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*ISOLATION OF THE NUCLEIC ACID OF MOUSE MAMMARY TUMOR VIRUS (MTV)**

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The mouse mammary tumor virus (MTV) was one of the first tumorigenic viruses found in mammals.[1, 2] Nevertheless, little progress has been made on the elucidation of the molecular structure of the virus.[3] The absence of a rapid bioassay has hindered progress in virus purification, and the difficulty in growing the virus in tissue culture has made use of radioisotopes impractical.[4] MTV is normally transmitted through the mother's milk. This establishes a lifelong infection, and virus can be recovered from tissue or milk at any time. The ultimate development of a mammary tumor in an infected female is dependent not only upon the presence of the virus but also upon genetic and hormonal factors.

Previous chemical studies have shown that MTV contains 30 per cent lipid and its nucleic acid is RNA.[5] Electron micrographs indicate[2, 6, 7] that its size and structure are similar to the other large, lipid-containing RNA viruses such as the avian tumor viruses, the mouse leukemia viruses, and the myxoviruses. The purification of virus and isolation of high-molecular-weight RNA from other RNA tumor viruses such as Rous sarcoma and Rous-associated virus (RSV + RAV),[8] avian myeloblastosis virus (AMV),[9] and the Rauscher mouse leukemia virus (MLV),[10, 11] as well as the recent development of a fast serological assay for MTV,[12] suggested that virus purification and RNA isolation could be accomplished with MTV. This report describes such a study. A high-molecular-weight single-stranded RNA similar to that obtained from other RNA tumor viruses was found and its properties were studied.

Materials and Methods.—Virus: P^{32}- or H^3-labeled virus was used in all experiments. The virus was obtained from mouse milk which is a rich source of virus.[13] Milk was collected from lactating female A/Crgl or BALB/cfC$_3$HCrgl mice approximately 1 week post partum; this milk contains infective MTV.[14] In a control study of milk from mice not infected with MTV, milk was obtained from BALB/cCrgl females. Twenty-four hr before milking, each mouse was injected with 1.5–2.5 mc carrier-free $P^{32}O_4$ or 1.5 mc H^3-orotic acid (0.23 mc/μM) in 0.2–0.4 ml Tris buffer pH 7.4 containing 0.1 *M* NaCl. The females were not permitted to suckle their young for 12 hr prior to milking. Each mouse received 0.1 ml of Pitocin (Parke-Davis Co.) intraperitoneally

10 min before milking. Milking was accomplished with a pulsating suction device;[15] the yield was approximately 0.5–1.0 ml per mouse. The collection chamber of the milking machine was kept in crushed ice during the milking procedure.

Virus purification: The virus was purified in two steps, both carried out in the cold (0–4°C). Tris buffer pH 7.4 containing 0.01 *M* Tris HCl, 0.1 *M* NaCl, and 0.001 *M* EDTA was used unless otherwise specified.

Milk was diluted with 3 vol of the standard Tris buffer and then sufficient 0.5 *M* EDTA solution pH 7.4 was added to make a final concentration of 0.03 *M* EDTA.[5] This solution was incubated at 0°C for 15 min and then centrifuged for 10 min at 10,000 rpm in a Servall centrifuge. Most of the surface lipid layer on the top of the centrifuge tube and the small pellet were discarded. Virus was then isolated from 5 to 20 ml of clarified supernatant by the following procedure:

(1) *Centrifugation to a density interface:* A sucrose solution with a density of approximately 1.32 gm per ml containing 65% (w/v) sucrose, 0.01 *M* Tris HCl pH 7.4, 0.1 *M* NaCl, and 0.001 *M* EDTA was prepared in D_2O. About 0.35 ml of this solution was placed at the bottom of a 5.4-ml tube for the Spinco SW50 rotor and overlaid with 1.5 ml of a 15% (w/v) sucrose-H_2O solution containing the same buffer. The remainder of the tube was then filled with the virus solution and it was centrifuged for 30 min at 50,000 rpm. After centrifugation a visible band was seen on the top of the sucrose-D_2O solution. This band was found to contain viral antigenicity. The tube was then punctured at the bottom and the band collected in approximately 0.3 ml. If more than one tube was used, the resulting bands were pooled, diluted with Tris buffer, and concentrated again by the same centrifugation step to a single band.

(2) *Sucrose density gradient:* The virus-containing band from the first step of purification was diluted with the standard buffer to a final volume of approximately 1.0 ml to reduce the solution density. This was layered on top of a linear sucrose and D_2O gradient[16] (65% sucrose in 100% D_2O to 16% sucrose in approximately 20% D_2O) in Tris buffer pH 7.4, and centrifuged in a Spinco SW50 rotor at 50,000 rpm for 2 hr. A band containing the viral antigen could be seen or detected by radioactivity at about the middle of the gradient. In addition, a diffuse, light-scattering band appeared between the virus band and the top of the gradient. The gradient was fractionated by puncturing the tube from the bottom and collecting 5–6 drops per fraction.

Isolation of the RNA: The appropriate fractions from the final sucrose density gradient step of the virus purification were pooled, combined with viruses containing the desired known RNA markers such as tobacco mosaic virus (TMV), RAV + RSV, and MLV; the RNA was then extracted by the phenol-SDS method as previously described.[16]

Assay of MTV antigenicity: The various gradient fractions were analyzed for the presence of MTV-associated antigenicity by the recently developed immunodiffusion assay.[12] Agar plates were prepared with 0.6% Noble agar, 0.85% NaCl, and 1:10,000 Merthiolate in distilled water, pH 7. Wells were cut in the agar with a 3-mm diameter cork borer. Each gradient fraction was diluted with the standard Tris buffer and added to the antigen wells of the agar plate on days 1, 2, and 3. Standard rabbit antisera, individual, undiluted, and unabsorbed, were added to the antiserum wells on day 2. The plates were maintained at room temperature. Precipitate lines were visible within 4–5 days, but the plates were routinely examined for a period of 2 weeks.

All fractions of several density gradient preparations were tested several times in the immunodiffusion assay. Each fraction was tested against different standard rabbit antisera, both from rabbits immunized with MTV-infected tissue extracts, and from rabbits immunized with MTV-free tissue extracts (the controls). Most of these antisera were among those described previously.[12] Such antisera produce several precipitate lines when reacted in immunodiffusion with antigen preparations of MTV-infected tissue. Most of these precipitate lines result from the interaction of specific rabbit antibodies with mouse tissue antigens. One characteristic precipitate line, however, is formed only when antiserum from rabbits immunized with MTV is used;[12] aggregations of type B virus particles have been found in this precipitate line.[17]

Assay of MTV infectivity: Fractions of two different density gradient preparations of A/Crgl milk diluted with 2 vol of Tris buffer were inoculated into 3-week- and 5-week-old female BALB/cCrgl mice. These mice are not infected with MTV, and after infection hyperplastic alveolar nodules and tumors develop in their mammary glands.[18] Each mouse received an amount of material equivalent to that obtained from either 0.1 ml or 0.01 ml of fresh milk.

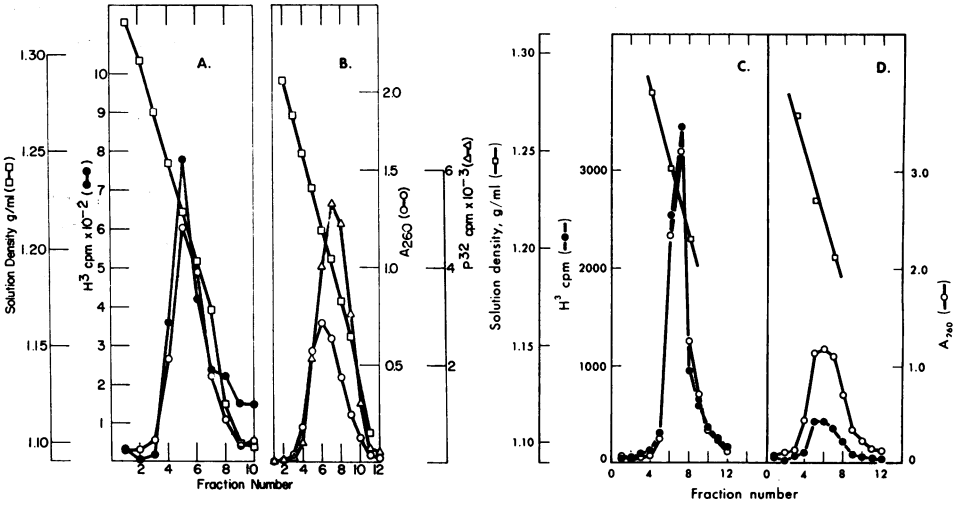

FIG. 1.—Equilibrium density (□-□-□) gradient centrifugations of purified milk-MTV (*A–C*) and a control experiment using the milk of uninfected mice (*D*). One aliquot of each fraction was counted in a Tri-Carb spectrophotometer using Bray's scintillation fluid. Another aliquot was diluted with buffer to determine the optical density, viral antigenicity, or infectivity. (*A*) shows the distribution of radioactivity and absorbancy at 260 mμ (○-○-○) after purification of 2.5 ml H^3-orotic acid-(●-●-●)-labeled milk of the A/Crgl strain of mice; (*B*) shows the result obtained in an analogous experiment using $P^{32}O_4$-(△-△-△)-labeled milk for virus purification; (*C*) and (*D*) are the distributions of radioactivity and absorbancy at 260 mμ (○-○-○) after the purification of 2.5 ml H^3-orotic acid-(●-●-●)-labeled milk of MTV-infected (*C*) and uninfected (*D*) BALB/c mice.

Results and Discussion.—Properties of purified virus: Figures 1*A*, *B*, and *C* show the results of three experiments comparing the final sucrose density gradient step of the purification of P^{32}- and H^3-orotic acid-labeled MTV. The virus in each case was isolated from about 2 to 3 ml milk obtained from one of two different strains of MTV-infected mice that had been injected with the radioactive precursors 24 hr previously (see *Methods*). An easily visible band appeared in the middle of the gradients followed by a light-scattering zone up to the density of 1.15 gm/ml. Using H^3-orotic acid as a specific RNA precursor, the absorbancy peak at 260 mμ and radioactivity peak were found to coincide (Figs. 1*A* and *C*). Viral antigenicity (see below) was found in fractions 4 and 5 (in Fig. 1*A*) in the order of intensity 5 > 4. Thus the maxima of viral antigenicity, RNA-specific radioactivity, and absorbancy at 260 mμ coincide at fraction 5 in Figure 1*A*. The observed density of approximately 1.22 gm/ml at fraction 5 (Fig. 1*A*) and at fraction 7 (Fig. 1*C*) is in agreement with the previously reported density of MTV in a RbCl density gradient.[7]

When $P^{32}O_4$ was used to label the virus, the radioactivity peak was in a position of lower density in the gradient than the optical density peak (Fig. 1*B*). This displacement of radioactivity and optical density may be accounted for by the presence of P^{32} in nonviral phospholipid material. The result of a control purification of 2.5 ml H^3-orotic acid-labeled milk of uninfected BALB/c mice, as shown in Figure 1*D*, supports this explanation. It can be seen that this milk also contains some fast-sedimenting material which appears in a relatively wide density range in the final sucrose gradient. Although it is unknown whether the milk of MTV-infected mice contains the same relative proportion of this material, its presence may account for

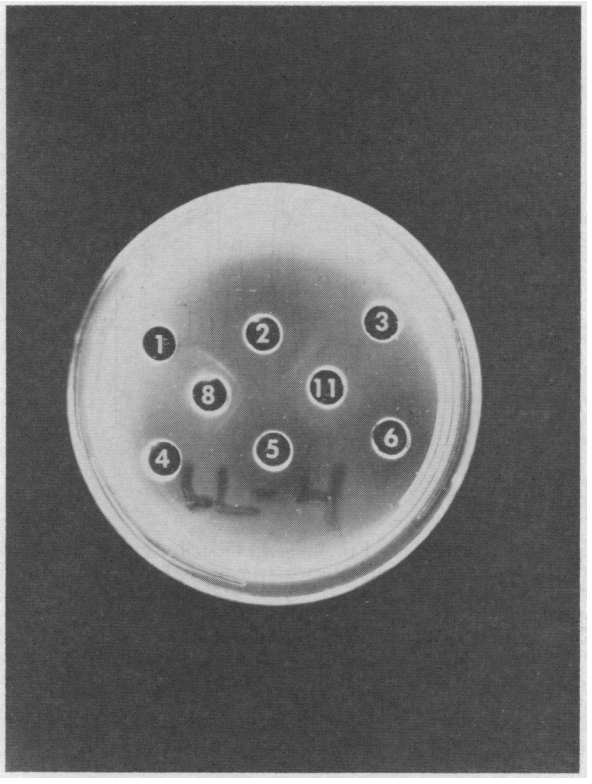

FIG. 2.—Assay for MTV antigen in two fractions of a sucrose density gradient. On days 1, 2, and 3, well 8 of the agar plate was filled with gradient fraction 8, and well 11 was filled with gradient fraction 11. On day 2, well 2 was filled with antiserum from a rabbit immunized with an MTV preparation from a C3H/Crgl mammary tumor, and well 5 was filled with antiserum from a rabbit immunized with an MTV preparation from a DBA/Crgl mammary tumor. Also at day 2, wells 4 and 6 were filled with antiserum from a rabbit immunized with an MTV-free preparation of BALB/cCrgl mammary tissue. This antiserum served as a negative control. Wells 1 and 3 were left empty. The precipitate line characteristic of MTV has appeared between well 8 and wells 2 and 5, indicating the presence of MTV antigen in fraction 8. No precipitate line has formed where fraction 8 was tested against control antiserum lacking antibodies against MTV (well 4). No MTV-specific precipitate line has been formed by fraction 11. However, another precipitate line can be observed between well 11 and all three wells containing rabbit antisera. This line represents a mouse tissue antigen.

most of the absorbancy in the virus peaks shown in Figures 1*A*, *B*, and *C*. The RNA-specific radioactivity of the UV-absorbing material in Figure 1*D* (isolated from the milk of uninfected mice) is much lower than in the comparable virus peaks, Figures 1*A*, *B*, and *C*. No MTV-antigen and no 70 $S_{20,w}$ MTV-RNA (see below) were found in the gradient (Fig. 1*D*). Therefore, the described MTV purification is incomplete but sufficient for the experiments reported in this study.

The sucrose gradient purification technique was used in the present experiments because previous studies with several related viruses have shown that infectivity is not significantly reduced by this method.[8–10, 16] Purification by RbCl density gradient, on the other hand, both affects the morphological integrity of the virus particles as indicated in electron micrographs[7] and reduces viral antigenicity,[17] although the material is still infectious.[5, 19] In contrast, the preparations obtained by sucrose density gradient showed antigenic integrity, as indicated by the forma-

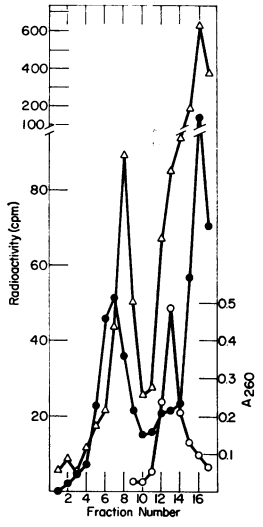

FIG. 3.—Distribution of H^3 (●-●-●), P^{32} (△-△-△), and absorbancy at 260 mμ (O-O-O) after velocity sedimentation of H^3-labeled MLV-RNA, P^{32}-labeled MTV-RNA, and carrier TMV-RNA. The RNA's dissolved in 0.15 ml buffer containing 0.01 *M* Tris HCl pH 7.4, 0.1 *M* NaCl, and 0.001 *M* EDTA, were layered on top of a 5.2-ml linear sucrose gradient 5–20% (w/v) in the same buffer and centrifuged for 65 min at 50,000 rpm in a Spinco SW50 rotor at 5°C. Fractions were diluted to determine the optical density and then counted directly in Bray's scintillation fluid.

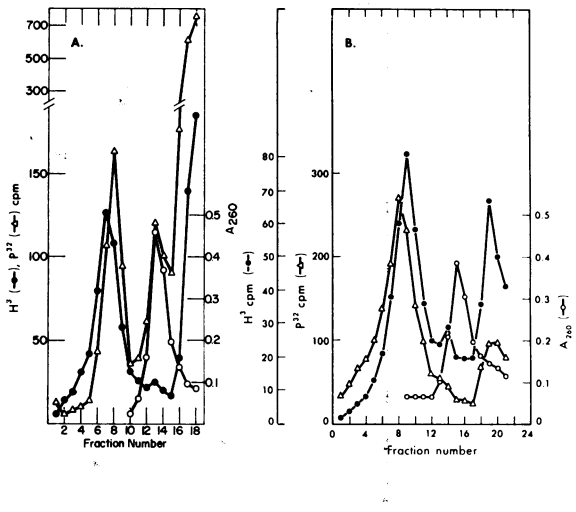

FIG. 4.—(*A*) Distribution of H^3 (●-●-●), P^{32} (△-△-△), and absorbancy at 260 mμ (O-O-O) of H^3-labeled RSV + RAV-RNA, P^{32}-labeled MTV-RNA derived from A/Crgl mice, and carrier TMV-RNA.

(*B*) Distribution of H^3 (●-●-●), P^{32} (△-△-△), and absorbancy at 260 mμ (O-O-O) of H^3-labeled MTV-RNA derived from BALB/cfC_3H mice, P^{32}-labeled RSV + RAV-RNA, and carrier TMV-RNA.

Centrifugation was for 75 min as described for Fig. 3. The RNA was precipitated with 200 μg yeast RNA by the addition of a $^1/_{10}$ vol of 50% TCA, collected on Millipore filters, and counted in toluene-based scintillation fluid.

tion of a very sharp and strong MTV precipitate line when reacted in immunodiffusion with known rabbit antisera. Infectivity studies of the sucrose density gradient purified material are in progress.

Antigenicity of purified virus: Figure 2 shows a typical immunodiffusion plate from the assay of an MTV density gradient preparation of A/Crgl milk, which was collected in 16 fractions. MTV antigen was found by immunodiffusion (see *Methods*) in fractions 8 through 10. Most of the viral antigenicity was associated with fraction 8, which had the highest absorbancy at 260 mμ and a solution density of approximately 1.22 gm/ml. The decrease in viral antigenicity paralleled the decrease in optical density in each fraction $8 > 9 > 10$. In fractions of lower density, as demonstrated by fraction 11 in Figure 2, only mouse tissue antigen was detected.

In the preparation diagramed in Figure 1*A*, the precipitate line characteristic of MTV antigen was formed by fractions 4 and 5. More viral antigen, as measured grossly by time of appearance and intensity of the precipitate line, was observed in fraction 5 than in fraction 4. Viral antigenicity was not detected in other fractions of this preparation, either because it was absent, or else in low concentration. Precipitate lines characteristic of mouse antigens were observed in fractions 5 through

10, which probably represent most of the optical density and radioactivity in fractions 6 through 10.

As a control, the density gradient fractions of milk obtained from MTV-free BALB/c mice were also examined in the immunodiffusion assay; as expected, no precipitate line characteristic of MTV was observed.

Properties of the MTV-RNA: The sucrose gradient fractions in the density range from approximately 1.24 gm/ml to 1.18 gm/ml containing the P^{32}- or H^3-labeled purified virus (as shown in Fig. 1) were pooled, and the nucleic acid was isolated as described, using TMV-RNA as carrier. The nucleic acid was then fractionated by sucrose gradient centrifugation in the presence of different RNA's of known sedimentation constants. The results shown in Figures 3 and 4 were obtained by centrifuging the RNA solutions in linear sucrose gradients containing 0.1 *M* NaCl.

It can be seen in Figures 3 and 4 that there are two major radioactively labeled components in each of the described fractionations. One sediments rapidly near the bottom of the gradient. The other sediments much more slowly and is near the top of the gradient. A minor peak or shoulder of radioactive material can be seen to sediment exactly with TMV-RNA in these experiments. All three radioactive components are rendered completely TCA-soluble by pancreatic ribonuclease in

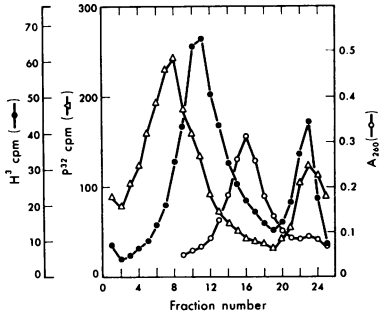

FIG. 5.—Distribution of H^3 (●-●-●), P^{32} (△-△-△), and absorbancy at 260 mμ (O-O-O) after velocity sedimentation of P^{32}-labeled RSV + RAV-RNA, H^3-labeled MTV-RNA (derived from BALB/cfC$_3$H mice), and TMV-RNA in low ionic strength. The RNA's dissolved in 0.15 ml standard buffer were layered on top of a 5.2-ml linear sucrose gradient 5–20% (w/v) containing 0.001 *M* Tris HCl pH 7.4, 0.00025 *M* EDTA, and centrifuged for 150 min at 50,000 rpm in a Spinco SW50 rotor at 5°C. Counting was as described in Fig. 4.

0.2 *M* salt,[8] indicating that they consist of single-stranded RNA. The fast-sedimenting component is considered to be intact MTV-RNA. The small peak or shoulder of radioactivity sedimenting with TMV-RNA probably represents association of labeled RNA with the carrier TMV-RNA as previously described for MLV-RNA,[10] rather than a separate component of MTV-RNA. The slowly sedimenting component is probably degraded viral RNA as previously described in the cases of RAV + RSV,[8] NDV,[16] AMV,[9] and MLV;[10] however, RNA from other sources could also be present.

Sedimentation velocity: The sedimentation properties of MTV-RNA were determined by comparison with the known RNA's of RSV + RAV and MLV. The RNA's of MTV, RSV + RAV (Figs. 4 and 5), or MLV (Fig. 3) were isolated and fractionated simultaneously in a sucrose gradient. TMV-RNA was added as carrier and as a 32 $S_{20,w}$ RNA-sedimentation marker.

The experiment shown in Figure 3 demonstrates that MTV-RNA has a lower sedimentation constant than MLV-RNA, which has a sedimentation constant 74 $S_{20,w}$ under the same conditions.[10] Figures 4*A* and *B* compare the sedimentation

velocity of MTV-RNA isolated from the viruses of two different strains of mice with that of RSV + RAV-RNA, which has a sedimentation constant of 71 $S_{20,w}$.[9] It can be seen in these experiments that both MTV-RNA's move at the same rates during centrifugation, as compared to the respective RNA markers. The MTV-RNA peak in both cases is only slightly displaced to the right of the RSV + RAV-RNA peak indicating that the sedimentation constant of intact MTV-RNA is only a little less than 71 $S_{20,w}$. Thus, a sedimentation constant of approximately 70 $S_{20,w}$ may be estimated for MTV-RNA in 0.1 M salt.

Since dependence of the sedimentation constant on ionic strength has been shown to be characteristic of single-stranded RNA,[20, 21] a sedimentation experiment with H^3-labeled MTV-RNA, P^{32}-labeled RSV + RAV-RNA, and carrier TMV-RNA in 0.001 M salt was carried out. All RNA's show (Fig. 5) a strong reduction in their sedimentation constant in low salt. MTV-RNA, however, exhibits a considerably lower sedimentation constant than RSV + RAV-RNA (27 $S_{20,w}$ under these conditions),[9] although these two RNA's have almost the same sedimentation constant in 0.1 M salt (Fig. 4). A parallel sedimentation experiment in low salt comparing MTV-RNA from a different strain of mice (A/Crgl) with RSV + RAV and TMV-RNA confirmed the results shown in Figure 5. Thus, MTV-RNA is probably single-stranded and probably different from RSV + RAV-RNA in secondary structure. But MTV-RNA's obtained from viruses of different strains of mice have proved to be of similar, maybe identical, structure.

If it is assumed that the viral RNA is single-stranded, as suggested by the susceptibility to ribonuclease and the dependence of the sedimentation constant on salt concentration, an estimate of its molecular weight can be made with the empirical relation mol wt = 1550 $(S_{20,w})^{2.1}$ determined by Spirin[22] for TMV-RNA in 0.1 M NaCl, 0.01 M EDTA. A single-stranded RNA with $S_{20,w}$ = 70 in 0.1 M NaCl would have a molecular weight of 12×10^6. This value, however, must be considered an estimate for reasons that have been discussed previously.[8–10, 16] A more detailed chemical characterization of MTV-RNA was not possible in the present study because of the extremely low labeling efficiency of the virus in mice. One ml mouse milk, from mice which had been previously labeled with 2.5 mc $P^{32}O_4$ each (see *Methods*), contained only enough intact 70 $S_{20,w}$ viral RNA for one sedimentation experiment such as those described in Figures 3–5.

It can be concluded from the present data that the RNA tumor viruses of mice, namely, MTV and MLV, have RNA's of different sedimentation constants. In this respect they are unlike the two avian tumor viruses RSV + RAV and AMV, which have RNA's of identical sedimentation behavior[9] and which are biologically very closely related. AMV is known to be a helper virus for RSV.

With the isolation of the RNA of MTV, an RNA from one representative of each of the known groups of RNA tumor viruses have been described. These are: RSV + RAV, which induces sarcomas in chickens; AMV, which induces leukemia in chickens, both belonging to the avian leukosis group of viruses; and two mouse tumor viruses—the Rauscher MLV, which is a representative of the murine leukemia virus group, and MTV. These viruses contain single-stranded RNA of uniform size, larger than the known RNA's from other groups of viruses.

Summary.—A single-stranded 70 $S_{20,w}$ RNA was isolated from purified MTV derived from two different strains of mice. The MTV-RNA is shown to be com-

parable in size and structure to the RNA of the other known tumor viruses RSV + RAV and MLV.

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