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The RNA of Human Coronavirus OC-43

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A homogeneous RNA complex with a sedimentation coefficient of 70 S and an apparent molecular weight of approximately 6.1×10^6 was released from purified ^{32}P-labeled, mouse-brain-derived OC-43 virus after treatment with 1% sodium dodecyl sulfate (SDS) for 15 min at 23°. The complex was highly susceptible to heat, releasing 4 S RNA fragments at 37° and breaking down to fragments of 4–70 S at 60°; it was also degraded by centrifugation through dimethyl sulfoxide gradients. Unlike tobacco mosaic virus or Rous sarcoma virus RNA, OC-43 RNA prepared by extraction with phenol–SDS or phenol–chloroform degraded into a range of fragments with coefficients of 15–55 S; 4 S RNA was also present as a minor component. This suggests that (a) extensive nicking of a large RNA molecule has occurred during viral growth, due to ribonucleases which are inactivated during phenol extractions; (b) heterogeneity for OC-43 RNA is not due to internal ribonuclease activity and fragments are held together by noncovalent linkages much weaker than those present in the 70 S retroviral RNA complex, or by small proteins; or, most probably, (c) a combination of extensive nicking and weak noncovalent linkages results in the heterogeneous denaturation products.

INTRODUCTION

The Coronaviridae were described as a separate taxonomic group by Almeida *et al.* (1968) but, despite their ubiquity and economic importance, few biophysical characterization studies have been reported. The RNA of avian infectious bronchitis virus (IBV) was first described by Tannock (1973), who obtained from purified virions a highly heterogeneous array of RNA fragments using a phenol–sodium dodecyl sulfate (SDS) extraction procedure. Fragments ranged in molecular weight from 0.5×10^6 to more than 3.0×10^6; a smaller, more homogeneous class of a size similar to ribosomal 4 S RNA was also present.

More recently, Watkins *et al.* (1975) described IBV RNA, after its release from virions by SDS lysis, as a single species having a molecular weight of 9.0×10^6 and a sedimentation coefficient of 50 S. They reported, but without supporting evidence, that by phenol extraction a range of RNA fragments could be obtained which was similar to that Tannock (1973) described.

Garwes *et al.* (1975) examined the RNAs of two porcine coronaviruses, transmissible gastroenteritis virus (TGEV) and hemagglutinating encephalomyelitis virus (HEV), after preparation by SDS lysis. They obtained a single large RNA peak with electrophoretic mobility similar to that of 70 S Rous sarcoma virus (RSV) RNA. Like RSV RNA (Duesberg, 1968), the RNAs of TGEV and HEV could be readily dissociated to 35 S and 4 S subunits, if prepared by SDS lysis at temperatures above 60°.

In the present work, we attempt to resolve apparent differences in coronaviral RNA profiles obtained by different methods of preparation, using human coronavirus OC-43 as the model for study. OC-43

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was first isolated in human tracheal organ culture, but it has been adapted to grow to high titer in suckling mouse brain (McIntosh *et al.*, 1967). Unlike 229E, a human coronavirus strain which can be propagated only in cell culture but is extremely labile (Hierholzer, 1976), OC-43 is a relatively stable virus and possesses a hemagglutinin (Kaye *et al.*, 1970; Hierholzer *et al.*, 1972). It is, therefore, a more convenient model for study.

MATERIALS AND METHODS

Solutions and reagents. The following buffers were used: NET (0.1 *M* NaCl, 0.01 *M* EDTA, 0.01 *M* Tris, pH 7.4), Loening's electrophoresis buffer [0.03 *M* $NaH_2PO_4 \cdot H_2O$, 0.001 *M* EDTA, 0.036 *M* Tris pH 7.8 (Loening, 1969) with 0.2% SDS], phosphate-buffered saline (PBS; Hierholzer *et al.*, 1969), and PC extraction buffer (0.5% SDS, 0.14 *M* LiCl, 0.01 *M* sodium acetate, pH 4.9). Other reagents included deoxyribonuclease Type 1 (Nutritional Biochemicals Corp.), pancreatic ribonuclease (5 × crystalline, Calbiochem), diethyl pyrocarbonate (Sigma), and mercaptoethanol (Eastman). Ribonuclease-free sucrose (Sigma), and dimethyl sulfoxide (DMSO; Baker) were used in gradient experiments.

Acrylamide and bisacrylamide (Eastman) for polyacrylamide gel electrophoresis (PAGE) were recrystallized and prepared in electrophoresis buffer at 2.2 and 0.15%, respectively. Polymerization was accomplished with 0.4% *N,N,N'N'*-tetramethylethylenediamine and 0.1% ammonium persulfate. Phenol (Fisher, reagent grade) for RNA extraction was twice distilled. Yeast carrier RNA was prepared according to the method of Tannock (1973).

Isotopes. Carrier-free [^{32}P]orthophosphate and [5,6-^{3}H]uridine (40–50 Ci/mmol) were obtained from New England Nuclear Corp.

Viral and RNA markers. [^{3}H]Uridine tobacco mosaic virus (TMV) and [^{3}H]uridine ribosomal RNA were prepared by the method of Tannock *et al.* (1970). Some ribosomal RNA preparations used for PAGE contained significant amounts of ribosomal precursor RNA, which served as an additional marker. Preparations of purified [^{3}H]uridine vesicular stomatitis virus (VSV) and nondefective, [^{3}H]uridine Rous sarcoma virus (RSV) were generously provided by Drs. J. F. Obijeski, Center for Disease Control (CDC), Atlanta, Georgia, and P. H. Duesberg, University of California, Berkeley, California, respectively. RNA was extracted from each by the PC method (see below).

Viral hemagglutinin (HA) assay. The microtiter method of Hierholzer *et al.* (1969), with 0.5% chicken erythrocytes, was used.

Virus growth. Four-day-old suckling mice from the CDC pathogen-free colony were inoculated intracerebrally with 300–320 μCi of [^{32}P]orthophosphate, followed at 8 hr with $10^{4.22}$ LD_{50} of OC-43 virus (14th mouse brain passage) by the same route. Brains were harvested 40 hr later, suspended in PBS to approximately 20%, and stored at −70°.

Virus purification. Virus was released from cells in the suspension by freezing and thawing four times, and particulate matter was removed by centrifuging at 5,000 *g* in a Spinco 40 rotor for 20 min. The supernatant was retained in an ice bath while the pellet was resuspended in PBS to 20% of the original volume and recentrifuged; both supernatants were then combined for purification. Virus was partially purified by adsorption to and elution from human "O" erythrocytes according to the method of Kaye *et al.* (1970). Virus-adsorbed erythrocytes were washed three times with ice-cold PBS, and virus was finally eluted in two steps at 37° into approximately 20% of the original volume of PBS. In some early purifications, the eluate was then treated with ribonuclease and deoxyribonuclease (each at 50 μg/ml final concentration) in order to degrade any cellular nucleic acids adherent to the virus. When later work revealed that the profile of RNA extracted from virus treated in this manner was similar to that from untreated virus, we omitted the step.

Virus in the eluate was then concentrated by centrifuging through a 15% sucrose interface to a 0.75-ml cushion of 65% sucrose in NET at 43,200 *g* for 30 min in a

Spinco SW 36 rotor. The concentrates were pooled, diluted with NET, and centrifuged to equilibrium for 15 hr at 97,100 *g* through a 10-ml 25–65% sucrose gradient in NET at 7° in an SW 36 rotor. Thirty-drop fractions were collected, and aliquots of each were assayed for total radioactivity, hemagglutinin, density (from refractive index), and absorbance at 280 nm. A typical profile is seen in Fig. 1A. Two visible bands are present: (1) a lower opalescent band in the density range 1.168–1.202 g/ml containing all hemagglutinin activity and (2) an upper, more flocculent band of membranous material, having a density of 1.074–1.133 g/ml and no hemagglutinin.

Fractions 6–10 containing the lower band were then pooled, diluted with NET, and centrifuged in a velocity gradient in the SW 36 rotor for 1 hr at 43,200 *g* in a 9.0-ml 15–65% sucrose gradient in NET. Fractions were collected and assayed for hemagglutinin and radioactivity. The profile in Fig. 1B clearly shows that the single hemagglutinin-rich band obtained by equilibrium centrifugation may be further resolved into (1) a fast-sedimenting peak with radioactivity but little hemagglutinin, and (2) an upper heterogeneous peak of coincident hemagglutinin and radioactivity. The lower peak, which is not usually visible, probably consists of internal cell membranes and has been observed by Pocock and Garwes (1975) in the purification of TGEV. Heterogeneity in the upper peak is consistent with the high degree of pleomorphism observed for coronaviruses (Almeida *et al.*, 1968). Fractions from the upper peak, which were shown by electron microscopy to contain highly purified virus, were pooled, diluted to 5% sucrose with NET, and held at 4° for RNA analysis. Final virus preparations contained approximately 11 cpm/HA unit and purification resulted in a 14-fold enrichment of hemagglutinin per unit of protein.

RNA extraction. The following methods were used. (1) In the warm phenol–SDS (WPS) method (Tannock, 1973), the viral suspension was shaken for 10 min at 37°

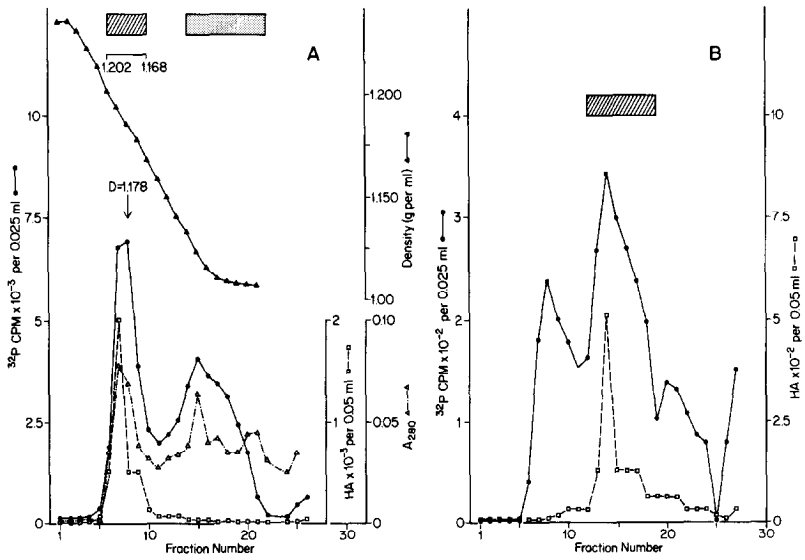

FIG. 1. (A) Equilibrium gradient centrifugation of partially purified ^{32}P-labeled OC-43. A preparation of OC-43 was partially purified by adsorption to and elution from human "O" cells, concentrated and centrifuged through a 10-ml 25–65% sucrose gradient in NET for 15 hr at 97,100 *g* in a SW 36 rotor at 7°. The distributions of radioactivity (●——●), hemagglutinin (□- - -□), absorbance at 280 nm (△—·—△), and density (▲——▲) are shown. (B) Velocity gradient centrifugation of equilibrium-purified virus. Fractions 6–10 in Fig. 1A were pooled, diluted with NET, and centrifuged for 1 hr at 43,200 *g* through a 9-ml 15–65% sucrose gradient in NET in an SW 36 rotor at 7°. The distributions of radioactivity (●——●) and hemagglutinin (□- - -□) are shown. The upper peak (fractions 12–18) was pooled for use in RNA studies.

with phenol containing 1% SDS. The two phases were separated by centrifugation, and the extraction was repeated. After a suitable marker RNA, 100 μg of carrier RNA, and sodium acetate to 0.15 *M* were added, 2 vol of ethanol were added and all RNAs were allowed to precipitate for 16 hr at −15°. The precipitate was collected by centrifuging at 43,200 *g* for 30 min in an SW 36 rotor and then dissolved in a suitable volume of NET. All RNAs were stored −70° for analysis. (2) In the SDS-lysis (SL) method (Garwes *et al.*, 1975), SDS was added to 1% (w/v) to a purified virion suspension, and the mixture was held at 23° for 15 min. The RNAs released were mixed with suitable marker RNA and immediately analyzed in preformed sucrose gradients or in polyacrylamide gels. (3) In the phenol–chloroform (PC) method for influenza modified from Pons (1975) by using the ribonuclease inhibitor diethyl pyrocarbonate (DEP) in place of bentonite, the virus sample was mixed with 5% DEP, 0.1 vol 2-mercaptoethanol, 1 vol PC extraction buffer, and 1 vol of phenol:chloroform (1:1) and shaken for 3 min at 23°. The phases were separated by centrifugation, and the extraction of the aqueous phase was repeated. Precipitation in the presence of marker and carrier RNAs and centrifugation were as described for the WPS method.

RNA sucrose gradient analysis. RNA preparations prepared by phenol extraction were centrifuged through 4.4 ml of 15–30% sucrose gradients in NET in Spinco SW-50.1 rotor in a Model L-2 for 2.25 hr at 189,000 *g* and 7°. Those prepared by SDS lysis were centrifuged in a Beckman Model L-4 ultracentrifuge for 2 hr at 189,000 *g* and 7° (Tannock and Griffith, 1975).

RESULTS

Sucrose Gradient Analysis of OC-43 RNA Prepared by Extraction Methods Used for Other Coronaviruses

RNA was prepared from two samples of ^{32}P-labeled OC-43 virus by the WPS and SL methods. Profiles of OC-43 and marker RNAs from each gradient after centrifugation are shown in Fig. 2. With the WPS method (Fig. 2A), most RNA was distributed broadly in the 15–50 S region of the gradient, and a smaller quantity of 4 S RNA was also present. With the SL

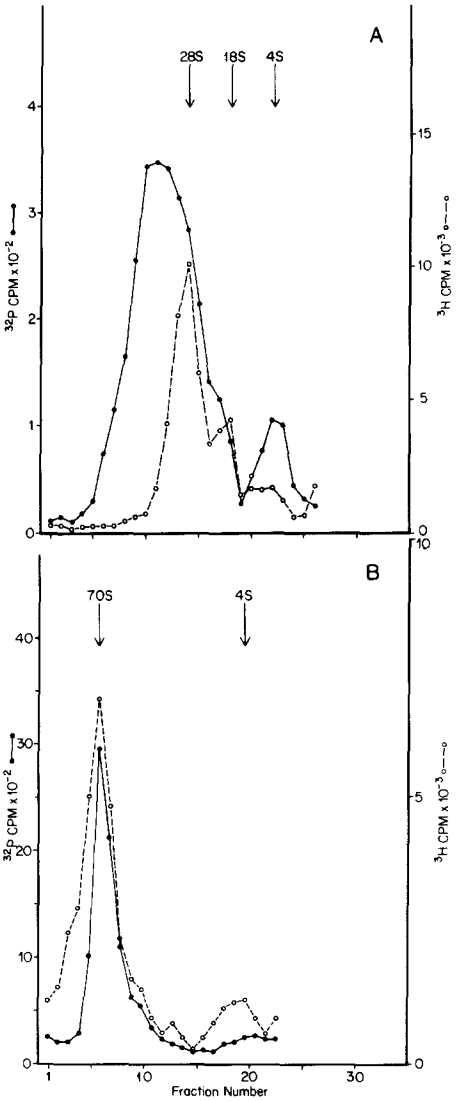

FIG. 2. Sucrose gradient analysis of OC-43 RNA prepared by extraction methods used for other coronaviruses. RNA was extracted from ^{32}P-labeled OC-43 virus by the WPS method (A), carrier and [^{3}H]uridine ribosomal RNA were added, and all RNAs were precipitated with ethanol. Each precipitate was dissolved in 0.5 ml of NET and analyzed by ultracentrifugation in sucrose gradients as described under Materials and Methods. RNA from a further sample (B) was released by the SL method, mixed with 0.1 ml of [^{3}H]uridine RSV RNA, and analyzed by centrifuging through sucrose gradients, as above. The profiles of acid-insoluble radioactivity for OC-43 (●——●) and RSV (○- - -○) are shown.

method (Fig. 2B), a single large homogeneous RNA was obtained with a sedimentation coefficient identical to the major (70 S) component of RSV RNA. The PC method was used for all subsequent extractions involving phenol because of the greater RNA yields obtained (Tannock and Hierholzer, unpublished observation) and the observation (Molloy *et al.*, 1974) that phenol-chloroform mixtures conserved polyadenosine sequences in RNA.

PAGE of OC-43 RNA

The profiles obtained for OC-43 RNA by sucrose-gradient centrifugation were compared with those obtained by PAGE. Figure 3A shows a typical profile in which OC-43 RNA, prepared by the SL method, migrates as a homogeneous complex with an electrophoretic mobility similar to that of both 45 S ribosomal precursor RNA and (not shown in this figure) 70 S RSV RNA. Semilogarithmic plots of molecular weight versus electrophoretic mobility from replicate experiments gave a mean apparent molecular weight of 6.1×10^6 for this undegraded complex (Fig. 4). Acrylamide gels electrophoresed at 23° revealed considerable breakdown of the complex; this thermolability of OC-43 RNA was confirmed in later experiments (see Fig. 5). No discrete viral 4 S RNA can be seen in Fig. 3A and the increasing of ^{32}P radioactivity towards the end of the gel corresponds to the non-

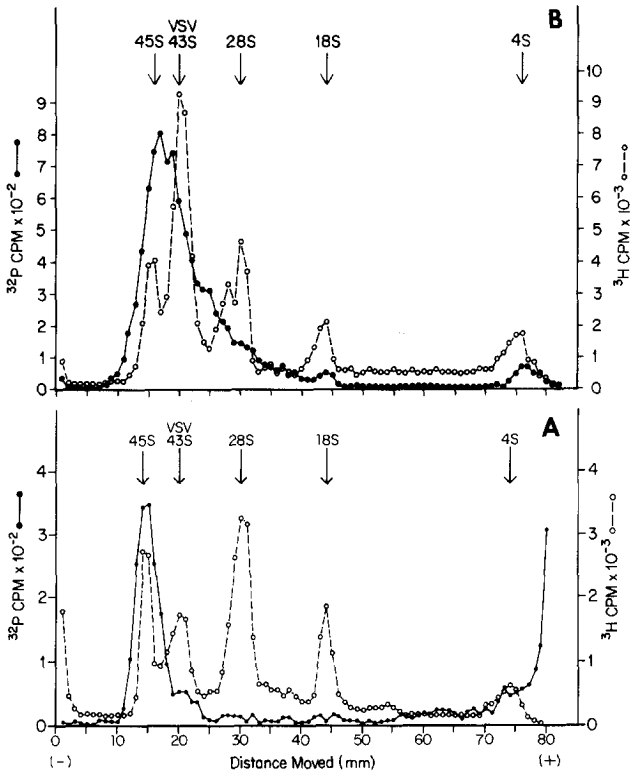

FIG. 3. PAGE of OC-43 RNA. (A) A mixture was prepared in 100 μl of NET consisting of ^{32}P-labeled OC-43 RNA released by the SL method and [^{3}H]uridine ribosomal and [^{3}H]uridine VSV RNA. Sucrose and bromophenol blue were added at final concentrations of 10% and 0.01%, respectively; a 60-μl aliquot was electrophoresed at 5° in a 9-cm 2.2% polyacrylamide gel for 2 hr at 70 V and 9 mA per tube. The distributions of radioactivity for OC-43 (●——●) and ribosomal and VSV (○- - -○) RNAs are shown. (B) A preparation of ^{32}P-labeled OC-43 RNA was extracted by the PC method as described under Materials and Methods. [^{3}H]Uridine ribosomal and [^{3}H]uridine VSV RNA and 50 μg of carrier RNA was added to each, and all RNAs were precipitated with ethanol. The precipitate was dissolved in 100 μl of NET; sucrose and bromophenol blue were added, and a 60-μl aliquot was electrophoresed as above. The distributions of radioactivity for OC-43 (●——●) and ribosomal and VSV (○- - -○) RNAs are shown.

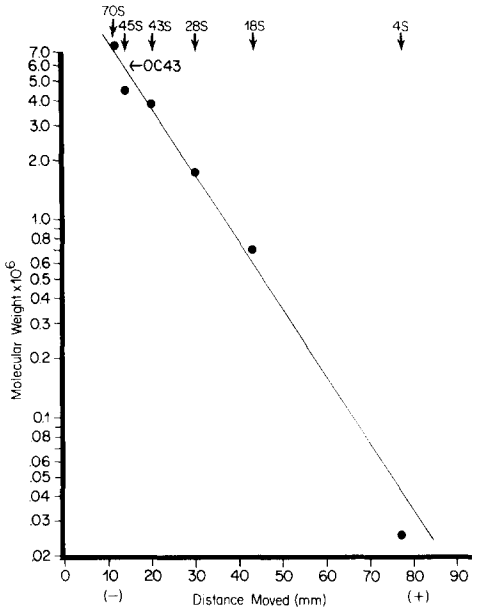

FIG. 4. Determination of apparent molecular weight (MW_a) for 70 S OC-43 RNA. Molecular weight values for the marker RNAs in Fig. 3 were: 70 S RSV RNA, 7.6×10^6 (King, 1976); 45 S ribosomal precursor RNA, 4.50×10^6 (McConkey and Hopkins, 1969); 43 S VSV RNA, 3.82×10^6 (Repik and Bishop, 1973); 28 and 18 S ribosomal RNA, 1.75 and 0.70×10^6, respectively (Loening, 1968); 4 S ribosomal RNA, 0.025×10^6 (Staynov *et al.*, 1972). The position of the OC-43 RNA complex released by the SL method is indicated.

RNA components found at the top of gradients for total radioactivity (see Fig. 7A). The electrophoretic profiles of OC-43 RNA, after preparation by the PC method, were determined at 5°. Clearly, the PC method resulted in considerable breakdown of the large complex into a range of RNA fragments with electrophoretic mobilities between 45 S and 18 S (Fig. 3B). A minor 4 S component was also present; it was similar to that found for IBV RNA after extraction by the WPS method (Tannock, 1973).

RNA Complexes of OC-43 Produced by SDS Lysis at Elevated Temperatures

Garwes *et al.* (1975) reported that RNA complexes from the porcine coronaviruses TGEV and HEV were degraded if extracted by SDS lysis at temperatures above 60°, as has been noted for the RNAs of Retroviridae (Duesberg, 1968). To confirm this with OC-43 RNA, preparations of OC-43 virus were treated with 1% SDS by the SL method at 23°, 37°, and 60°. The distributions of acid-insoluble radioactivity for each gradient suggest that at 37° there was some breakdown of the 70 S complex into smaller 4 S fragments, and at 60° a more generalized breakdown to a range of intermediate species occurred (Fig. 5). These results confirm those of Garwes *et al.* (1975), who demonstrated

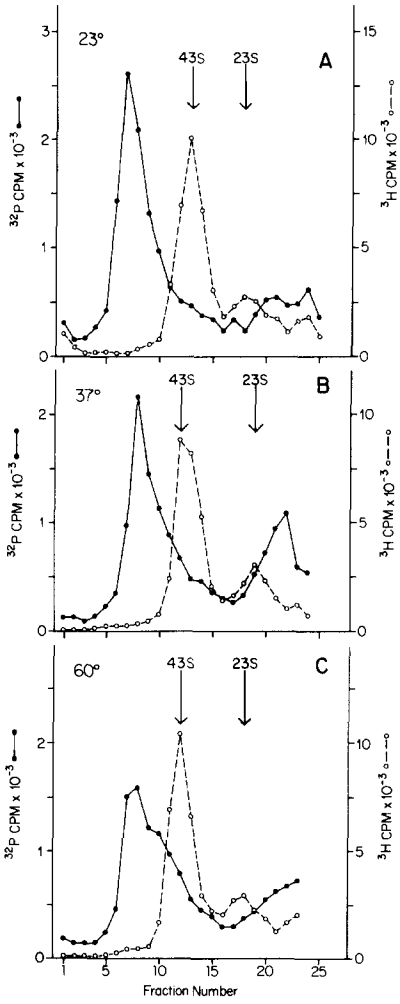

FIG. 5. Effects of heat upon 70 S OC-43 RNA. RNA from ^{32}P-labeled OC-43 was released by the SL method at (A) 23°, (B) 37°, and (C) 60°. Each extract was chilled at 0°, mixed with [^{3}H]uridine VSV RNAs, and analyzed in 15–30% sucrose gradients, as described under Materials and Methods. The distributions of acid-insoluble radioactivity for OC-43 (●——●) and VSV (○- - -○) RNAs are shown.

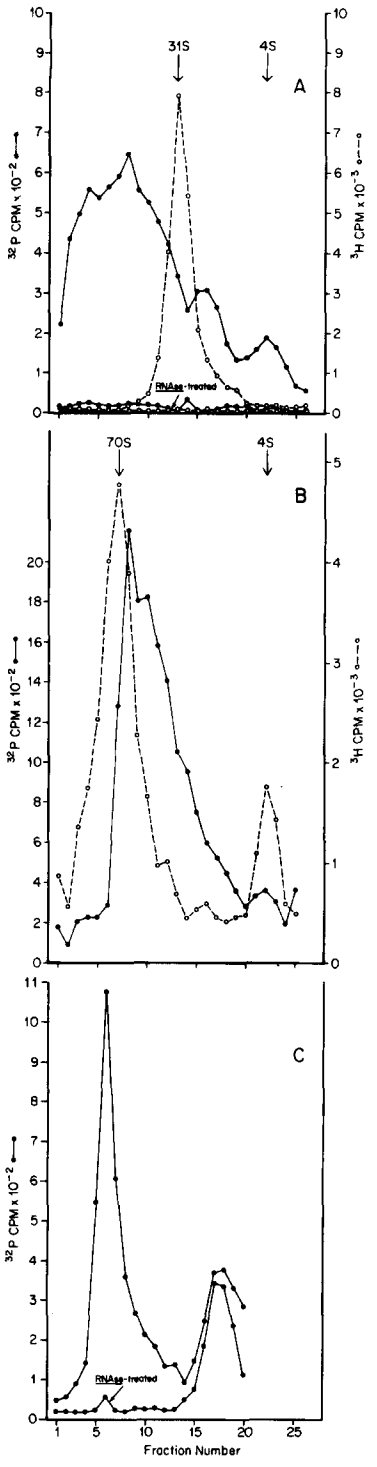

FIG. 6. Phenol extraction of OC-43 RNA in the presence of TMV or RSV RNA; susceptibility of OC-43 to ribonuclease. RNAs were extracted from mixtures of ^{32}P-labeled OC-43 and either (A) [^{3}H]uridine TMV or (B) [^{3}H]uridine RSV by the PC method. RNA was released from a third OC-43 sample by the SL method (C), and all preparations were analyzed in 15–30% sucrose gradients as described under Materials and Methods. Half of each fraction in gradients A and C was treated with 5 μg of pancreatic ribonuclease in 25 μl of distilled water for 30 min at 25°. The distributions of acid-insoluble radioactivity for OC-43 (●——●) and TMV and RSV (O- - -O) RNAs are shown.

that HEV and TGEV RNAs are heat-labile, although little dissociation occurs at temperatures of less than 60° in a buffer consisting of 0.036 *M* Tris (pH 7.8), 0.03 *M* NaH_2PO_4 and 0.001 *M* EDTA. However, in NET buffer at slightly lower pH and a higher salt concentration (0.1 *M* NaCl, 0.01 *M* Tris, pH 7.4, 0.01 *M* EDTA) the OC-43 RNA complex seems even more labile than that of the porcine coronaviruses.

Preparation of OC-43 RNA by the PC Method in the Presence of TMV and RSV

Heterogeneity in the major RNA component of OC-43 after phenol extraction (Figs. 2, 3) may have been caused by (1) mechanical disruption by phenol of noncovalent bonds linking the OC-43 RNA fragments which form the 70 S complex, or (2) specific release or activation of virion ribonucleases in the extraction procedure. To examine these possibilities, we coextracted RNAs from mixtures of ^{32}P-labeled OC-43 virus and [^{3}H]uridine TMV or [^{3}H]uridine RSV by the PC method. TMV RNA contains a single piece of RNA with a sedimentation coefficient of 31 S. The major 70 S RNA of RSV is comprised of smaller fragments of variable size and linked by noncovalent bonds; a number of minor RNA components are also present (Duesberg, 1968; Bishop *et al.*, 1970a, b; Baltimore, 1974; King, 1976). RNAs in each mixture, after preparation, were precipitated with ethanol in the presence of carrier RNA and analyzed in sucrose gradients.

The profiles of acid-insoluble radioactivity for each gradient are shown in Fig. 6. As a test for identity of the viral nucleic acid, half of each fraction in gradient A

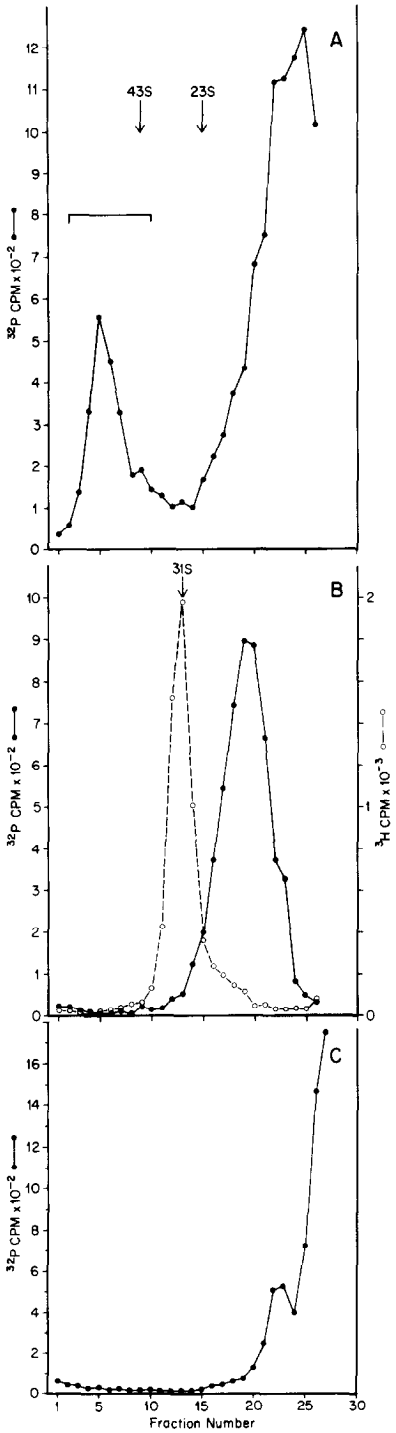

FIG. 7. Susceptibility of 70 S OC-43 RNA to PC extraction and centrifugation in DMSO gradients. OC-43 RNA was released from ^{32}P-labeled OC-43 virions by the SL method and isolated by centrifugation in 15–30% sucrose gradients. Samples of each gradient fraction were assayed for total radioactivity, and the profile is shown in A. Fractions 3–7, containing 70 S RNA, were pooled and reextracted in the presence of 0.1 ml of WPS-extracted [^{3}H]uridine TMV RNA. All RNAs present were then reprecipitated in ethanol after 100 μg of carrier RNA was added. The precipitate was dissolved in 0.5 ml of NET and analyzed in 15–30% sucrose gradients. The profile of acid-insoluble radioactivity for OC-43 (●——●) and TMV (○- - -○) RNAs is shown in B. Fifty microliters of a preparation of ^{32}P-labeled OC-43 was treated to release 70 S RNA by the SL method. The preparation was then centrifuged through 5–20% sucrose gradients in DMSO containing 10 μM LiCl for 5 hr at 189,000 g and 22°; the profile of total radioactivity is shown in C.

was mixed with 25 μl of NET containing 5 μg of ribonuclease for 30 min at 25°; the acid-insoluble radioactivity remaining was then determined after drying on paper strips. Figures 6A and 6B suggest the same heterogeneity for the major class of OC-43 RNA fragments already noted by the phenol extraction method (Figs. 2, 3), and again confirm the presence of a minor 4 S component. Both TMV and the major 70 S RNA of RSV remain undegraded after extraction. This suggests either (a) that the heterogeneity of the major RNA class is caused not by the activation of ribonucleases but by disruption of noncovalent linkages between RNA fragments which are much weaker than similar linkages within 70 S RSV RNA, or (b) a large RNA genome is extensively nicked by virion ribonucleases during viral growth and these enzymes are inactivated by phenol extraction. Sedimentation coefficients for the major RNAs released by phenol (15–55 S; Fig. 6B) were less than those for the homogeneous 70 S peak obtained by the SL method (Fig. 2B).

All ^{32}P-labeled structures throughout the gradient in Fig. 6A are clearly RNA, as judged by their uniform susceptibility to ribonuclease. A similar experiment was then carried out to extend this finding for ^{32}P-labeled material released by the SL method. SL-extracted virus was centrifuged to release the RNA as a 70 S complex (Fig. 2B), and half of each gradient fraction was treated with ribonuclease, as above. The profiles for acid-insoluble ra-

dioactivity (Fig. 6C) indicate that the major 70 S complex released by SDS lysis is fully susceptible to ribonuclease, whereas the ^{32}P-labeled material occurring at the top of the gradient remains fully resistant. The latter probably represents structural phospholipids which remain at the top of the gradient after SDS lysis and are distinct from ribonuclease-sensitive 4 S RNA obtained by the PC method (Fig. 6A).

Lability of OC-43 70 S RNA in the Presence of Phenol and DMSO

Further evidence that OC-43 70 S RNA is a complex of fragments held together by weak, noncovalent bonds was obtained by (1) isolation of 70 S OC-43 RNA by SDS lysis and centrifugation, followed by extraction by the PC method in the presence of TMV RNA, and (2) isolation of RNA by the SL method and centrifugation in DMSO gradients. OC-43 70 S RNA was lysed with SDS and centrifuged to isolate the RNA complex as described (see Fig. 7 legend). The profile for total (acid-soluble and -insoluble) radioactivity from aliquots of each gradient fraction reveals a sharp peak of ^{32}P radioactivity in the 70 S region (the RNA complex) and a much larger peak of low density material at the top of the gradient (Fig. 7A). Similarly located but much smaller peaks for acid-insoluble, ribonuclease-resistant radioactivity are seen in Figs. 2B, 5A, and 6C. It therefore seems likely that SDS lysis separates RNA from large amounts of acid-soluble components, some acid-insoluble phospholipids, and perhaps other components in the outer virion coat.

Fractions 2–10 were extracted by the PC method in the presence of [^{3}H]uridine TMV RNA, precipitated with ethanol in the presence of carrier RNA, and analyzed. The profile for acid-insoluble radioactivity reveals that such treatment completely degraded OC-43 70 S RNA, whereas TMV RNA remained intact (Fig. 7B). This experiment thus confirms earlier evidence that the 70 S complex is held together by weak noncovalent bonds which are destroyed by phenol extraction (Figs. 2, 3) or gentle heating (Fig. 5). The breakdown of isolated 70 S RNA by the PC extraction method is more complete than by similar extraction of purified virions. It does not appear to be due to ribonuclease activity because TMV RNA within the same mixture remains intact, although phenol extraction could denature OC-43 70 S RNA and so reveal breaks in an otherwise intact large RNA molecule which were produced by such activity.

Since DMSO has been used as a critical test for noncovalent linkages in RSV 70 S RNA (Duesberg, 1968), we investigated its effect on 70 S OC-43 RNA prepared by SDS lysis. The profile for total radioactivity (Fig. 7C) shows a complete breakdown of the 70 S RNA complex to smaller fragments which sediment slightly faster than structural phospholipids at the top of the gradient (Fig. 7A). Duesberg (1968) noted similar instability for RSV 70 S RNA, whose constituent RNA fragments are held together by noncovalent bonds less susceptible to phenol extraction than OC-43 RNA (Fig. 2B).

DISCUSSION

The OC-43 RNA complex released by SDS lysis and 70 S RSV RNA have identical sedimentation coefficients. The latter is a complex of several noncovalently linked subunits (MW 2.5–3.3 $\times$ 10^6) which are freed by heating or treatment with DMSO (Duesberg, 1968; King, 1976). In addition to 70 S RNA, a range of smaller fragments, including a 4 S species, is present in phenol extracts of RSV (Robinson *et al.*, 1965; Duesberg, 1968; Bishop *et al.*, 1970a, b; Dahlberg *et al.*, 1974). The 70 S OC-43 complex may also consist of noncovalently linked RNA subunits which are disrupted by DMSO, but unlike 70 S RSV, are highly susceptible to phenol. The complex is more readily broken down by heating than are similar complexes isolated from RSV (Duesberg, 1968) and the TGEV and HEV coronaviruses (Garwes *et al.*, 1975). Its apparent molecular weight, determined comparatively by coelectrophoresis with other RNAs, is 6.1 $\times$ 10^6; this value is only considered to be approximate, however, since 2.2% gels do not adequately resolve high molecular-weight RNAs (Loening, 1969; King, 1976).

Native OC-43 RNA is readily denatured into fragments by organic solvents and heat, much like that observed with B77 sarcoma virus RNA by Stoltzfus and Snyder (1975). However, the sizes of the OC-43 RNA fragments from the disrupted complex seem to vary considerably. Phenol extraction of purified virions produces fragments ranging between 15 and 50 S, and a small amount of a homogeneous 4 S RNA is also present (Figs. 2, 3, 6). A range of fragments of molecular weight 0.7–5 $\times$ 10^6 and a smaller species was obtained after PAGE, similar to that described by Tannock (1973) for IBV RNA. Coextraction experiments with TMV and RSV (Fig. 6) did not implicate internal ribonuclease activity as a cause of this heterogeneity. When the 70 S complex released by SDS lysis was isolated and reextracted with phenol–chloroform, only 5–10 S RNA fragments were obtained (Fig. 7). Why fragments extracted in this manner should be smaller is unknown. Again, however, ribonucleases could not be detected, because a preparation of TMV RNA, when included in the extraction mixture, remained undegraded (Fig. 7B). Possibly, RNA extracted by phenol from purified virions contains more secondary structure. Alternatively ribonucleases could have been present throughout viral growth, producing nicks in an otherwise intact RNA molecule and subsequently being destroyed by phenol–chloroform extraction.

The release of 4 S RNA from the 70 S complex by heating has also been observed for TGEV and HEV RNAs (Garwes *et al.*, 1975). Recent studies have shown that polyadenylate residues are present in 15–55 S but not in the 4 S RNAs obtained by PC extraction (Tannock and Hierholzer, in preparation), indicating a functional difference between these RNA classes. Detailed studies are needed to determine what these functions are.

Although SDS is recognized as an effective RNA deproteinizing agent (Boatman and Kaper, 1973), the susceptibility of the 70 S OC-43 complex to phenol suggests that small protein linkers could be required to hold the RNA fragments together and that they are not removable by SDS lysis. Evidence against this comes from cesium sulfate gradient experiments in which the buoyant densities of the OC-43 complex prepared by SDS lysis and phenol-extracted TMV RNA were identical (Tannock and Hierholzer, unpublished observations). Additionally, Lowry protein determinations and 8% acrylamide gels (protein-stained) on purified RNA samples containing 120 μg RNA did not detect protein, implying that any protein linkers present must constitute less than 2% of the RNA structure. The complexing of a few small proteins to a large RNA molecule, however, may produce no detectable difference in buoyant density, as Sen *et al.* (1976) have noted for Rauscher murine leukemia viral RNA and phosphoprotein P12, with a type-specific RNA binding affinity.

Extensive nicking of retroviral 70 S RNA due to ribonuclease activity has been observed after extended viral growth in cell culture (Bader and Steck, 1969; King and Wells, 1976) and during virus purification (Scheele and Hanafusa, 1972). The integrity of the nicked 70 S RNA is still maintained after extraction from virions by phenol, being only apparent after treatment with RNA denaturants. Extensive changes in OC-43 RNA conformation produced by phenol suggest that, even if extensive nicking of the 70 S complex occurs during viral growth, there are marked differences in its secondary structure compared to that of 70 S retroviral RNA.

To determine whether a genuine subunit structure is present in the coronaviral complex, it would be highly desirable to examine the virion RNA present at different times after maturation in a more strictly defined milieu than that of the mouse brain. However, for human coronaviruses, the lack of convenient model systems is a severe impediment to this work.

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