JOURNAL OF VIROLOGY, Nov. 1975, p. 1161-1170

Vol. 16, No. 5
Printed in U.S.A.

Structure of B77 Sarcoma Virus RNA: Stabilization of RNA After Packaging

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Received for publication 3 July 1975

Extracellular maturation of Bratislava 77 (B77) sarcoma virus RNA involves a stabilization of linkage between 35*S* subunits since the T_m of 60 to 70*S* RNA in the presence of 0.5 M NaCl increases from 56 to 67.5 C as the age of the virus increases. This stabilization process is strongly temperature dependent; the rate at 45 C is increased fourfold over the rate at 37 C. As a result of the instability of the immature RNA, denaturation to subunits occurs at room temperature during phenol extraction. This dissociation can be prevented by increasing the NaCl concentration during the extraction. The data support a model which proposes that RNA subunits of oncornaviruses exist as assembled 60 to 70*S* RNA even in immature virions but that the linkages between subunits are stabilized as a function of time. These linkages appear to be maintained by nucleotide base pairing rather than by protein. However, isolated RNA does not undergo stabilization, suggesting that some other component of the virion is necessary for the process to occur.

The genome of oncornaviruses is composed of a number of 35*S* RNA subunits which are usually associated in a 60 to 70*S* aggregate structure (5). It has been reported, however, that species of RNA obtained from some oncornaviruses harvested at several-minute intervals (rapid harvest virus) differ from RNA isolated from virus harvested at longer intervals (2, 3). In one study (3) a major 55 to 60*S* RNA intermediate was isolated from the Prague strain of Rous sarcoma virus (Prague RSV) harvested at 5-min intervals. In addition, a minor 35*S* peak, a peak at 28*S* and a 4 to 12*S* RNA species were also observed. As the extracellular age of the virus increased, the 35*S* and 28*S* peaks disappeared and the major peak sedimented at 67*S* rather than at 60*S*. In another study (2), 3-min Prague RSV was found to contain a major 35*S* RNA species, lesser amounts of 60 to 70*S* RNA, and also 4 to 10*S* RNA. Incubation of the 3-min virus at 40 C resulted in the apparent conversion of the free 35*S* RNA subunits to 60 to 70*S* RNA. Similar results have recently been reported for Visna virus (1). Other oncornaviruses have also been reported to contain free 35*S* subunits (12, 15, 23). The appearance of free subunits in rapid harvest virus and the ability to convert 35*S* RNA to 60 to 70*S* RNA suggested that the assembly of 35*S* RNA subunits may occur after virions bud from cells and has been cited as evidence to indicate that the 35*S* RNA is a precursor to 60 to 70*S* RNA (2). Free 35*S* subunits, however, have not been isolated from murine oncornaviruses harvested at several-minute intervals (7, 19). In an effort to resolve these apparently discordant results, we have investigated the RNA structure and maturation of another member of the avian sarcoma virus group, B77 sarcoma virus. This paper is concerned with observations on the stability of the immature RNA from B77 virus.

MATERIALS AND METHODS

Virus and cells. A stock of B77 avian sarcoma virus was kindly supplied by Peter K. Vogt, University of Southern California. A cloned stock of virus was isolated from a single focus of transformed chicken embryo cells. Primary chicken embryo fibroblasts were isolated from 10- to 12-day-old embryos (Spafas, Inc., Norwich, Conn.) essentially according to the procedure of Vogt (27). After the cells on the primary plates were confluent (4 to 5 days), they were detached with 0.08% (wt/vol) trypsin and resuspended in Medium 199 (GIBCO, Grand Island, N.Y.), supplemented with 10% tryptose phosphate broth, 5% calf serum (secondary growth medium) and 2 μg of polybrene per ml (Aldrich Chemical Co., Milwaukee, Wisc.) (24). The cells were added in a volume of 10 ml to 100-mm plastic petri dishes (approximately 2×10^6 cells per plate), incubated for 1 h at 38 C in a 5% CO_2 atmosphere, and infected with 0.1 to 0.5 focus-forming units of virus per cell of cloned B77 sarcoma virus. The following day, the medium was removed and replaced with secondary growth medium. On day 4 or 5 after infection, the cells

appeared to be morphologically transformed and were either used at this point for labeling or were diluted 1:2 and used when the transformed cells were again confluent. After the cells appeared to be transformed, the medium was replaced with secondary growth medium containing 1% Me_2SO.

Radioactive labeling of virus. Plates of cells transformed and producing B77 virus were labeled for 9 h with 40 μCi of [5-^{3}H]uridine per ml (22 Ci/mmol; Schwarz/Mann, Orangeburg, N.Y.) in Medium 199 containing 10% tryptose phosphate broth, 2% calf serum, and 1% Me_2SO. After this time the cells were washed with the same medium and the virus was harvested at various time periods depending on the experiment (see legends to figures). The harvested medium was stored at 4 C during the 6-h collection period.

^{32}P-labeled virus was isolated by labeling transformed cells with 200 μCi of [^{32}P]phosphate per ml, carrier free (ICN Pharmaceuticals, Cleveland, Ohio), for 12 h in phosphate-free minimal Eagle medium containing 2% dialyzed calf serum and 1% Me_2SO. After this time the labeled medium was removed and replaced with fresh medium. The medium was harvested at 3-h intervals.

Purification of virus. All steps were carried out at 4 C. Virus-containing medium was centrifuged at 7,000 rpm in the Beckman J-21 centrifuge to remove cells and other debris. The supernatant was layered over a discontinuous gradient prepared in an SW27 centrifuge tube and composed of 1 ml of 70% (wt/vol) sucrose (Schwarz/Mann; RNase free) in a solution containing 0.1 M NaCl, 0.01 M Tris, pH 7.5, and 0.001 M EDTA (NET) overlaid with 5 ml of 20% (wt/vol) sucrose in the same buffer. After centrifugation at 23,000 rpm for 1 h in the Beckman SW27 rotor, the virus, which appeared at the 20% and 70% sucrose interface, was isolated by collection from the bottom of the centrifuge tube. Aliquots were removed from the fractions and counted for 5% trichloroacetic acid-precipitable radioactivity. The virus-containing fractions were pooled, diluted at least twofold with NET buffer, and applied to linear 20 to 70% sucrose gradients. The gradients were centrifuged for 4 h at 23,000 rpm in the Beckman SW27 rotor. One-milliliter fractions were collected from a hole pierced in the bottom of the tube. Aliquots were counted to locate the sharp peak of radioactivity. The fractions containing the virus peak were pooled and stored at −70 C.

Isolation of RNA. All buffers used for RNA work were autoclaved. As further precaution against nucleases, buffers were treated with a suspension of bentonite. The bentonite was removed by centrifugation at 12,000 rpm in the Beckman J21 centrifuge. Glassware was heated at 180 C for at least 2 h before use. Viral RNA was isolated by modifications of the standard phenol extraction technique. Purified virus isolated from the continuous sucrose gradient was diluted two- to threefold with NET buffer (or at other salt concentrations to be described below), made to a final concentration of 0.1% 2-mercaptoethanol (vol/vol) and 0.5% sodium dodecyl sulfate (SDS) (wt/vol) (BDH Chemical Co., Poole, England), followed immediately by addition of equal volumes of 90% (vol/vol) redistilled phenol and chloroform which together equaled the final volume of the diluted virus. Yeast tRNA (100 μg) was added as a carrier to the preparation. The mixture was then rapidly brought to room temperature for subsequent extraction, agitated by hand for 5 min at room temperature (or various extraction temperatures), cooled to 4 C, and centrifuged for 10 min at full speed in a Clay Adams Dynac centrifuge at room temperature. The aqueous phase was removed, NET buffer equal in volume to the original diluted virus was added, and the extraction process was repeated. The aqueous phases were combined, residual phenol was removed by ether extraction, and the RNA was precipitated at −20 C with 2 volumes of ethanol. Greater than 90% of the denatured (heated for 3 min at 80 C) [^{3}H]uridine-labeled 65*S* RNA from 3-h virus bound to an oligo(dT)-cellulose column, indicating that RNA prepared in this manner is essentially intact. Denatured RNA also migrated as a single band when subjected to gel electrophoresis, as expected for a cloned sarcoma virus (6).

Measurements of radioactivity. Radioactive samples were counted in a scintillation solvent containing toluene, naphthalene, methyl cellosolve, and BBOT (Research Products Corp., Elk Grove, Ill.) at appropriate channel settings in the Packard 3375 liquid scintillation counter.

RESULTS

Effect of salt concentration on the species of RNA isolated from immature B77 virions. Immature B77 sarcoma virus (3-min virus) labeled with [^{3}H]uridine was extracted at room temperature with phenol-chloroform in the presence of 0.5% SDS at two different NaCl concentrations, 0.5 M and 0.1 M. The RNA extracted in 0.5 M NaCl and sedimented on a 5 to 30% glycerol gradient (Fig. 1A) contains two major peaks of radioactivity: one peak which cosediments with ^{32}P-labeled RNA extracted with phenol-chloroform from virus harvested at 3-h intervals (65*S*); and another major peak, containing approximately 26 to 30% of the total [^{3}H]uridine radioactivity, which sediments at 4 to 10*S* and presumably contains 4*S*, 5*S*, and 7*S* RNA. The latter material is also present in mature virus in about the same proportion (10). Denaturation of high-salt RNA at 70 C in 0.5 M NaCl results in the disappearance of most material sedimenting at 65*S* and in the production of a slower sedimenting peak of radioactivity which cosediments with heat-denatured RNA from 3-h virus (Fig. 1B), previously shown to sediment at approximately 35*S* (8). It should be noted that stronger denaturation conditions (80 C for 3 min) than those used in Fig. 1B are required to completely convert 65*S* RNA to 35*S* subunits when the denaturation is carried out in 0.5 M NaCl (see Fig. 4). In Fig. 1B, this

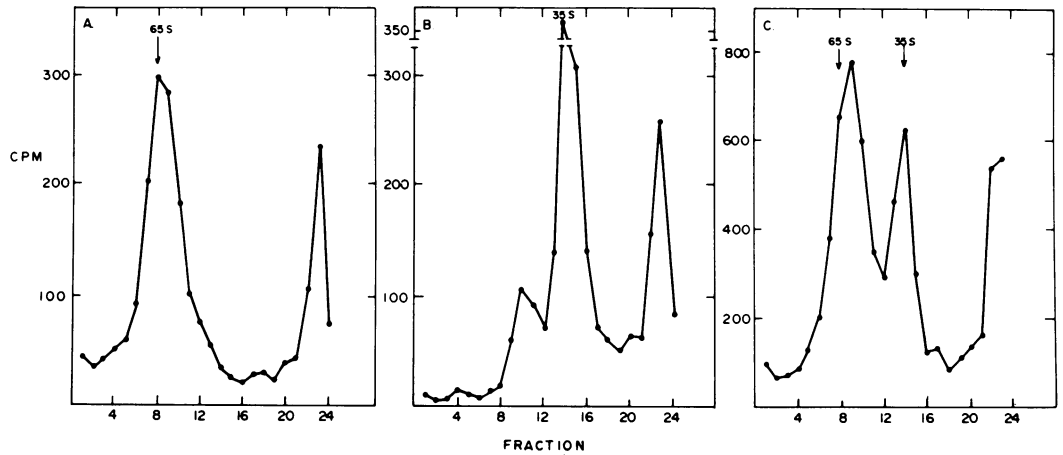

FIG. 1. *Sedimentation analysis of RNA extracted from immature B77 sarcoma virus at different NaCl concentrations. (A) B77 sarcoma virus was labeled with [^{3}H]uridine, harvested at 3-min intervals, and purified according to procedures described in Materials and Methods. The virus was extracted with phenol-chloroform at room temperature according to the procedures described in Materials and Methods except that the aqueous phase was brought to a final concentration of 0.5 M NaCl, 0.01 M Tris, pH 7.5, 0.001 M EDTA (0.5 M NaCl-TE). Alcohol-precipitated RNA was centrifuged at 12,000 rpm for 30 min in the Beckman J-21B centrifuge. The precipitates were dissolved in 0.5 M NaCl-TE and a 200-μl aliquot containing approximately 1,200 counts/min was warmed to room temperature and layered on 12-ml gradients of 5 to 30% glycerol (vol/vol) in 0.1 M NaCl, 0.005 M EDTA, 0.02 M Tris, pH 7.5. The gradients were centrifuged at 4 C for 3.5 h at 35,000 rpm in the SW41 rotor. Fractions of 0.5 ml were collected and counted directly for radioactivity in 15 ml of scintillation solvent. The position of native [^{32}P]B77 RNA extracted in 0.1 M NaCl from virus harvested at 3-h intervals is shown in the figure. Sedimentation is carried out from right to left in this and subsequent figures. (B) The same procedure as in A was followed except that a 200-μl aliquot of RNA in 0.5 M NaCl-TE was heated to 70 C for 3 min before layering on 5 to 30% glycerol gradients. The position of [^{32}P]B77-labeled heat-denatured RNA (3 min at 80 C in TE buffer) extracted from 3-h virus is shown in the figure. (C) The same procedure as in A was followed except that approximately 4,000 counts/min of [^{3}H]uridine-labeled virus was extracted in NET buffer at 25 C. The positions of the native and heat-denatured RNAs extracted from 3-h virus are also shown in the figure.*

accounts for the minor peak of radioactivity which sediments faster than the 35*S* RNA. In contrast to the results given in Fig. 1A, RNA isolated from immature virus extracted with phenol-chloroform in 0.1 M NaCl at room temperature (Fig. 1C) is composed of three major peaks of radioactivity: one peak sedimenting at about 60*S*, an intermediate peak sedimenting at approximately the position of 35*S* RNA, and a 4 to 10*S* peak. (The shift of the fastest sedimenting peak from 64*S* to 60*S* is also seen when mature 65*S* RNA is dissociated with low concentrations of formamide. This observation will be developed further in a following communication.) Upon denaturation of the low-salt RNA at 70 C, a profile similar to Fig. 1B was obtained (data not shown).

Incubation of [^{3}H]uridine-labeled 5-min virus in 0.5 M NaCl at 4 C followed by dilution to 0.1 M NaCl before extraction or addition of NaCl to 0.5 M after 1 min of exposure of the virus to phenol in 0.1 M NaCl resulted in distributions of radioactivity in 60 to 70*S* RNA and 35*S* RNA similar to those shown in Fig. 1C (Table 1). Free 35*S* subunits (about 40% of the total genome RNA) appear only when 0.1 M NaCl is present at the time the virions are disrupted with phenol and the profile is unaffected by prior or subsequent exposure to 0.5 M NaCl. Consequently the effect of high salt cannot be explained either as an assembly of free subunits to a 60 to 70*S* structure before extraction, as an aggregation of subunits in 0.5 M NaCl after extraction or a selective loss of 35*S* subunits during high-salt extraction. The fact that the same recovery of radioactivity was achieved using either extraction condition also eliminated the latter as a possible explanation for the results.

Effect of Pronase digestion on the stability of immature RNA. There seem to be two possible remaining explanations for the observed results. First, the RNA subunits in the immature virus might be held together by protein linkers which are stable to phenol extraction in 0.5 M NaCl but sensitive in 0.1 M NaCl. The second possibility is that the RNA subunits are associated by regions of base pair-

TABLE 1. *Effect of NaCl concentration before, during or after phenol-chloroform extraction on distribution of radioactivity from 5-min [^3H]uridine-labeled B77 virus*[a]

NaCl concn (M)			% Distribution of RNA	
1 min before extraction	During extraction	1 min after extraction	60 to 70S	35S
0.5	0.5	0.5	100	0
0.1	0.1	0.1	58	42
0.5	0.1	0.1	56	44
0.1	0.1	0.5	59	41

[a] Purified 5-min virus in NET buffer was either extracted directly at room temperature according to procedures in Materials and Methods or was brought to a concentration of 0.5 M NaCl at 4 C, extracted at 25 C 1 min later either with or without prior dilution to 0.1 M NaCl. In the indicated case, 1 min after addition of phenol and chloroform, the solution was brought to 0.5 M NaCl and carried through the remainder of the extraction at this salt concentration. Recovery of RNA and sedimentation analysis were carried out as described in legend to Fig. 1A and the proportion of counts in the 60 to 70S peak and 35S peak was determined.

ing which spontaneously melt out during some extraction conditions but are protected when higher salt concentrations are used. The first possibility was investigated by disrupting the virus in 1% SDS together with 500 μg of self-digested Pronase per ml for 30 min at 25 C before phenol extraction at high (0.5 M) or low (0.1 M) NaCl concentrations. The extracts were then analyzed by density gradient centrifugation. The results were virtually identical to those shown in Fig. 1A and C for high- and low-salt extraction, respectively (Table 2). Little, if any, nicking of RNA occurred during the Pronase treatment. Thus, the putative protein linkers, if they exist, must be stable to both high-salt phenol extraction and to Pronase digestion. As shown in the following experiments, the second possibility, that the RNAs are linked by unstable base-paired regions, seems to be the more likely explanation.

Effect of temperature on the stability of immature RNA. It is well known that an increase in salt concentration results in an increase in thermal stability of double-helical nucleic acid molecules. This is true also for the bonds holding together the RNA subunits in the 60 to 70S RNA. There is a shift in the midpoint of the thermal transition of immature 60 to 70S RNA to 35S RNA (T_m) from 56 to 45 C as the salt concentration is lowered from 0.5 to 0.1 M NaCl (Fig. 2). If the RNA is reextracted with phenol-chloroform at various temperatures in 0.1 M NaCl, however, the results shown in Fig. 3 are obtained. The proportion of 60 to 70S RNA to 35S RNA is dependent on the temperature of extraction. A melting curve is generated by these data and is plotted in Fig. 2. In the

TABLE 2. *Effect of prior Pronase treatment on distribution of radioactivity from phenol-chloroform extraction of 5-min [^3H]uridine-labeled B77 virus*[a]

NaCl concn (M) during extraction	% Distribution of RNA	
	60 to 70S	35S
0.5	95	5
0.1	62	38

[a] Pronase (Calbiochem, Fullerton, Calif.) was self-digested for 4 h at 37 C. Purified 5-min [^{3}H]uridine-labeled virus in NET buffer was incubated in the presence of 1% SDS, 500 μg of Pronase per ml for 30 min at 25 C, followed by high- and low-salt phenol extraction and sedimentation analysis as described in legend to Fig. 1.

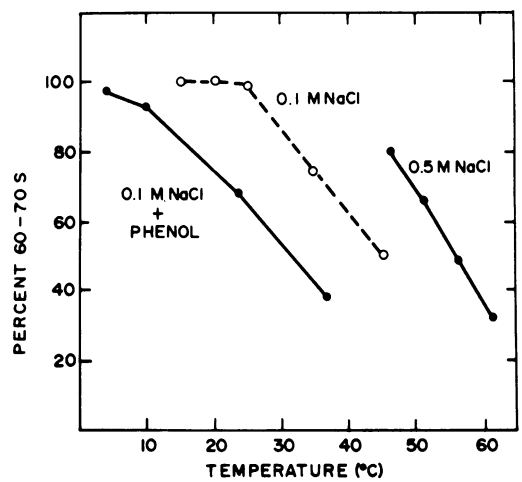

FIG. 2. *Effect of salt and phenol on the thermal dissociation of immature B77 60 to 70S RNA. B77 sarcoma virus was labeled with [^3H]uridine, harvested at 5-min intervals, purified and extracted with phenol according to procedures described in Fig. 1A. Precipitated RNA was dissolved in either 0.5 M NaCl-TE or NET buffer, 200-μl aliquots were incubated at the temperatures specified in the figure for 3 min after which the sample was immediately quenched in ice. Glycerol gradient sedimentation was carried out as described in Fig. 1. The percentage of 60 to 70S RNA was determined by summing the radioactivity in the 60 to 70S peak and in the 35S peak. The ratio of 60 to 70S RNA radioactivity to this sum was then determined. The procedure for preparation of "0.1 M NaCl + phenol" curve is given in legend to Fig. 3.*

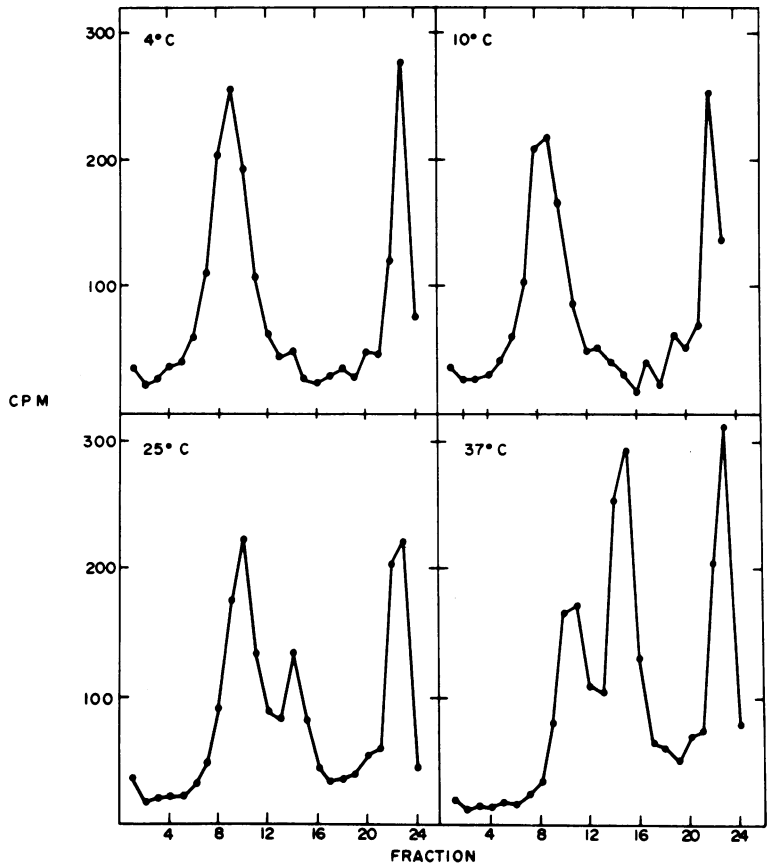

FIG. 3. *Sedimentation analysis of RNA from immature B77 sarcoma virus reextracted with phenol at various temperatures. B77 [^{3}H]uridine-labeled RNA from 5-min virus was isolated by similar procedures as those described in legend to Fig. 2. Aliquots of the RNA were then reextracted with phenol in NET buffer at various temperatures (4, 10, 25, and 37 C, shown in figure), and the RNA was recovered by ethanol precipitation and sedimented according to procedures given in legend to Fig. 1. The proportion of 60 to 70S RNA was determined and these data are plotted in Fig. 2.*

presence of phenol, a 13 C shift in T_m occurs, resulting in considerable dissociation of the 60 to 70S RNA at room temperature. Massie and Zimm have noted previously that phenol lowers the T_m of double-stranded DNA (17) such that a 7.7% phenol concentration lowers the T_m of DNA by approximately 20 C. The solubility of phenol in 0.1 M NaCl in the presence of chloroform under conditions used in our experiments is approximately 4% at 25 C, whereas at higher NaCl concentrations its solubility decreases (20). Consequently, the presence of higher salt in the phenol extraction has two possible effects on the stability of the RNA complex: (i) it raises the T_m due to known effects of salt concentration on the stability of base-paired regions; (ii) it lowers the phenol concentration in the aqueous phase and thus raises the apparent T_m. It is concluded that the reason for the difference in the two profiles of Fig. 1A and C is that the immature viral RNA is linked by bonds which spontaneously dissociate under standard conditions of phenol extraction.

Direct lysis of immature virus. If the presence of phenol lowers the T_m of unstable base-paired regions, release of RNA by direct lysis of the virus vould be expected to result in significantly less RNA denaturation. Therefore, 5-min virus was disrupted with 1% SDS and 0.1 M NaCl at 25 and 37 C in the presence and absence of Pronase. The distribution of radioactivity in the 60 to 70S peak relative to the 35S peak (Table 3) was unaffected by Pronase digestion, consistent with previous results given in Table 2. The incubation temperature, however, did affect the resulting distribution. Little dissociation of 60 to 70S RNA occurred at 25 C, but at 37 C there was considerable conversion to

TABLE 3. *Distribution of radioactivity from 5-min [^3H]uridine-labeled B77 virus after direct disruption in 1% SDS, 0.1 M NaCl*[a]

Temp (C)	Pronase (500 μg/ml)	% Distribution of RNA	
		60 to 70S	35S
25	+	89	11
25	-	87	13
37	+	57	43
37	-	55	45

[a] Virus was pelleted at 40,000 rpm for 1 h in an SW50 rotor, resuspended in NET buffer containing 1% SDS and incubated for 30 min at the indicated temperature in the presence and absence of Pronase. Sedimentation was carried out in 5 to 30% glycerol gradients containing 0.1% SDS at 20 C for 3 h at 35,000 rpm in the SW41 rotor.

35S subunits. From the results given in Fig. 2, such dissociation was expected in 0.1 M NaCl at 37 C.

Stability of 60 to 70S B77 RNA as a function of virus age. Recognition of the effects of phenol, salt concentration, and temperature on the linkage of subunits in immature 60 to 70S RNA led us to re-examine the question of extracellular maturation of oncornavirus RNA in terms of the stability of the putative base-paired regions. Denaturation curves for RNA isolated by high-salt (0.5 M NaCl) phenol extraction of virus harvested at various intervals from the medium of B77-infected cells are given in Fig. 4. There is a gradual shift in the T_m from 56 C for 5-min virus to 67.5 C for 12-h virus (T_m^∞) (Fig. 4, inset). A plot (T_m^∞ - T_m) versus age of virus yields a straight line (Fig. 5) indicating that RNA maturation over the first 80% of the observed change follows apparent first order kinetics with a half-life of 45 min. Extrapolation to zero time indicates a total change in T_m of 11.5 C (the 3-min virus has a T_m virtually identical to 5-min virus, consistent with expected results from such an extrapolation). The remaining 20% of the maturation occurs at a slower rate (see Fig. 4).

It was possible that the observed change in T_m with increasing age might be due to selection for particular populations of virions. To test for this possibility, synchronous maturation of purified immature virus was carried out. Aliquots of purified 5-min virus were incubated at 37 C for various times and extracted with phenol in the presence of 0.5 M NaCl, and the amount of dissociation of 60 to 70S RNA at 56 C was measured (Fig. 6). This latter value was found empirically to be inversely proportional to T_m and thus could be used to assay the extent of maturation. Again, approximately 80% of the maturation appears to proceed with apparent first order kinetics (Fig. 7), followed by a slower rate for the subsequent 20%. The half-life for the initial processing of the virus at 37 C was 23 min. It will be recalled that the half-life for the rate of maturation of virus collected at various intervals is 45 min, approximately twice that observed for purified virus. However, since the average age of virus collected in an interval is t/2, the observed half-life would be expected to be twice that occurring in the synchronous maturation. Thus, the results are consistent with this expectation.

The temperature dependence of the rate and extent of maturation is given in Fig. 6. The rate at 45 C is increased about fourfold when compared with the rate at 37 C; the rate of maturation at 25 C is slower and proceeds to an extent of only about 25% in 2 h. RNA from 5-min virus, which was incubated at 37 C in the presence of 0.5 M NaCl for times up to 6 h, does not undergo detectable maturation. It should be noted that in this time period no RNA degradation was observed.

DISCUSSION

We have obtained evidence that the intersubunit linkages in immature B77 60 to 70S RNA are unstable and spontaneously dissociate when the virus is extracted with phenol at room temperature in the presence of 0.1 M NaCl. This linkage can be stabilized by extracting the virus in the presence of 0.5 M NaCl, lowering the temperature during phenol extraction, or directly disrupting the virus with SDS. Although our data do not exclude the presence of protein linkers which are resistant to phenol, SDS and Pronase, the most likely explanation for the observed results is that phenol affects the stability of short regions of base pairing between subunits and that such base pairing can be stabilized by an increase in the NaCl concentration. Further studies on the nature of the putative intersubunit base pairs will be required to confirm this hypothesis. Since phenol extraction is a widely used method for isolating RNA from viruses and cells, the results reported above may be generally applicable to studies investigating possible physical association of polynucleotide subunits through regions of base pairing. Short regions of base pairing are possibly involved, for instance, in the specific association of RNA subunits during assembly of viruses with segmented genomes such as the reoviruses and influenza viruses (21) as well as oncornaviruses.

We suggest that the observed increase in T_m of B77 RNA with age of virus reported above

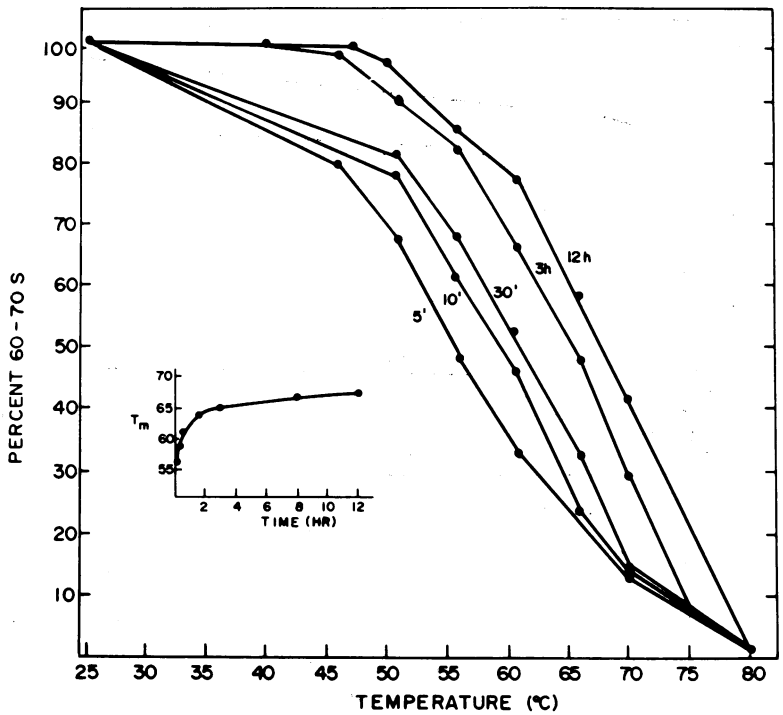

FIG. 4. *Thermal denaturation of B77 sarcoma virus RNA isolated from virus harvested at various intervals. B77 sarcoma virus was labeled with [^{3}H]uridine, harvested at the indicated intervals, and purified according to procedures described in Materials and Methods. Isolation of RNA was carried out as described in legend to Fig. 1A. Incubation of RNA at the indicated temperatures was carried out for 3 min in 0.5 M NaCl-TE after which the sample was immediately quenched in ice. Sedimentation analysis and determination of percentage of dissociation of 60 to 70S RNA was carried out as described in legend to Fig. 2. Approximately 1,200 counts/min 60 to 70S RNA were used for each determination.*

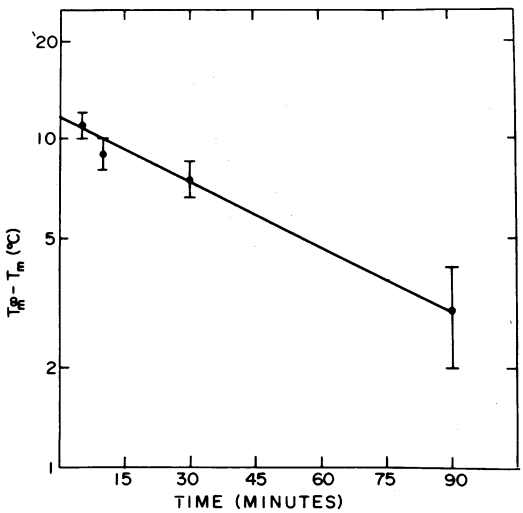

FIG. 5. *Plot of log (T_m^∞ – T_m) versus time of harvest.*

results from an increase in stability of base-paired regions. The reason for such a shift in T_m is not known. It may occur (1) as a result of an increase in the length of base-paired regions between subunits, either reflecting a rearrangement of subunits in the virion or an increase in the number of associated 4*S* or 5*S* RNAs which might serve as linkers of 35*S* subunits in the 60 to 70*S* complex or (2) a replacement of some adenylic-uridylic base pairs with stronger guanylic-cytidilic pairs (16, 26). Because the RNA alone does not undergo the T_m shift (Fig. 6) it suggests that other components of the virions are necessary to mediate RNA stabilization. One possibility is that rearrangements may occur in the protein capsid of the virus which modify or promote associations between the RNA subunits. Changes in the capsid polypeptide composition of immature versus mature virions have been previously observed (3) and may be related to such a rearrangement. Also, the strong temperature dependence suggests that a rearrangement of capsid proteins may be involved in the RNA maturation. If low-molecular-weight RNAs function as linkers they may also be necessary in the high local concentrations in which they are present in the intact virion.

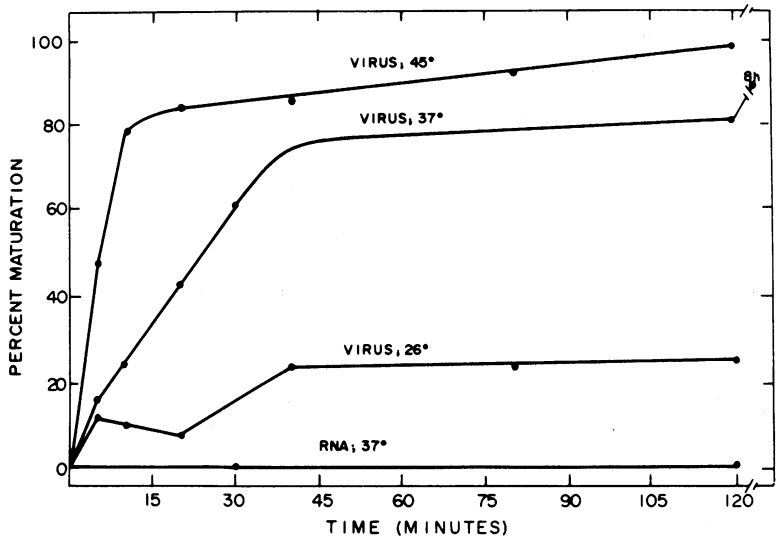

FIG. 6. *Maturation of purified virus as a function of time and temperature. B77 sarcoma virus was labeled with [^3H]uridine, harvested at 5-min intervals and purified according to procedures described in Materials and Methods. Aliquots of virus (approximately 3,000 counts/min) which had been isolated from isopycnic sucrose gradients were diluted twofold with NET buffer and incubated for various time periods at the temperatures specified in the figure. RNA from 5-min virus extracted according to conditions described in Fig. 1A was also incubated at 37 C in 0.5 M NaCl-TE buffer. Quenching in ice terminated the incubations. Each sample was then extracted with phenol in 0.5 M NaCl-TE buffer. The precipitated RNAs were pelleted, redissolved in 0.5 M NaCl-TE buffer, heated to 56 C for 3 min, and quenched in ice. The samples were then sedimented on 5 to 30% glycerol gradients and the proportion of 60 to 70S RNA was determined as described in legend to Fig. 2. Percentage of maturation was determined in the following manner:*

$$\%\ maturation = 1 - \frac{\Delta\%\ dissociation\ of\ incubated\ sample}{\Delta\%\ dissociation\ of\ unincubated\ sample} \times 100$$

where Δ% dissociation is defined as the difference between percent dissociation at 56 C in 12-h 60 to70S RNA (fully mature RNA) and the dissociation at 56 C of RNA from virus incubated for the indicated times.

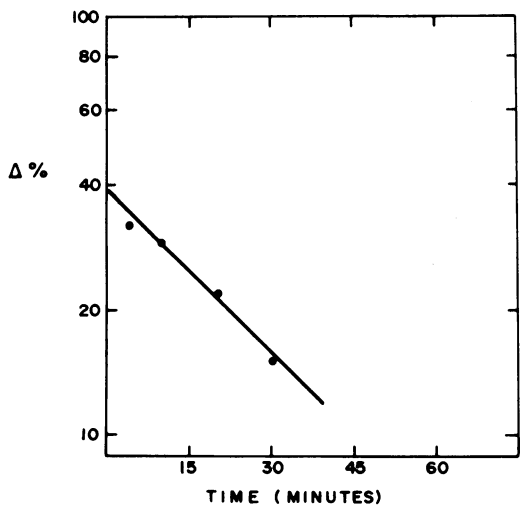

FIG. 7. *Plot of log Δ% dissociation versus time of incubation at 37 C.*

The possible functional significance of the RNA stabilization process is currently under investigation. It will be of interest to know whether the observed age-dependent changes in specific infectivity (22) and endogenous RNA dependent RNA polymerase activity (3) are directly related to changes in the structure of the 60 to 70*S* RNA as monitored by the T_m shift.

Our results suggest that the reason for the reported differences in the RNA species isolated from immature Prague RSV is related to the different methods of RNA extraction used in these two studies (2, 3). In one study (3) a major 55 to 60*S* (versus 67*S* for the mature RNA) and minor 35*S* RNAs were observed. The method of RNA extraction used in this study was direct lysis of the virus at relatively low ionic strength (0.01 M sodium acetate, pH 5.0) in the presence of 1% SDS at 20 C. In the other study, where a major 35*S* RNA component was reported, room temperature phenol extraction was used to

isolate the viral RNA from the immature virions (2). This 35S component was found to have a slightly smaller electrophoretic mobility than heat-denatured RNA from mature virus. We have obtained similar results with B77 sarcoma virus when extracted using these two different procedures and have found that the properties of the "intermediates" observed are similar to products obtained by controlled formamide denaturation of mature RNA (Stoltzfus, manuscript in preparation). The types of RNA molecules observed in immature virus are therefore dependent on the extent of dissociation of an unstable 60 to 70S RNA complex which in turn is dependent on the extraction process used.

It is interesting that free 35S subunits have not been extracted from murine leukemia viruses harvested at frequent intervals (7, 19), although intermediate forms have been reported (7). Based on the results presented here, we would expect that either the intersubunit linkages in their RNAs are maintained by more stable bonds than in the avian oncornaviruses or that RNA stabilization after budding occurs more rapidly.

The results presented above do not contradict the hypothesis that 35S RNA subunits are precursors to the 60 to 70S RNAs (2). Regarding this point, we have confirmed the results of other studies on the structure of avian oncornavirus RNAs (13, 18), that all 35S subunits of B77 RNA contain poly(A) sequences of approximately the same length (unpublished observations). Since poly(A) is thought to be added post-transcriptionally to the 3'-ends of mRNA (4), it suggests that 35S RNAs are synthesized separately and assembled at some time after synthesis. However, from the results presented above, we conclude that the subunits in B77 sarcoma virus are physically linked, even in immature virions. Thus, the question of when after RNA synthesis this linkage occurs is still open. Since most of the studies which have been carried out to determine the size of intracellular RNA of oncornaviruses (9, 11, 14, 25) have used phenol extraction as a method of isolation, it is not surprising, in view of the results reported here, that 35S subunits have been found as a predominant species. It is interesting that Leong et al. (14) found significant differences in the sizes of RSV-specific RNA obtained when they extracted RNA from infected cells at 60 C versus room temperature. Fan et al. (9), who in some of their studies isolated RNA by techniques which did not involve phenol extraction, found both 60 to 70S and 35S RNA in cells infected with murine leukemia virus. They concluded, however, that a majority of the 60 to 70S RNA was located at the cell surface since most of it could be selectively removed by treating the cells with trypsin or EDTA. Further studies of these kinds should help resolve the question of when and where the 35S RNA subunits are linked.

ACKNOWLEDGMENTS

This study was supported by Public Health Service grant no. CA-16262 from the National Cancer Institute.

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