysis of viral polypeptides. Labeled virus was adjusted to 2% concentration with each of SDS and 2-mercaptoethanol and to 0.1 M with phosphate buffer (pH 7.2) and heated in a boiling-water bath for 2 min. PAGE analysis was carried out on 10% gels (22). After electrophoresis gels were fractionated on a Gilson gel crusher (Gilson Medical Electronics Inc.), and 1-mm fractions were solubilized with 0.2 ml of 30% hydrogen peroxide (Fisher Scientific Co.) at 55°C for 6 h. Radioactivity was determined on a liquid scintillation spectrometer.

Assay of transcriptase and methylase activities in avian reovirions. Purified avian virus at 1 mg/ml in 0.05 M Tris-chloride, pH 8.0, was incubated at 37°C for 60 min with 100 μg of chymotrypsin per ml to convert the virions to cores. These cores were used immediately in the enzyme assays without further purification. In a volume of 4 ml the reaction mixture contained cores equivalent to 1 mg of virions, 15 μmol of $MgCl_2$, 4 μmol of Tris-hydrochloride (pH 8.0), 20 μmol of phosphoenol pyruvate, 200 μg of pyruvate kinase, 100 μg of purified bentonite, 2 nmol of *S*-adenosyl-L-[*methyl*-^{3}H]methionine, 1 mmol (50 μCi) of α-[^{32}P]GTP, and 3 mmol each of ATP, CTP, and UTP (7, 23). Incubation was at 45°C. Small portions were removed at intervals, trichloroacetic acid was added to 10% concentration, the resulting precipitates were collected by filtration through membrane filters (Millipore Corp.), and their radioactivities were assayed.

Preparation of rabbit antiserum against avian reovirus. Purified avian reovirus, 0.5 mg, was emulsified with Freund adjuvant and injected subcutaneously into a rabbit (8). Two weeks later a booster of 0.25 mg of virus in adjuvant was given subcutaneously. After a further month the rabbit was bled, the blood was allowed to clot, and the serum was removed and used in neutralization tests.

RESULTS

Purification of avian reovirus. The growth curve of avian reovirus in CEF is shown in Fig. 1. Purification of avian reovirus labeled with [^{3}H]uridine was done using the procedure de-

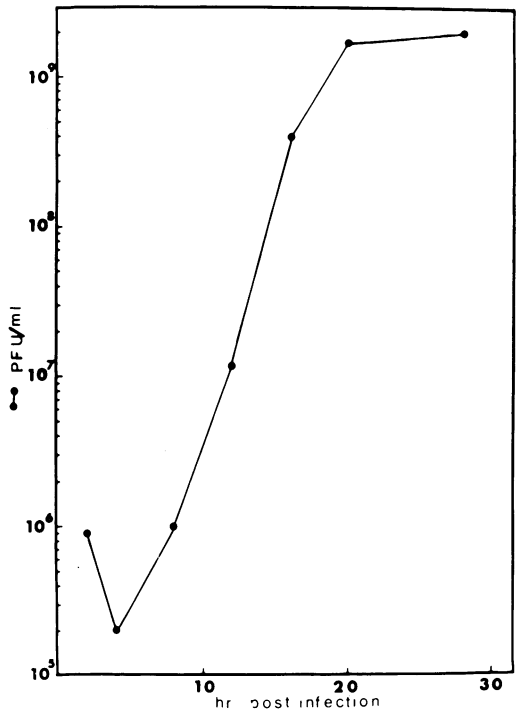

FIG. 1. *Growth curve of avian reovirus. Monolayer cultures of CEF in plastic dishes (60 mm) were infected with 10 PFU/cell. After 2 h at 20°C to permit adsorption of virus, the cells were washed twice with 2 ml of PBS and then 5 ml of MEM containing 2% heat-inactivated fetal calf serum was added. At intervals duplicate cultures were frozen at −70°C, thawed, subjected to sonic oscillation, and plaque titered on CEF monolayers.*

scribed for the human reoviruses (21, 22). The virus was propagated in monolayers of CEF, and when the infectious cycle was complete virus was released from the cells by homogenization with Freon 113, and the aqueous extract was centrifuged over a cushion of CsCl. A broad band of virus was collected from near the center of the cushion, dialyzed against SSC, and the virus was pelleted, resuspended, and then sedimented to equilibrium in a CsCl gradient. Two clearly separated bands were seen at ρ = 1.37 g/ml and at approximately 1.29 g/ml, the denser band containing virtually all the infectivity of the preparation. Each band was collected, separately subjected to sedimentation through sucrose gradients to further purify the material, and the two resulting bands were isolated from the respective gradients by side puncture of the tubes. Part of each preparation was stained with 2% phosphotungstic acid and examined under the electron microscope. The material with ρ = 1.37 g/ml had the structure shown in Fig. 2, and in size and morphology the particles are indistinguishable from R_2 virions examined in the same way. The lower density component was comprised of empty virions corresponding to the top component found in most mammalian reovirus preparations (20).

A sample of the denser component was mixed with purified ^{14}C-labeled R_2 virions and subjected to isopycnic centrifugation in CsCl, obtaining the results shown in Fig. 3. Both the avian and R_2 viruses banded at the same density.

Nucleotide composition of avian viral RNA. RNA was extracted from purified virions labeled with ^{32}P and passed through a column of Sephadex G-50. Approximately 75% of the ^{32}P was found in the void volume of the column and the remainder was in a well-separated trailing peak. Nucleotide analysis of the two fractions are shown in Table 1. The RNA fraction excluded by Sephadex G-50 had an (A + G)/(C + U) ratio of unity and a nucleotide composition similar to that of the dsRNA of type 3 reovirus. The excluded fraction was rich in adenosine (A), corresponding to a similar oligonucleotide population in mammalian reoviruses (1), and was not further studied. These results are similar to those of Koide et al. (11).

Secondary structure of avian viral RNA. In this and the succeeding sections attention is confined to the major fraction of viral RNA excluded by Sephadex G-50. This fraction was first tested for resistance to RNase digestion at different electrolyte concentrations. RNA is completely resistant to RNase action in SSC but is rapidly digested at a concentration of 0.1× SSC or lower (Fig. 4).

In Fig. 5 are shown the results for the thermal denaturation of the viral RNA at different electrolyte concentrations. Denaturation occurs over a narrow temperature range, and at concentrations of 0.01×, 0.1×, and 1× SSC the melting temperature values are 76, 85, and 95°C, respectively. This behavior is similar to that of mammalian reoviral dsRNA (16). Clearly the avian viral RNA is also double stranded.

Size of the avian viral dsRNA. The avian viral dsRNA was analyzed by sedimentation through a sucrose gradient with the results shown in Fig. 6. Three size classes of dsRNA were observed sedimenting at approximately 14*S*, 12*S*, and 11*S* as has been found for mammalian reoviral dsRNA (27).

Avian viral dsRNA was analyzed by PAGE in two ways. First, samples of ^{3}H-labeled dsRNA obtained from purified avian and R_2 reoviruses were analyzed in separate wells of a

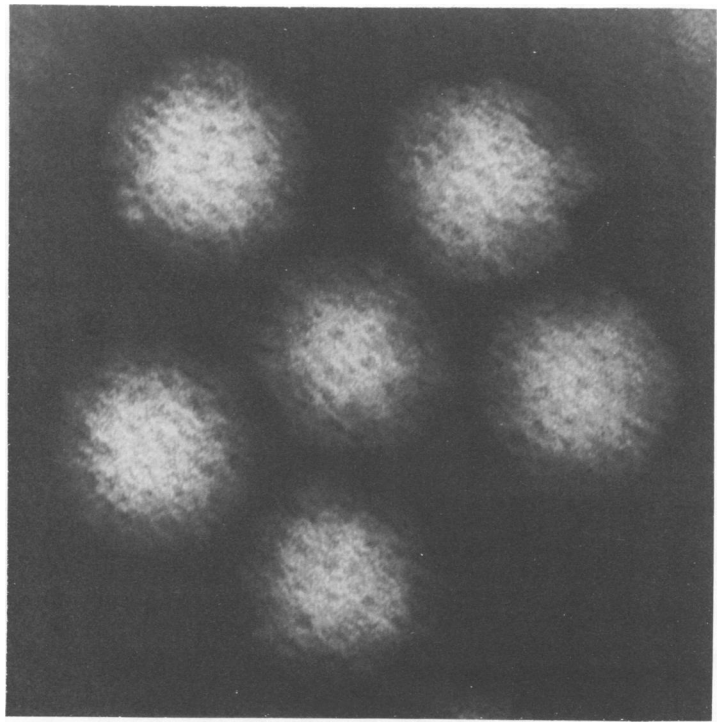

FIG. 2. *Electron micrograph of purified avian reovirus. ×373,000.*

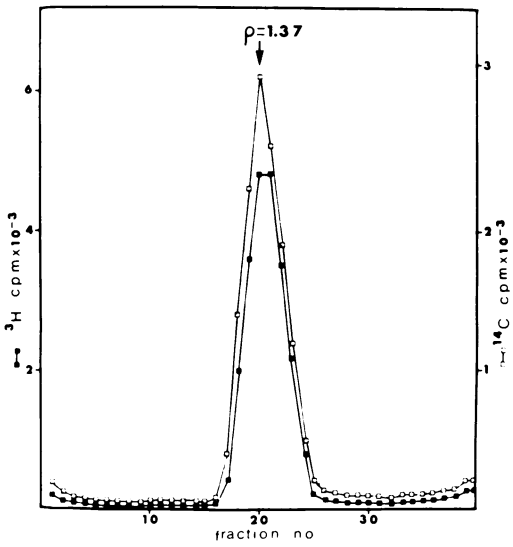

FIG. 3. *Isopycnic centrifugation in CsCl of [^{3}H]uridine-labeled avian reovirus. The avian virus was mixed with [^{14}C]uridine-labeled R_2 virus prior to sedimentation as a density marker. The average density of CsCl was 1.37 g/ml, and the centrifugation was for 16 h at 37,500 rpm at 4°C in an SW40 Beckman rotor. Symbols: (■) ^{3}H-labeled avian virus; (□) ^{14}C-labeled R_2 virus.*

TABLE 1. *Base composition of avian viral RNA fractions obtained by Sephadex G-50 chromatography*

RNA preparation	C	A	G	U	G + C	(A + G)/ (C + U)
Excluded fraction	22.7	28.2	22.1	29.0	42.8	1.01
Retained fraction	2.1	82.4	5.3	10.2	7.4	7.13

polyacrylamide gel slab, and the positions of the bands were identified by fluorography as shown in Fig. 7. Ten segments of avian viral dsRNA were seen, all slightly displaced from the corresponding segments of the R_2 virus.

To be more certain of these segment displacements a sample of [^{3}H]uridine-labeled avian dsRNA was mixed with a sample of [^{14}C]uridine-labeled R_2 dsRNA, and the mixture was analyzed by PAGE. In every case the segments of avian viral dsRNA were more or less displaced from the corresponding segments of R_2 viral dsRNA as the results in Fig. 8 show. The molecular weights of the individual segments can be determined from the distances they migrated in this gel. When the average molecular weights of the three size classes of R_2 viral dsRNA (2.6×10^6, 1.7×10^6, and 0.8×10^6 for L, M, and S, respectively) are plotted

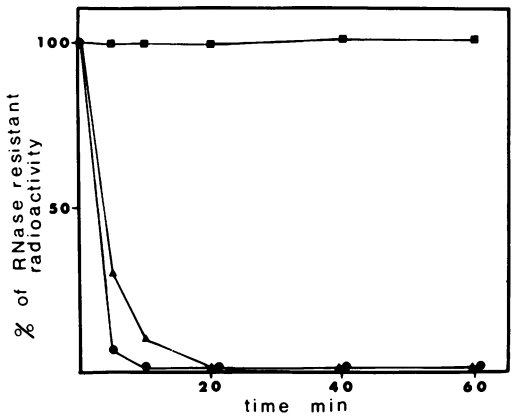

FIG. 4. *The effect of ionic strength on the digestion of avian viral dsRNA with RNase.* ^{32}P*-labeled RNA (5,000 cpm) was contained in 1-ml quantities of SSC, 0.1 × SSC, or 0.01 × SSC to each of which 5 μg of RNase was added. Samples were withdrawn at intervals for the determination of trichloroacetic acid soluble* ^{32}P*. Symbols: (●) 0.01 × SSC; (▲) 0.1 × SSC; (■) SSC.*

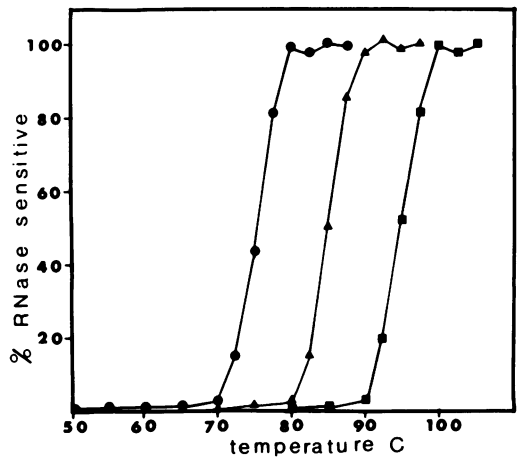

FIG. 5. *The effect of ionic strength on the thermal denaturation of avian reoviral dsRNA. Equal amounts of avian viral dsRNA labeled with* ^{32}P *in SSC, 0.1 × SSC or 0.01 × SSC were heated for 10 min at different temperatures and then quickly chilled in ice. Each sample was then adjusted to a concentration of 1 × SSC in 1 ml, 5 μg of RNase was added, and after 30 min at 37°C the amount of trichloroacetic acid-soluble* ^{32}P *was determined. Symbols: (●) 0.01 × SSC; (▲) 0.1 × SSC; (■) SSC.*

against average distances migrated they fall on a straight line (19). The molecular weights of the ten avian viral dsRNA segments were obtained from this line and were successively 2.7×10^6, 2.6×10^6, 2.4×10^6, 1.7×10^6, 1.5×10^6, 1.3×10^6, 1.2×10^6, 0.8×10^6, 0.74×10^6, and 0.68×10^6 for the largest to the smallest segment.

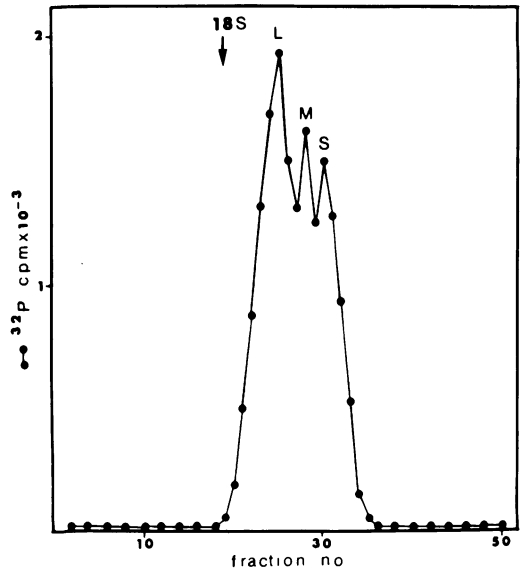

FIG. 6. *Sucrose gradient sedimentation analysis of avian reoviral dsRNA. A 15 to 30% linear sucrose gradient was employed in 0.3 M STES buffer. Sedimentation was for 24 h at 26,000 rpm in an SW27 Beckman rotor at 20°C. Direction of sedimentation is from right to left.*

Polypeptide composition of the avian viral capsid. The polypeptides of the avian reovirus capsid were labeled with ^3H-labeled amino acid mixture during viral growth. After purification of the virus it was mixed with [^{35}S]methionine-labeled R_2 virus to provide migration markers, the mixture was dissociated and analyzed by PAGE as described in Materials and Methods. The results are shown in Fig. 9.

Eight avian viral polypeptides were observed migrating fairly close to the R_2 viral polypeptide markers, i.e., λ_1, λ_2, λ_3, μ_1, μ_2, σ_1, σ_2, and σ_3. Core particles derived from avian reovirus by chymotrypsin digestion and analyzed in a similar way contained only λ_1, λ_2, λ_3, μ_1, and σ_2 polypeptides as did the marker R_2 viral cores (data not shown). The molecular weights of the avian viral polypeptides were determined from their distances of migration on the gel and the data of Smith et al. (20), and were λ_1 = 140,000, λ_2 = 125,000, λ_3 = 115,000, μ_1 = 85,000, μ_2 = 72,000, σ_1 = 40,000, σ_2 = 36,000, and σ_3 = 32,000.

Transcriptase and methylase activities in avian reovirions. Digestion of R_2 reovirions with chymotrypsin converts them to core particles thereby activating two enzymatic activities, a transcriptase that can transcribe the 10

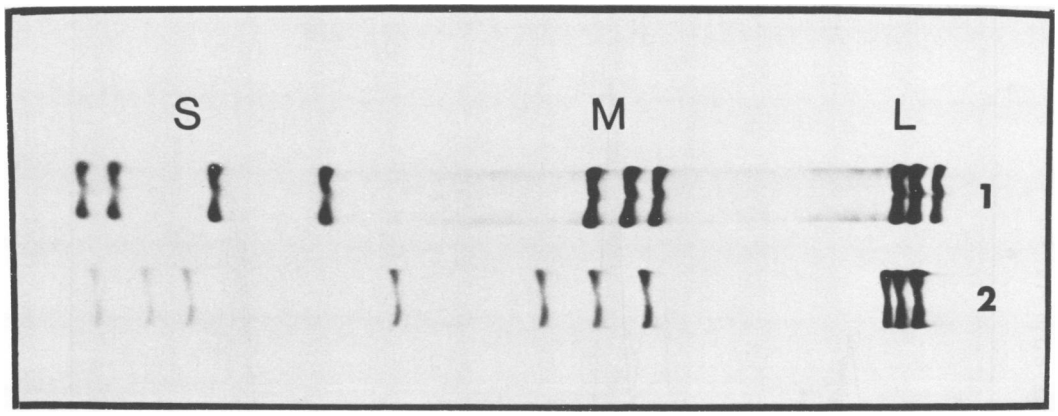

FIG. 7. *Fluorogram of a PAGE analysis of [^{3}H]uridine-labeled avian reoviral dsRNA (2) and R_2 viral dsRNA (1).*

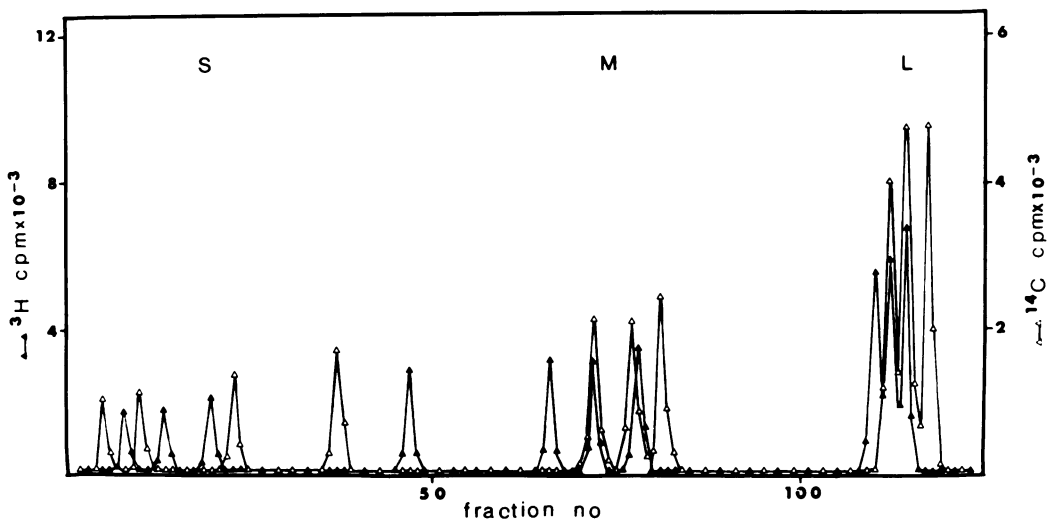

FIG. 8. *PAGE analysis of a mixture of [^{3}H]uridine-labeled avian viral dsRNA and [^{14}C]uridine-labeled R_2 viral dsRNA. L, M, and S represent the large, medium, and small size classes of genomic segments. Symbols: (▲) avian reovirus; (△) R_2 virus.*

segments of the viral genome and a methylase that methylates the transcripts in the presence of *S*-adenosyl-L-methionine (7, 17, 18). To determine whether avian reovirions had similar activities, chymotrypsin cores were prepared and placed in a standard reaction mixture containing α-[^{32}P]GTP and *S*-adenosyl [^{3}H]methionine. The kinetics of incorporation of both isotopes into trichloroacetic acid-insoluble RNA are shown in Fig. 10, and they are similar to those found with mammalian reoviral cores (7).

Heat inactivation of avian reovirus. To get some idea of the stability of the avian virus, samples of purified virus were exposed to three different temperatures along with samples of purified R_2 virus to act as comparisons. At intervals samples were removed, and the surviving virus was assayed. The results are shown in Fig. 11, and the kinetics of inactivation of the avian virus are seen to be similar to those of R_2 virus at all three temperatures.

Test for neutralization of avian reovirus by type 3 reovirus antibody. Several strains of avian reovirus have been shown to have some common antigens with type 3 reovirus. To determine if this was the case with the S1133 strain of avian virus studied here, neutralization tests were carried out with both the avian and R_2 reovirus strains and the antiserum to human type 3 reovirus. Type 3 virus was inactivated with linear kinetics at two dilutions of antiserum, but there was no neutralization of

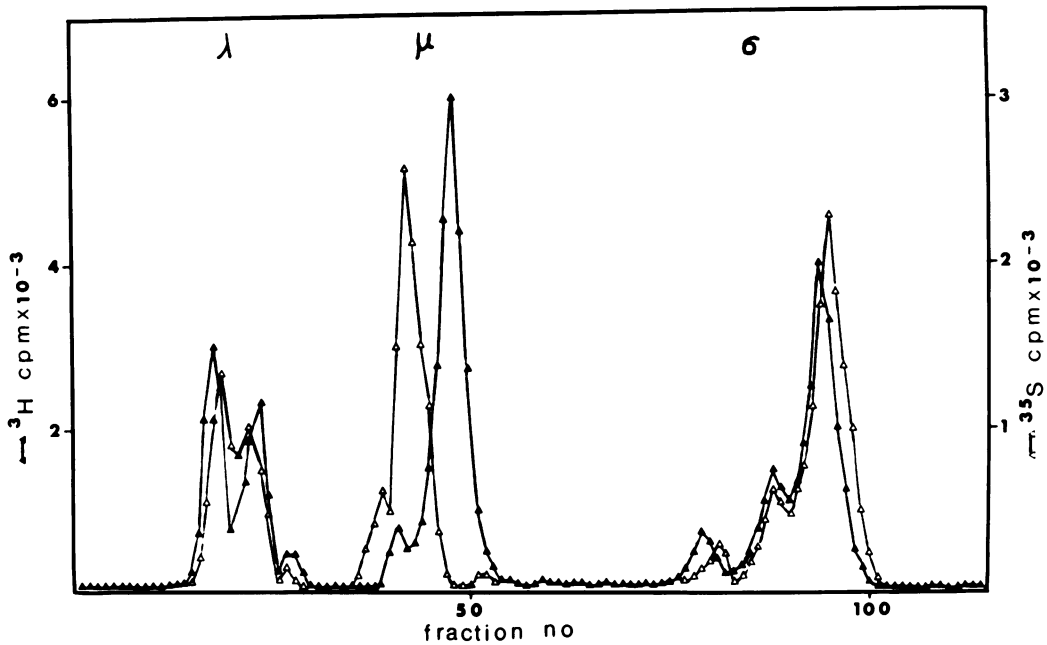

FIG. 9. *PAGE analysis of the capsid proteins of avian reovirus. Symbols: (▲) ^{3}H-labeled avian viral polypeptides; (△) ^{35}S-labeled polypeptides of R_2 virus added as migration markers.*

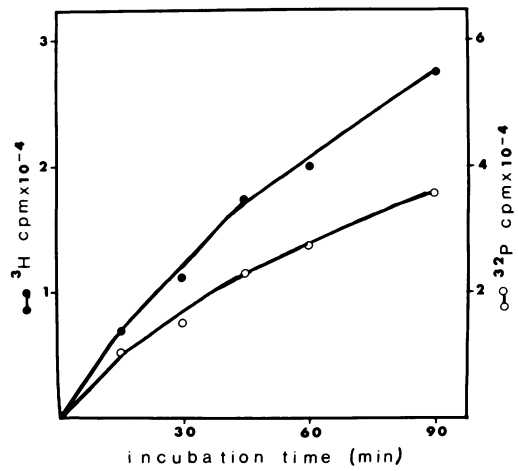

FIG. 10. *Kinetics of incorporation of α-[^{32}P]GTP and S-adenosyl [^{3}H]methionine into the transcripts synthesized in vitro with avian reoviral cores. The curves measure the incorporation of (●) ^{3}H and (○) ^{32}P into trichloroacetic acid-insoluble material.*

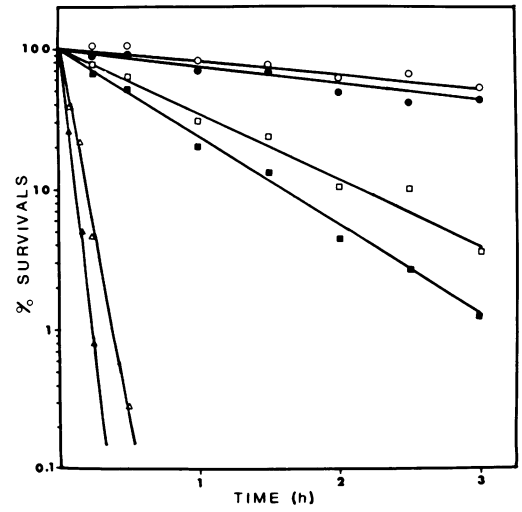

FIG. 11. *Thermal inactivation of avian and type 3 reoviruses. Samples of purified virus (10^7 PFU/sample) in MEM containing 2% fetal calf serum were heated for different intervals at the required temperatures and then plaque titered. Symbols: (○, □, △) avian reovirus at 37, 45, and 55°C, respectively; (●, ■, ▲) R_2 virus at 37, 45, and 55°C, respectively.*

the avian reovirus (Fig. 12) by type 3 antiserum. The results in Fig. 12 were obtained with commercially prepared chicken antiserum. Similar results were obtained with rabbit antiserum prepared as described in Materials and Methods. These two viruses do not have any common neutralizing antigens, and this observation is consistent with the almost complete lack of sequence homology between the two dsRNA genomes (23).

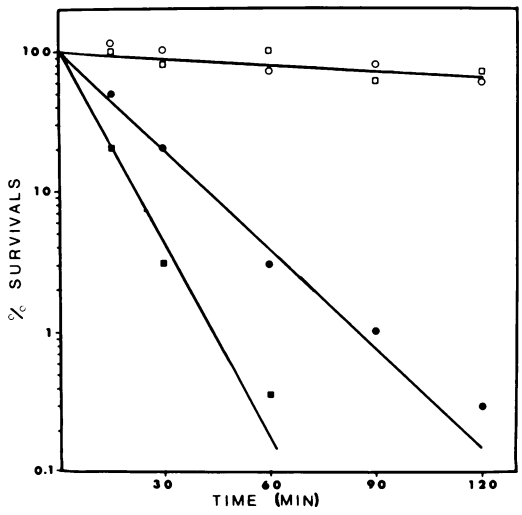

FIG. 12. *Kinetics of neutralization of avian and R_2 reoviruses with type 3 viral antiserum. Symbols: (●) Inactivation of R_2 virus with 10^{-3} dilution of commercially obtained antiserum (chicken); (■) inactivation of R_2 virus with 2×10^{-3} dilution of antiserum; (○, □) inactivation of avian reovirus with 10^{-3} and 2×10^{-3} dilutions, respectively, of type 3 antiserum.*

DISCUSSION

The results of the analyses presented here indicate that the S1133 agent is an avian reovirus strikingly similar to human reoviruses in its general properties. Its genome is comprised of 10 segments of dsRNA, it has the same capsid structure and polypeptide components as type 3 reovirus and contains a transcriptase and a methyl transferase as an integral part of the viral core. On the other hand, all 10 dsRNA segments of the avian virus can be distinguished from those of type 3 virus by PAGE; several of the capsid components of the two viruses can be distinguished from each other by the same means, and there is no cross-reaction between the two viruses in serum neutralization tests. These superficially minor differences between the human and avian strains of reovirus, however, can supply extremely useful markers in mixed infection experiments with the two viruses as will be shown in later papers.

ACKNOWLEDGMENTS

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