Isolation of the Mouse Mammary Tumor Virus: Chemical and Morphological Studies 1,2

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SUMMARY—The course of purification of the mammary tumor virus from milk of infective Columbia RIII strain mice by methods involving velocity gradient (zone) centrifugation in Ficoll, a chemically inert, high molecular weight, synthetic polysaccharide, is described. On a dry weight basis, an approximate 1,000-fold enrichment of virus was achieved. A fraction consisting of what appeared to be incomplete virus was separated in a zone above the main viral particle zone in the gradient tube. The viral particles had an average lipid content of 27 percent and a ribonucleic acid (RNA) content of 0.8 percent on a dry weight basis. Experiments pointed to the essential single-stranded character of the RNA, the mononucleotide composition of which, in moles percent, was: uridine monophosphate, 28.9; guanosine monophosphate, 30.2; cytidine monophosphate, 21.6; adenosine monophosphate, 19.3. The value of $3.7 \times 10^6$ daltons was determined for the average RNA content of the virions. In certain morphological features and in aspects of their maturation, largely relating to the arrangement of the nucleic acid-containing structure within the particles, they differed significantly from the myxoviruses, a group which they otherwise resemble in general morphology and gross chemical composition. Substantial tumorigenicity for the mammary gland was found to reside in viral particle preparations, purified by isopycnic gradient centrifugation in rubidium chloride and gradient electrophoresis, methods previously described—the purity of which was attested by electron microscopic examination.—J Nat Cancer Inst 35: 549–565, 1965.

THE ONCE contentious issue regarding the identification of the mouse mammary tumor virus (MTV) apparently is no longer in serious dispute. A recent review (7) presented a number of confluent lines of evidence indicating that particles with the morphological attributes of virus, which were consistently found in fixed thin sections of spontaneous mammary tumors and high-speed pellets of infective milk—the so-called B particles of Bernhard et al. 1 Received December 16, 1964.

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represented the mature form of the mammary tumor virus. The aim of the present work was to isolate the viral particles in as pure a state as possible, to examine their chemical, physical, and morphological characteristics and to determine whether such purified particles, when injected into female mice for assay, would produce mammary cancer.

Previously, particles in a satisfactory state of purity were isolated from infective milk and tumor tissues by such procedures as equilibrium density gradient centrifugation in rubidium chloride (RbCl) (3). It became apparent, however, that the morphological integrity of the particles could be maintained only with great difficulty during their handling from the density gradient zones, so that other less destructive methods of isolation were sought. In one such method, 10 to 20 ml quantities of infective milk were subjected to gradient electrophoresis in a specially constructed vessel, and viral particles, in an excellent state of preservation and free from contaminants, were recovered from the fast-migrating edge of the main particle zone (4). The present paper describes the course of purification of the MTV from infective milk by a procedure, designed to achieve a maximal yield of viral particles, involving velocity gradient centrifugation in Ficoll, a chemically inert, high molecular weight, synthetic polysaccharide. Preliminary work on the nature of the viral nucleic acid is presented, and the morphology of the viral particles, insofar as it is understood, is discussed. Bioactivity data are available on two preparations of particles purified by the RbCl density gradient and by the gradient electrophoresis methods. These are presented here.

MATERIALS AND METHODS

Isolation of crude virus from mouse milk.—Milk from the Columbia University (New York, N.Y.) colony of RIII strain mice was used. Lots of about 100 ml were spun at 0° C for 1 hour in an International centrifuge at 600 × g and the cream and sediment discarded. The skim milk was diluted with 3 volumes of TBS (0.01 M Tris buffer, pH 7.4, + 0.14 M NaCl) and 1 volume of 0.15 M Na Versenate, pH 7.4. This step resulted in some clarification of the diluted skim milk through dissociation of protein aggregates. It also improved the yield of crude virus, which otherwise would have been pellet out with milk solids in the succeeding step, entailing centrifugation at 0° C in the Servall centrifuge at 10,000 × g for 20 minutes. The resulting supernatant was centrifuged for 1 hour at 21,000 rpm in the Spinco #40 rotor at 0° C. The pellets were resuspended in TBS to give turbid suspensions from which aggregated material was removed by being spun for 10 minutes at 600 × g in an International centrifuge. The resulting suspensions in 0.5 ml portions were layered on top of preformed Ficoll density gradients.

Ficoll density gradients.—Ficoll (Pharmacia, Uppsala, Sweden) was dissolved in distilled water and passed through a column of Sephadex G-25 (coarse) gel (Pharmacia) or dialyzed against repeated changes of distilled water for 2 to 3 days in the cold. It was then lyophilized and solutions of the desired concentration were made in TBS. Usually 1 ml of 40 percent w/v Ficoll solution was placed at the bottom of 5 ml ultracentrifuge tubes and carefully overlaid with 0.5 ml of 35 percent solution, followed by successive 0.5 ml portions of 30, 25, 20, 15, and 10 percent. After standing for 4 days in the cold room at 4° C, by which time the boundaries between the layers had disappeared (as determined by inspection with the schlieren system of an electrophoresis apparatus), the crude virus suspensions were layered on top, and the whole was centrifuged in the SW39 rotor for 1 hour at 39,000 rpm. After centrifugation, light-scattering zones were removed from the tube, diluted tenfold with TBS, and centrifuged for 1 hour at 21,000 rpm in rotor #40. The pellets were resuspended in TBS, cleared of aggregated material by a low-speed spin, and then recentrifuged at 21,000 rpm. This cycle of ultracentrifugation was repeated once more. Portions were taken for electron microscopic examination, dry weight, lipid, and nucleic acid estimations. Density was measured on 20 to 30 μl droplets in calibrated bromobenzene-kerosene density columns (5).

Electron microscopy.—The RCA model EMU 3F electron microscope was used and the preparation of specimens was as previously described (4).

Dry weights.—Portions of 0.2 ml were pipetted onto light aluminum preweighed foil discs and dried in the vacuum desiccator over P₂O₅ in the
cold room for 2 days. The vacuum desiccator with samples was then placed in an oven at 90°C for 1 to 2 hours. Samples were weighed in a Sartorius microbalance to the nearest 1/100 mg. Dry weights were corrected for salt content.

Lipid content of particles.—Samples, dried and weighed on aluminum foil discs, were refluxed for about 10 minutes with chloroform-methanol, 2:1. The extracts were cooled, filtered, and transferred to tubes where the solvent mixture was evaporated, at about 50°C, with a stream of nitrogen. The residue was re-extracted at room temperature with light petroleum ether. The petroleum ether extract was carefully placed on a preweighed aluminum foil disc, dried, and weighed. Over 90 percent of the initial chloroform-methanol extract was extractable with petroleum ether.

Nucleic acid extraction.—Previous work (6) had indicated that the virus contained ribonucleic acid (RNA). Suspensions were treated for 30 minutes with 10 μg ribonuclease (Worthington Biochemical Corp., Freehold, N.J., crystalline enzymes) at 25°C, after which the particles were sedimented and resuspended in 1.0 ml volumes of TBS. One-tenth ml of a 4 percent Bentonite suspension (7) was added, followed by 0.12 ml of a 10 percent sodium dodecyl sulfate solution (SDS). The SDS had been twice recrystallized from hot 95 percent ethanol. A significant clarification of the opalescent suspensions occurred on addition of the detergent. After 1 minute an equal volume of redistilled phenol (Mallinckrodt Chemical Works, St. Louis, Mo., AR) saturated with aqueous 1 percent SDS was added, and the whole shaken by hand for 10 minutes at 25°C. Following centrifugation for 10 minutes at 10,000 × g, the (upper) aqueous phase was removed and the interface and lower phenol phase re-extracted with Tris buffer. After centrifugation, the aqueous extract was added to the original aqueous phase and the whole shaken with one half volume of water-saturated phenol. The aqueous phase was again recovered and cleared of dissolved phenol when shaken with peroxide-free ether 4 to 6 times. The ether was removed by nitrogen bubbled through the solution at 0°C for 5 minutes. The solution was made 2 percent with respect to sodium acetate. Cold absolute ethanol, 2½ volumes, was added and the mixture kept for about 2 days at -10°C. The nucleic acid precipitate was recovered by centrifugation at 600 × g for 20 minutes. RNA was estimated from the value of its optical density at 260 mμ. A purified yeast RNA (8) was used as a standard. The phosphorus content was 8.3 percent and the optical density at 260 mμ of a solution containing 50 μg per ml at pH 7.0, with a 1 cm light path, was 1.20.

Mononucleotide composition.—The RNA, after dialysis against distilled water, was hydrolyzed in 0.3 N KOH for 18 hours at 37°C. The resulting mononucleotide mixture was separated on a Dowex 50-H⁺ column, according to the method of Katz and Comb (9). A 2.0 × 0.9 cm column was used.

The ratio of the viral RNA was compared with cellular RNA derived from the normal mammary tissue of RIII females. The tissue was homogenized at room temperature in the presence of 0.5 percent SDS in 0.01 M sodium acetate at pH 5.2 and buffer-saturated phenol. A VirTis “45” homogenizer, running at maximum speed for two 10- to 15-second bursts, was employed. The RNA was isolated and purified according to the method of Gierer and Schramm (10). A 5.0 × 0.9 cm Dowex 50-H⁺ column was used to separate the hydrolyzed mononucleotides.

Thermal denaturation of viral RNA.—A cuvette holder with a thermostated heat-exchanger accessory was attached to a Beckman DU spectrophotometer. The cuvette containing the analytical solution was kept vapor-tight, as was an adjacent cuvette containing water, in which a thermocouple, calibrated over the range 0 to 100°C, was placed. Changes in absorption readings at 260 mμ were recorded over the temperature range 24 to 94°C.

Bioassays.—Virus particles isolated both from milk and tumor were assayed in young (15–25 days of age) C57BL mice by intraperitoneal inoculation of 0.2 ml samples of virus suspension in phosphate-buffered saline (PBS). The colony of C57BL mice used has been maintained by Dr. Cushman Haagensen at Columbia University since he obtained them from E. C. MacDowell approximately 30 years ago. Although the response to MTV inoculations has never been greater than a 60 percent tumor incidence, these mice, nevertheless, have the advantage of a zero spontaneous tumor incidence. Furthermore, they have failed to develop tumors after hormonal stimulation.
or force-breeding. By all the criteria known, they are not carriers of a latent MTV, but they do respond to injections of the virus. For a complete bioassay, more than 2 years must elapse from the date of inoculation to the final assessment of the tumor potency of the inoculum on the death of the last of the mice.

RESULTS

Isolation of Virus From Mouse Milk

The whole-milk samples contained an average of 140 mg of dried solids per ml and the skim milk 100 mg per ml.

It was anticipated that the preformed Ficoll density gradient would be distorted during centrifugation, since the larger molecular species in the medium tended to sediment. Text-figure 1 shows the degree of distortion observed under the conditions used for particle separation. Text-figure 2 is a diagrammatic representation of the density gradient tube with 5 separated milk-particle fractions numbered 1 to 5 from the top of the gradient tube. The particles in the separated fractions were isolated; a morphological description is given in table 1.

Electron micrographs of 4 fractions are presented in figures 1, 2, 3, and 4.

The quantitative distribution of the fractions in terms of dry weight, from an average starting volume of 100 ml milk (14.0 g dry weight), is given in table 2. The lipid and the RNA content, in percentage of dry weight, are also given.

Since 14.4 mg of purified virus particles was obtained in fraction 4 from an original starting weight of 14.0 g, an enrichment by a factor of 972 had been achieved. The lipid content of the fractions decreased from 40 to 25 percent on descending the gradient; the lipid content of the virus (27%) was of the same order as that previously reported (11). In the same report an average RNA content of 1.2 percent was recorded for defatted virus, as analyzed by the method of Schmidt and Thannhauser (12). The different procedure used in the

![Text-Figure 1. Ficoll density gradient before (broken line) and after 1 hour centrifugation at 39,000 rpm in SW39 rotor.](https://academic.oup.com/jnci/article-abstract/35/3/549/931485)

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Distance from bottom of tube (mm)</th>
<th>Average density (g/ml)</th>
<th>Morphological identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>28-34</td>
<td>0.90</td>
<td>Vesicles and nonviral membranous structures ranging in size from 20 to 500 m.(\mu). The relatively low density of the fraction may be due to its high lipid content</td>
</tr>
<tr>
<td>2</td>
<td>33-30</td>
<td>1.00</td>
<td>Viral membrane fragments having characteristic spines; terminal bulbs of viral tail-like extension, 20 to 40 m.(\mu) in diameter</td>
</tr>
<tr>
<td>3</td>
<td>22-18</td>
<td>1.05</td>
<td>Immature or incomplete viral particles, with heads 60 to 80 m.(\mu) in diameter and tails 150 to 250 m.(\mu) long; viral membranous fragments</td>
</tr>
<tr>
<td>4</td>
<td>12-7</td>
<td>1.14</td>
<td>Main virus zone. Characteristic particles having head of diameter 75 to 90 m.(\mu) and flexible tail-like extension with terminal bulb apparent in some particles; some damaged and fragmented particles also present</td>
</tr>
<tr>
<td>5</td>
<td>7-4</td>
<td>1.16</td>
<td>Aggregated viral particles, similar to those in fraction 4</td>
</tr>
</tbody>
</table>
TABLE 2.—Total dry weight, lipid, and RNA content of various fractions from Ficoll gradient ultracentrifuge tubes illustrated in text-figure 2*

<table>
<thead>
<tr>
<th>Fraction number</th>
<th>Average density</th>
<th>Total dry weight of fraction (mg)</th>
<th>Lipid (% dry wt)</th>
<th>RNA (% dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (Start of gradient)</td>
<td>0.90</td>
<td>4.8 ± 1.1</td>
<td>40 ± 3</td>
<td>Not detected</td>
</tr>
<tr>
<td>2</td>
<td>1.00</td>
<td>7.1 ± 0.9</td>
<td>33 ± 3</td>
<td>Not detected</td>
</tr>
<tr>
<td>3</td>
<td>1.05</td>
<td>3.4 ± 0.9</td>
<td>30 ± 2</td>
<td>0.1</td>
</tr>
<tr>
<td>4</td>
<td>1.14</td>
<td>14.4 ± 1.2</td>
<td>27 ± 2</td>
<td>0.8</td>
</tr>
<tr>
<td>5</td>
<td>1.16</td>
<td>3.8 ± 1.4</td>
<td>25 ± 2</td>
<td>0.8</td>
</tr>
</tbody>
</table>

*Average of 4 experiments; 100 ml of milk (mean dry weight = 14.0 g) used for each experiment. The stated volumes of milk are after a three- to fivefold dilution resulting from the nipples being continuously moistened with saline.

present experiments resulted in a value of 0.8 percent.

Viral Ribonucleic Acid

The viral RNA preparation had an absorption maximum at 260 m\(\mu\) and a minimum at 234 m\(\mu\). The ratios \(\text{OD}_{260}/\text{OD}_{234} = 1.4\) and \(\text{OD}_{260}/\text{OD}_{280} = 1.78\) were found for solutions in PBS at pH 7.0. Although it seemed likely that the RNA preparations had not been completely deproteinized, a more rigorous purification of the nucleic acid was not attempted in the present experiments. An increase in optical density at 260 m\(\mu\) of 38 percent was found on hydrolysis in 0.3 N KOH for 18 hours at 37° C. After ribonuclease hydrolysis a total increase of approximately 30 percent in optical density at 260 m\(\mu\) was observed.

Text-figure 3 shows the curves for the thermal denaturation and renaturation of the nucleic acid. Hyperchromicity gradually increased to a total of over 18 percent with increase in temperature. The effect was almost completely reversible and was indicative of the melting out or destruction and reformation of the helical regions of a single-stranded RNA.

The dry weight density of the virus was estimated by the pycnometric method (73). A value of 1.19 was obtained. The average diameter of the virus particles from fixed thin section was 100 \(\mu\). The volume of the virus was calculated and multiplied by the dry weight density and Avogadro's number to give the mass of the virus in molecular weight units (daltons). A value of \(3.7 \times 10^8\) was obtained. Since approximately 1.0 percent of this represented RNA, the average RNA content of the virus was \(3.7 \times 10^6\) daltons.

Mononucleotide Composition of Viral Ribonucleic Acid

The mononucleotide composition of the viral RNA and of RNA from samples of normal mam-
mary tissue of the same mouse strain from which the virus was derived is presented in table 3. Differences were observed, especially in the concentration of uridine monophosphate (UMP), which was present at a much higher level in the viral RNA.

The quanine (G) + cytosine (C) content of the viral RNA was 51.8 percent and the ratio of purines to pyrimidines 0.98. Text-figure 4 depicts the separation and relative concentration of the mononucleotides from the viral RNA.

### Table 3.—Comparison of mononucleotide composition of ribonucleic acid of mouse mammary tumor virus and ribonucleic acid of normal mammary tissue (moles/100 moles)

<table>
<thead>
<tr>
<th>Experiment</th>
<th>UMP</th>
<th>GMP</th>
<th>CMP</th>
<th>AMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30.8</td>
<td>31.2</td>
<td>20.0</td>
<td>18.0</td>
</tr>
<tr>
<td>2</td>
<td>28.0</td>
<td>31.4</td>
<td>21.7</td>
<td>18.9</td>
</tr>
<tr>
<td>3</td>
<td>27.9</td>
<td>28.0</td>
<td>23.1</td>
<td>21.0</td>
</tr>
<tr>
<td>Average</td>
<td>28.9</td>
<td>30.2</td>
<td>21.6</td>
<td>19.3</td>
</tr>
</tbody>
</table>

Mammary tissue, average of 3 determinations

<table>
<thead>
<tr>
<th>Nucleotide*</th>
</tr>
</thead>
<tbody>
<tr>
<td>UMP</td>
</tr>
<tr>
<td>21.0</td>
</tr>
</tbody>
</table>

*UMP: uridine monophosphate; GMP: guanosine monophosphate; CMP: cytidine monophosphate; AMP: adenosine monophosphate.

Concurrently with this, an internal rearrangement occurs, from which apparently the nucleic acid-containing structure, arranged as a shell, condenses to form a compact particle, the nucleoid, that seems to be surrounded by a membrane. (This “inner membrane” is frequently not apparent in negatively stained particles.) The whole is enclosed by the outer envelope. A possible consequence of this rearrangement on the bioactivity of the particles is discussed later. The inset on figure 5 shows an array of particles joined by bridges formed by the outer envelope, such as is observed with certain filamentous forms of myxoviruses (14, 15) and with murine leukemia viruses (16, 17).

B particles are observed in negative contrast in figure 6. The spine-covered envelope of the particles slightly resembles that of influenza virions. Interestingly, two envelope-associated activities of the latter virus, hemagglutinating and neuraminidase activities, have not been observed with the B particles (Lyons and Moore, unpublished results). As was first noted by Parsons (18), the spines are hollow and perhaps composite structures; the phosphotungstate (PTA) can be shown to penetrate them, revealing hollows of about 15 Å in diameter (figure 6c, arrow).

Usually the spine-clad envelope obscures details of the nucleoid, the outline of which can be observed in particles which have been allowed to swell in hypotonic media (fig. 6e). A particle was observed, however, (fig. 6a, arrow) which was partially denuded of spines; this revealed a spherical nu-
Isolation of Mouse Mammary Tumor Virus

Isolated, 85 mμ in diameter, which appeared to possess a coiled helical component of diameter 50 A, approximately (arrow). A spontaneously disrupted viral particle is shown (fig. 6d) in which filaments (arrow) between 50 to 70 A in diameter—it was difficult to determine this measurement precisely—appeared to have emerged from the nucleoid in the process of unwinding. Possibly the filaments shown in figures 6a and 6d represent the viral capsid. Among the group of particles shown in figure 6b is one into which the PTA has penetrated to outline a spherical nucleoid with a small depression at its center filled with PTA.

Tumorigenic Potency of Purified Viral Particles

RII mammary tumor tissue was extracted and fractionated on RbCl as previously described (3). The B particle band in a volume of 0.8 ml was removed from the ultracentrifuge tube, dialyzed against TBS to remove the RbCl, and inoculated at 2 different dilutions, 10⁻¹ and 10⁻⁴, into groups of 22 and 26 mice, respectively. At the 10⁻¹ dilution, the first mammary tumor appeared at 12 months, at which time 18 mice had survived. Of these, 6 or 33 percent subsequently developed tumors. At the higher dilution, 2 tumors developed in 23 mice (9% incidence). A portion of the suspension used in the inoculum was treated with 1 percent buffered formalin and examined by the negative contrast method in the electron microscope (fig. 7). It consisted almost entirely of viral particles. Fractions taken from above and below the B particle band in the ultracentrifuge tube were without bioactivity.

In a second similar experiment where mammary tumor tissue from DBA/212 mice was fractionated by a similar procedure, three fractions from the ultracentrifuge tube after centrifugation to equilibrium in RbCl were assayed. Two light scattering fractions from above the band containing the B particles as well as the B particle band were assayed. The top band caused no tumors in 16 mice and the second band gave none in 22 mice, whereas the B particle band gave 3 tumors in 20 mice.

In another experiment, viral particles were separated from RIII milk in an electrophoresis system employing a sucrose gradient (4). Of 25 mice inoculated with the undiluted fraction, 18 had survived to 12 months when the first tumor appeared. Six mice developed tumors, and 5 still survive. An electron micrograph has been published [plate 161 in (4)] which certified to the high state of purity of the viral particles used in the inocula.

Discussion

Recently, increasing use has been found for macromolecular substances as dense media in the density gradient separation of cell particulates, whole cells, and viruses (19–22). With such high-molecular-weight materials as the polysucrose derivative Ficoll, glycogen, polyglucose, or albumen, low osmotic activities are obtained throughout the gradients; also, cation-binding artifacts are eliminated. These considerations were why we used the commercially available and readily purified Ficoll to separate viral particles known to be notably susceptible to damage from osmotic shock.

Much nonviral material and viral membrane fragments were separated from the viral particles in the gradient. Also separated was a population of particles generally smaller than the virus (see table 1), lacking or deficient in nucleic acid, and reminiscent of the incomplete forms in myxovirus systems. It is possible that incomplete MTV or degraded MTV membranes account for the phenomenon of autointerference (23) which is apparently operative when concentrated inocula of infective milk and tumor extracts are used in titration of the MTV (24). Also, incomplete or degraded MTV could conceivably meet the size requirements of the inhibitor deduced from filtration and diffusion studies (25).

The small quantities of nucleic acid encountered in the present experiments precluded a more complete physicochemical characterization. The mean content per particle of 3.7 X 10⁶ molecular weight units can be compared with the value of 9.8 X 10⁶ obtained for the chicken myeloblastosis virus by Bonar and Beard (26). It is not known whether the nucleic acid exists in one or more strands in the particle. The thermal denaturation profile was indicative of single-stranded RNA with a series of...
helical regions. The mean temperature of thermal unfolding (53°C) and the G + C content (51.8%) conform closely to the straight-line relationship of these two factors, established by Spirin (27) for a variety of RNA species. The base analyses did not allow the detection of minor components or an assessment of the degree of alkylation of any base, either of which factors could be an important determinant of infectivity in the macromolecule. It remains to be shown that the infectivity is ribonuclease-sensitive, as has been found for the viral genetic material isolated from mouse leukemic cells (28, 29), from Rous sarcoma cells (30), and from chicken leukemic tissue infected with erythroblastosis virus (31).

It would appear that the phenol-SDS extraction procedure permitted the recovery of the bulk of the RNA of the particles (see page 552). There was the possibility that low-molecular-weight oligonucleotides in the viral RNA preparation were not coprecipitated with the high-molecular-weight material in the alcohol-salt medium. This circumstance, however, would be expected to affect materially the base ratios only if the bases were nonrandomly distributed to a marked degree.

The arrangement of the nucleic acid within the particle probably undergoes a change with time. The newly formed, enveloped A particle or immature B particle has its nucleic acid arranged in the form of a shell, as judged by the differential degree of staining with uranyl or indium salts. As the particle leaves the microenvironment of the cell membrane with its special conditions of osmotic pressure and pH (32) and comes under the influence of the bulk environment in the intercellular space, it increases in volume and its nucleic acid-containing structure condenses. This rearranged particle is the mature B particle. Since this final stage of viral maturation is effected outside the cell, conceivably, the virus is simultaneously partially inactivated in that the nucleic acid in the condensed state is, to a degree, now unfavorably disposed, on entering a new target cell, to unwind and thus to facilitate its translation and replication.

Efforts to detect the viral capsid in the present experiments by ether disruption and detergent treatment of particles were generally unsuccessful. Of interest were the apparently helical filaments of between 50 to 70 Å in diameter issuing from what appeared to be the unraveling core of a spontaneously disrupting particle (fig. 6d). It is tempting to consider such filaments as representing viral capsid which in the intact particle are coiled, as represented in figure 6a. On the other hand, it is known that dehydration of lipoprotein systems can lead to the separation of the lipid phase which may then assume a helical configuration (33).

The infective mouse milk used in the present experiments was obtained from breeding females of the Columbia RIII strain which have had a spontaneous mammary tumor incidence approaching 90 percent over the past 2 years. No evidence was found during this period for the presence of a competing milk-transmissible leukemia virus such as has been observed in other colonies of RIII mice (F. Squartini, personal communication). It was apparent that any potential leukemia viral genetic material in the Columbia strain was being effectively repressed. The fractions separated on the Ficoll gradients are currently being bioassayed. The bioassay data presented here show that substantial tumorigenic potency for the mammary gland resided in (RIII) viral particle suspensions of a high degree of purity, as attested by electron microscopic examination.

It is concluded that the medium-sized, lipid-containing RNA virions, produced in a manner similar to myxoviruses and derived from the cells of the mammary gland, are causal agents of mammary cancer in mice.

REFERENCES


(5) Hvidt, A., Johansen, G., Linderstål-Lang, K., and Vaslow, F.: Exchange of deuterons and 18O be-


Fractions separated in Ficoll density gradient (text-fig. 2 and table 1) observed by the method of negative contrast with diluted suspensions in 2 percent neutral sodium phosphotungstate (PTA).

**Figure 1.**—Membranous structures and vesicles of cellular origin comprise the major part of this fraction. \( \times 57,500 \)

**Figure 2.**—Viral membranous fragments with characteristic spines (arrow) and nonviral structures (lacking spines). \( \times 112,000 \)
Figure 3.—Viral particles deficient in nucleic acid, as determined chemically (table 2). Head portion of particles (arrow) is much less spherical in outline and smaller in size than in the complete viral particles separated in fraction #4. \( \times 112,000 \)

Figure 4.—Main viral particle zone: A viral particle (v, arrow); a viral fragment (f, arrow). Typical and representative area of the field is shown in low magnification to indicate the general uniformity of the viral particle population and the slight degree of contamination with fragments. Damaged viral particles (d) appear flat with little buildup of PTA around them. \( \times 38,500 \)

Figure 5.—Section of the periphery of a mammary tumor cell showing a plasma membrane (right and below) and the acinar space next to it containing viral particles. Viral (B) particle about to escape from cell can be seen at p. Immature particles at q are contrasted with mature particles at r; the diameter of the former is about nine tenths that of the latter. The inner electron-dense shell of the immature particles has condensed in the mature particles to form the nucleoid which is surrounded by a thin inner membrane. \( \times 110,000 \).

Inset, from the same specimen, a group of 5 particles is visible which appear joined by bridges (arrow) formed by the outer envelopes of the particles. \( \times 100,000 \)

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Figure 6a.—Viral particles derived from an aqueous suspension in PTA. Particle at right has been partially denuded of spines and reveals a nucleoid, 85 mμ in diameter, in which what is suggestive of a coiled helical filament, approximately 50 Å in diameter, can be observed (arrow). × 200,000

Figure 6b.—Group of viral particles and viral fragments (?) from isotonic saline suspension. The PTA has penetrated one particle to delineate the internal nucleoid (arrow). × 160,000

Figure 6c.—Micrograph of particles (in PTA) in which the characteristic spines covering the particles have hollow interiors as judged from the penetration of the PTA (arrow). × 200,000

Figure 6d.—Micrograph of a spontaneously disrupting viral particle (in PTA) out of which parallel filaments (arrow) have emerged. These have a light and dark periodicity suggestive of a helix and may represent viral capsid, the nucleic acid-containing structure of which the nucleoid is composed. × 220,000

Figure 6e.—A viral particle (in PTA) from aqueous suspension in which the nucleoid is outlined (arrow). × 160,000
Fig. 7.—Viral particles (in PTA) from RIII tumor separated from a RbCl density gradient tube (see page 555). Particles were fixed in 1 percent phosphate-buffered formalin, pH 7.0. A number of pleomorphic forms are present. In contrast to the unfixed preparation (cf. fig. 6) the particles here do not possess the tail-like extension. One of the artifacts introduced by the fixative appears to be an alteration in the particle envelope. This alteration now allows the phosphotungstate to penetrate, as reflected by the dark shade of the interior of the particles which is in contrast to the light shade seen in the unfixed particles. The spines on the outside envelope make interpretation of the internal structure difficult (arrows). A small bulbous swelling (i) on some of the particles may represent the area from which the particle envelope became detached from the plasma membrane and from which the tail-like extensions arise in the unfixed particles. X 100,000