Title
BIOPHYSICAL STUDIES OF MAMMARY TUMOR VIRUS: PURIFICATION AND CHARACTERIZATION

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BIOPHYSICAL STUDIES OF MAMMARY TUMOR VIRUS: PURIFICATION AND CHARACTERIZATION

JaRue Stanley Manning
(Ph. D. Thesis)

October 1969

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LAWRENCE RADIATION LABORATORY
UNIVERSITY of CALIFORNIA BERKELEY
TABLE OF CONTENTS

Acknowledgments iii
Abstract 1
List of Abbreviations 3
Chapter
  I. Introduction and Proposal 5
  II. Bioactivity of Milk-borne MTV: Infectivity Titration and Stability to Physico-chemical Treatment 7
  III. Isopycnic-zonal Centrifugation and Characterization of MTV 30
  IV. Rate- and Isopycnic-zonal Purification of MTV: Further Characterization of Virus 54
  V. Recapitulation and Speculation on Future Work 101
  VI. Bibliography 105
  VII. Appendix 116
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BIOPHYSICAL STUDIES OF MAMMARY TUMOR VIRUS:
PURIFICATION AND CHARACTERIZATION

Abstract
JaRue S. Manning

Milk samples from primaparous and multiparous BALB/cfC3H/Crgl mice were examined for viral infectivity using the hyperplastic alveolar nodule bioassay. Both whole- and skim-milk preparations contained high titers of infectious MTV \((10^{-6} \text{ to } 10^{-9} \text{ infectious end-point titer per 0.1 milliliter})\). Milk-borne MTV remained infective following treatment with 0.08 M EDTA or at pH 3 for thirty minutes but was inactivated by exposure to chloroform. MTV skim-milk bioactivity was thermostable at 50°C for thirty minutes and at 37°C or 25°C for twenty-four hours.

MTV milk-samples were isopycnically banded in continuous density gradients of different composition. Analysis of gradient fractions indicated that MTV buoyant density was similar to that reported for other oncogenic RNA viruses. Electron microscopic evidence showed that the light scattering band at 1.170 gm/ml in sucrose density gradients contained complete MTV particles of 105 nm diameter. A second band in the same gradient at 1.163 gm/ml contained pleiomorphic particles suggested to be incomplete MTV.

MTV was purified from milk samples by sequential rate- and isopycnic-zonal centrifugation. Purity of the MTV preparation was based on
analyses of gradient fractions with respect to (a) viral and non-viral antigenicity, (b) U.V. absorbance, (c) $^3$H-RNA radioactivity and (d) particle morphology. Based on all these criteria, the combined centrifugation procedure resulted in a higher degree of viral purity than previously reported. MTV buoyant density in sucrose gradients was 1.17 gm/ml and the sedimentation coefficient $S_{20,w}$ was estimated to be 800 to 900s. Electron micrographs of purified MTV demonstrated both the homogeneity and the isotropy of the preparation. Other morphological studies suggested the outer viral envelope to be composed of discrete subunits. The viral nucleoid appeared as a poorly resolved tangle of filaments and no detailed information regarding its structure was obtained.
# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Å</td>
<td>angstrom unit</td>
</tr>
<tr>
<td>AMV</td>
<td>avian myeloblastosis virus</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>g</td>
<td>times gravity</td>
</tr>
<tr>
<td>GLV</td>
<td>gross leukemia virus</td>
</tr>
<tr>
<td>gm</td>
<td>gram</td>
</tr>
<tr>
<td>H</td>
<td>tritium</td>
</tr>
<tr>
<td>HAN</td>
<td>hyperplastic alveolar nodule</td>
</tr>
<tr>
<td>i.p.</td>
<td>interperitoneal</td>
</tr>
<tr>
<td>mC</td>
<td>millicurie</td>
</tr>
<tr>
<td>mgm</td>
<td>milligram</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
</tr>
<tr>
<td>μm</td>
<td>millimicron</td>
</tr>
<tr>
<td>MTV</td>
<td>mammary tumor virus</td>
</tr>
<tr>
<td>MTV-B</td>
<td>mammary tumor virus B-particle antigen(s)</td>
</tr>
<tr>
<td>MTV-s1</td>
<td>mammary tumor virus &quot;soluble&quot; antigen 1</td>
</tr>
<tr>
<td>NDV</td>
<td>Newcastle disease virus</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PFU</td>
<td>plaque forming units</td>
</tr>
<tr>
<td>PTA</td>
<td>phosphotungstic acid</td>
</tr>
<tr>
<td>RPM</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RLV</td>
<td>Rauscher leukemia virus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RSV</td>
<td>Rous sarcoma virus</td>
</tr>
<tr>
<td>s</td>
<td>Svedberg (10^{-13} sec)</td>
</tr>
<tr>
<td>S_{20,w}</td>
<td>sedimentation coefficient corrected to water at 20°C</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TES</td>
<td>tris-buffered EDTA-saline</td>
</tr>
<tr>
<td>U.V.</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>VSV</td>
<td>vesicular stomatitis virus</td>
</tr>
<tr>
<td>W/v</td>
<td>weight per volume</td>
</tr>
<tr>
<td>\lambda</td>
<td>1 microliter</td>
</tr>
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</table>
CHAPTER I
Introduction and Proposal

The development of mammary tumors in mice has been related to a milk-factor transmitted from the mother to the progeny (Bittner, 1936). Considerable evidence in the literature indicates that the milk-factor is a virus (Andrews, 1939; Dmochowski, 1953; Moore, 1963; Blair, 1968) commonly referred to as mammary tumor virus (MTV). The viral etiology of mouse mammary cancer is generally accepted and much is known about the genetics and endocrinology of the infection and tumor development (Dmochowski, 1953; Bern and Nandi, 1961; DeOme and Nandi, 1966; Blair, 1968). In contrast, with the exception of recent information on MTV nucleic acid (Duesberg and Blair, 1966; Duesberg and Cardiff, 1968), relatively little is known about the biophysical properties of the virus.

Due to the comprehensive reviews of the MTV literature by Dmochowski (1953) and Blair (1968) and discussion of the salient literature in the introduction to each of the subsequent chapters, a separate literature review is not included in this thesis.

A careful review of the literature revealed that (a) the concentration and biostability of infectious milk-borne MTV had not been established; (b) the buoyant densities reported for MTV did not agree with values for other, similar oncogenic viruses; (c) a purification procedure had not been reported which permitted a precise biophysical and/or immunological characterization of milk-borne MTV. Based on these findings,
the following series of investigations on milk-borne MTV was initiated:
(a) infectivity-titration and biostability studies of MTV in mouse milk
(Chapter II); (b) characterization of MTV banded isopycnically in several
gradient solutions (Chapter III); and (c) sequential rate- and isopycnic-
zonal centrifugation of MTV and further characterization of the purified
virus (Chapter IV).
CHAPTER II

Bioactivity of Milk-borne MTV:

Infectivity Titration and Stability to Physico-chemical Treatment

A. Introduction

B. Materials and Methods

1. Mouse milking apparatus
2. Virus assay
3. Test animals
4. Source of viruses: collection and storage
5. Preparation of MTV skim-milk
6. Infectivity-titration of whole- and skim-milk samples
7. Treatment of MTV skim-milk with EDTA
8. Exposure of MTV skim-milk to pH 3
9. Exposure of MTV skim-milk to chloroform
10. Exposure of MTV skim-milk to 50°C, 37°C and room temperature
11. Exposure of VSV to pH 3 and 50°C: effect of presence of MTV-free skim-milk

C. Results and Discussion

1. Titration of milk-borne MTV
2. Effect of EDTA on infectivity
3. Influence of pH 3 and chloroform on infectivity
4. Thermostability of MTV

D. Summary
CHAPTER II
Bioactivity of Milk-borne MTV:
Infectivity Titration and Stability to Physico-chemical Treatment

A. Introduction

Lactating mammary-gland extracts and milk from MTV-infected mice have been used in numerous biological and physical investigations of MTV (see reviews: Dmochowski, 1953; Blair, 1968). However, studies of only limited scope have been reported on the titration and stability of the MTV-infectivity from these same sources (Andervont and Bryan, 1944; Bittner, 1945; Graff et al., 1948, 1949, 1952; Huseby et al., 1950; Lasfargues et al., 1958). Past work on the tumor-inducing potential of virus-infected lactating-gland extracts has been restricted to a narrow range of dilutions, with the puzzling result that in some instances tumor incidence increased with higher extract dilution (Bittner, 1945). Since the highest dilution tested ($10^{-3.5}$) was found to be tumorigenic, no estimate of the infectivity titer was possible. Huseby et al., (1950) reported continuously decreasing tumor incidence when high dilutions of cell-free lactating-gland extracts were injected into test mice. However, the number of dilutions tested was insufficient to confirm the earlier finding of Bittner (1945) or to establish the concentration of infective virus. Lasfargues et al., (1958) reported that milk from virus-infected mice was tumorigenic at a dilution as high as $10^{-12}$. 
Very little information is available regarding the stability of MTV-bioactivity to various treatment. Andervont and Bryan (1944) reported that the infectivity of lactating mammary-gland extracts was thermodabile; no infectivity was found following heating at 61°C for thirty minutes. Lasfargues et al., (1958) reported that milk-borne MTV samples were inactivated upon incubation at 37°C for four days.

No studies have been reported on the stability of milk-borne MTV-bioactivity to treatment with acid or organic solvents. (Lyons and Moore, 1962, noted that MTV-particles, obtained from a milk preparation, were disrupted by ether, but no information on viral infectivity was included.) Barnum et al., (1944) reported that mammary tumor extracts were inactivated during exposure to pH 4.5 for two hours but that other tumor extracts were not inactivated following extraction with acetone or petroleum ether.

Previous MTV characterization studies have shown the desirability of removing casein aggregates from MTV-infected milk samples (Graff et al., 1948, 1949, 1952). These authors demonstrated that treatment of milk with chymotrypsin or with citrate did not apparently alter the viral infectivity. Moore et al., (1962) reported on the use of the chelating agent, Versene, to disrupt casein aggregates, however the effect of this agent on MTV-infectivity was not clearly established.

The variability of the methods used in the above work and the preliminary nature of several of the studies preclude a comprehensive interpretation of the collected results. This points up the need to establish the milk-borne infectivity titer and the viral stability under
different conditions. In the titration and stability studies reported here, several pools of MTV-infected milk were used. MTV infectivity, determined using the hyperplastic alveolar nodule bioassay (Nandi, 1963; Nandi and DeOme, 1965), was tested following (a) serial dilution, (b) treatment with EDTA, chloroform, or pH 3, and (c) exposure to different temperatures. Non-viral milk components could conceivably have a protective effect on the virus. In order to evaluate this possibility, the infectivity of vesicular stomatitis virus (VSV), suspended in MTV-free mouse milk, was studied following exposure to pH 3 and 50°C.

B. Materials and Methods

1. Mouse milking apparatus. Several devices for obtaining milk from lactating mice have been described (Kahler, 1942; McBurney et al., 1964; Sykes et al., 1964; Feller and Boretos, 1967; Nowinski et al., 1967). Since all have certain disadvantages, a new mouse milking device was developed which incorporated the best features of several other milkers (McBurney et al., 1964; Feller and Boretos, 1967; Nowinski et al., 1967). The apparatus, shown in Figure 2.1a, is simple, inexpensive, and both easy to clean and to use. Most importantly, good milk yields were realized with its use.

The shafts of two sixteen-gauge stainless steel needles were inserted through the top of a number 15 vaccine vial cap. A short piece of PE 100 tubing, flared on one end, was fastened to a silicon rubber teat cup (kindly furnished by Dr. William Feller, National Cancer Institute). The other end of the tubing was attached to one of the needles in the cap.
Another piece of PE 100 tubing connected the second sixteen gauge needle with a vacuum source. Prior to milking, the vaccine vial cap was inserted into the top of a precleaned 2 dram vial. The use of this apparatus is described in Section 4.

2. Virus assay. MTV infectivity was determined using the assay developed by Nandi (1963), which is based on the occurrence of hyperplastic alveolar nodules in the mammary glands of MTV-infected mice following exogenous hormonal stimulation. Such stimulation was usually effected by two implants of hormone-pellets (0.32 mgm estradiol, 79.84 mgm corticosterone-acetate, 19.84 mgm cholesterol) at seven week intervals as described by Nandi and DeOme (1965). In some cases, the first and/or second implantation was replaced by daily intraperitoneal (i.p.) injections of the hormones (1 mgm estradiol, 500 mgm deoxycorticosterone acetate) in aqueous suspension. Formaldehyde-fixed mammary glands stained with iron-hematoxalin as described by DeOme et al., (1959) were examined for the presence of hyperplastic nodules using a dissecting microscope (3 to 7X magnification). The total number of nodules observed in all glands from each mouse was recorded. Noduligenic bioassays were performed by myself or by Melpar, Inc. (Falls Church, Va.).

VSV infectivity was determined using the standard chick-fibroblast plaque assay (McClain and Hackett, 1958), the details of which are not reported here. (For a discussion of plaque assays see Schmidt and Lennette, 1965).

3. Test animals. Test animals used for determining MTV infectivity were five week old BALB/cCrgl or BALB/c/MBA (Microbiological Associates) female mice. (BALB/c/MBA mice were used exclusively by
Melpar, Inc.) All mice were randomized among the cages and ear-tagged before use.

4. Source of viruses: collection and storage. MTV-infected milk was obtained from primaparous or multiparous BALB/c fC3H/Crgl mice, seven to eleven days postpartum. (Breeding females of this strain have a natural incidence of mammary tumors exceeding ninety per cent.) Twenty minutes prior to milking, mice which had been separated from their progeny one day before were injected, i.p., with 0.1 ml of oxytocin (Pitocin, Parke-Davis) to enhance milk secretion. Nipple regions of each mammary gland were washed with warm saline and the glands milked as shown in Figure 2.1b. The silicon rubber teat cups were sufficiently pliable to permit the operator to deliver a pulsating vacuum to the nipple, thus simulating a sucking action. No external control of the vacuum was necessary. Mice milked in this way each yielded an average of about 1.2 ml. Milk from several mice was usually collected into the same vial. The vials were tightly capped and frozen for storage at -70°C. The milking device was then rinsed thoroughly with saline and boiled in distilled water for one hour.

Vesicular stomatitis virus (VSV) (Indiana strain) was kindly furnished by Dr. A. J. Hackett (University of California, Berkeley).

5. Preparation of MTV skim-milk. MTV whole-milk was depleted of fat by centrifugation at 2000 RPM for forty-five minutes in an International PRI centrifuge (about 1000g). The milk remaining after discarding the fat pellicle and a small pellet is hereafter referred to as skim-milk. All milk dilutions were made using sterile isotonic saline.
6. **Infectivity-titration of whole- and skim-milk samples.** MTV-positive milk samples were serially diluted with sterile saline or PBS just prior to injection into test mice. Certain milk samples were rapidly frozen and then shipped at -70°C to Melpar, Inc. for subsequent dilution and bioassay.

7. **Treatment of MTV skim-milk with EDTA.** Skim-milk from multiparous MTV-infected mice was shipped, frozen, to Melpar, Inc., where it was thawed and divided into two aliquots. One aliquot was diluted with sterile saline and the other with EDTA-sterile-saline (0.08 M EDTA, 0.9% NaCl). Before injection into test mice (BALB/c/ATHA) serial dilutions were made of both of the solutions using sterile saline.

8. **Exposure of MTV skim-milk to pH 3.** A sample of MTV skim-milk was diluted with sterile saline and adjusted to pH 3 with a small volume of concentrated hydrochloric acid. After maintaining the skim milk at pH 3 and 4°C for thirty minutes it was quickly adjusted to pH 6.8 with a small volume of concentrated sodium hydroxide, then serially diluted with sterile saline. As a positive control for MTV-infectivity an untreated skim milk aliquot was held at 4°C for thirty minutes and serially diluted as above.

9. **Exposure of MTV skim-milk to chloroform.** Skim milk from the same pool used in the pH study (Section 5) was diluted 1:10 with sterile saline. One volume of reagent grade chloroform was added to seven volumes of the saline diluted skim milk and the mixture then shaken gently by hand.
for ten minutes at 4°C. Complete phase separation was accomplished by centrifugation at 1000g for ten minutes in a bench centrifuge. The aqueous phase plus a somewhat gelatinous material which appeared at the interphase boundary were removed together. A low pressure stream of dry nitrogen was passed over the surface of this material in order to remove any residual chloroform. Sterile saline was used as the diluent for the bioassays.

10. Exposure of MTV skim-milk to 50°C, 37°C and room temperature. Three one ml aliquots of MTV skim-milk (10⁻³ dilution, in sterile saline) were placed into separate two dram vials which were then purged of air with dry nitrogen before being sealed. (The air was removed from the sample vial in order to minimize possible oxidation effects.) Two of the vials were placed into a 50°C water bath; one vial was removed after ten minutes, the other after thirty minutes. The temperature of the vials was then immediately reduced by placing them in an ice bath. A third vial was maintained at 4°C and served as a positive control. All samples were allowed to warm to room temperature before injection into test mice.

One milliliter samples at 10⁻³ dilution (in sterile saline) were placed into two dram vials which were purged of air as described above. The vials were then sealed and placed into a 37°C incubator for two or four days, after which time the samples were tested for noduligenic activity.

Sealed vials containing undiluted MTV skim-milk were exposed to room temperature for two, three, four, twelve, twenty-four, and seventy-two hours and then rapidly frozen at -70°C. Frozen samples were shipped
to Melpar, Inc. for dilution with PBS and assay.

11. Exposure of VSV to pH 3 and 50°C; effect of presence of MTV-free skim-milk. A $10^{-2}$ dilution of MTV-free BALB/cCrgl skim milk was further diluted tenfold with a suspension of VSV (about $10^9$ PFU in saline-A; see Appendix). One aliquot of the VSV preparation was adjusted to pH 3 at 4°C for thirty minutes and then quickly readjusted to pH 7 (Section 9). A second aliquot of the virus-milk suspension was incubated at 50°C for thirty minutes as previously described in Section 10. Identical dilutions of VSV suspended in saline-A but without skim milk present were similarly exposed to acid (pH 3) and elevated temperature (50°C) and an untreated viral aliquot as a positive control was held at 4°C for thirty minutes. Both test and control samples were serially diluted with Saline-A for plaque assay (McClain and Hackett, 1958).

C. Results and Discussion

1. Titration of milk-borne MTV. The results of infectivity-dilution studies presented here have (a) confirmed the submaximal incidence of infectivity at high virus concentration (Bittner, 1945) and (b) extended previous virus-titration investigations (Bittner, 1945; Huseby et al., 1950), permitting an estimate of the concentration of the infectious units in MTV-infected milk samples. As is seen in Figure 2.2, milk-borne MTV from primaparous or multiparous milk pools showed maximum infectivity at dilutions of $10^{-2}$ to $10^{-3}$.

The phenomenon of submaximal infectivity at highest viral concentrations is a curious one, which to my knowledge has no parallel in
animal virology. Although the cause for such an irregular infectivity response has not yet been established, a number of possibilities including viral inhibitors have been proposed (Bittner, 1945; Moore, 1963). A different explanation may be that MTV tends to aggregate, perhaps with normal milk components, thereby reducing the effective viral concentration. Such an aggregation could also lead to a more rapid sequestration of the virus by the elements of the host's reticulo-endothelial system. Dissociation of such MTV aggregates and the concomitant higher degree of infectivity upon dilution would be similar to that noted for plant viruses (Bald, 1937; Lauffer and Price, 1945).

The MTV titration results of Figure 2.2 also demonstrate that both primaparous and multiparous milk from BALB/cfC3H mice contain high concentrations of infectious units (i.e., end-point dilution was approximately $10^{-6}$ to $10^{-9}$ per 0.1 ml). That whole milk from primaparous mice apparently contained more infectious particles than skim milk from multiparous mice suggests that some MTV was discarded with the fat pellicle during the preparation of the skim milk. Alternatively, such differences in infectivity could well be due to chance variations in the concentration of infectious MTV in the respective milk samples. No meaningful comparison between the infectious units of primaparous and multiparous mice can be made on the basis of results presented here. It has been shown elsewhere, however, that the concentration of MTV-B particles in the milk generally increases with the number of lactations (Blair, 1969).

The infectivity-titer for MTV reported here probably represents only a lower limit of the actual virus concentration, since the number of physical virus particles required to initiate a detectable lesion (hyper-
plastic nodule) could be much greater than one. The consistent finding of high viral infectivity in milk of MTV-infected mice clearly establishes the feasibility of using small volumes of milk as the source of virus for purification and characterization studies.

2. **Effect of EDTA on infectivity.** As seen in Figure 2.3, treatment of MTV skim-milk preparations with the chelating agent EDTA, had no detrimental effect on viral infectivity. The slight suggestion of enhanced viral bioactivity upon exposure to EDTA is not statistically significant. Both the treated and control samples showed maximum infectivity at a dilution of $10^{-2}$ and zero infectivity at $10^{-7}$, which was consistent with the results found for the untreated preparations (Figure 2.2). This suggests that if the submaximal response at low dilutions is due to aggregation with normal milk components then intact casein micelles are probably not involved since they are structurally unstable at low divalent ion concentrations (Noble and Waugh, 1965). The use of EDTA by Pitkannen (1965) in studies of the stability of Rous sarcoma virus (RSV) (similar in many respects to MTV, Robinson and Duesberg, 1968b) showed that RSV infectivity was stabilized under a variety of conditions by this agent. Further studies are required to determine if MTV infectivity is likewise stabilized by EDTA.

3. **Influence of pH 3 and chloroform on infectivity.** As shown in Table 2.1, the infectivity of MTV skim-milk exposed to pH 3 (83%) was not significantly different from that of the control milk sample (50%). This result is at variance with that reported for RSV and several Myxoviruses (Hamparian et al., 1963). However, Newcastle disease virus (NDV), a
Paramyxovirus similar in some respects to MTV (Bellett, 1967), has been shown to be stable to pH 4 and 8°C for more than a week. On the basis of this comparison it cannot be concluded that MTV shares with NDV an intrinsic stability to exposure at low pH. That is, MTV may have been protected against inactivation by the presence of non-viral milk products.

The protective influence of normal milk components on the acid-lability of an enveloped RNA virus can be seen in Table 2.3. A VSV suspension inactivated by treatment at pH 3 for thirty minutes was protected against inactivation if suspended in MTV-free skim-milk (Table 2.3). (VSV was used in this stabilization study since a purified preparation of MTV was unavailable.) To clearly establish the acid-stability or lability properties of MTV it would be desirable to study the infectivity of a purified viral preparation following exposure to low pH.

The complete inactivation of MTV in skim milk by chloroform (Table 2.1) is in agreement with the chloroform sensitivity of Myxoviruses and Paramyxoviruses (Feldman and Wang, 1961). (Although diethyl ether has often been used as a reagent for testing viral stability to lipid solvents (Andrews and Horstman, 1949), Hamparian et al., (1963) reported inactivation by chloroform was more consistent than that by diethyl ether.)

MTV inactivation with chloroform could well be due to the disruption of the lipoprotein viral envelope, which could thereby prevent the cellular adsorption and/or penetration of the virus.

4. Thermostability of MTV. Table 2.2 shows that MTV was not inactivated by incubation at (a) 50°C for thirty minutes, (b) 37°C for twenty-four hours or (c) 25°C for twenty-four hours, but it was inactivated after seventy-two hours at 37°C or ninety-six hours at 25°C. This
thermostability is not typical of most enveloped RNA viruses. The VSV study presented here is an example. As shown in Table 2.3, VSV was inactivated by exposure to 50°C for thirty minutes.

This result suggests that the thermo-resistance of MTV is independent of the presence of non-viral milk components. Also in contrast to the apparent thermostability of MTV, other RNA tumor viruses exhibit thermostability; the half-life of RSV infectivity is 240 minutes at 39°C and 8.5 minutes at 50°C (Dougherty, 1961). Similarly, Gross (1966) reported that passage A leukemia virus was inactivated after thirty minutes at 50°C. However, NDV does have thermostability properties similar to those found here for MTV. Most NDV strains tested, (28 out of 31), were shown to be infectious after heating to 56°C for thirty minutes or more (Hanson and Brandly, 1958). As with NDV, the thermostability of MTV could well be an inherent viral property. Alternatively, this viral stabilization may be due to the purging of air from the MTV milk samples prior to heating, thereby preventing oxidative degradation of viral envelope components essential for infectivity. In this regard the results of the RSV study by Pitkannen (1965) may be pertinent; she showed that mercaptoethanol, a reducer of disulfide groups, stabilized against RSV inactivation at 37°C.

In recapitulation, the thermostability studies of MTV presented above indicate that MTV is not easily inactivated at moderate temperatures. The reason for such stability is not yet understood.
D. Summary

Milk from MTV-infected BALB/cfC3H/Crgl mice, primaparous and multiparous, contains high titers of infectious MTV ($10^{-7}$ to $10^{-9}$ end-point dilution). Bioassays of infected milk samples when diluted with either saline or PBS showed maximum infectivity at intermediate dilutions of $10^{-2}$ to $10^{-3}$. Treatment of MTV skim-milk preparations with 0.08 M EDTA in saline did not significantly alter the infectivity-titration response. MTV skim- or whole-milk preparations were also not inactivated by treatment at (a) pH 3 and 4°C for thirty minutes or (b) 50°C for thirty minutes, 37°C for twenty-four hours or 25°C for twenty-four hours. However, treatment with chloroform or exposure to 37°C for ninety-six hours or 25°C for seventy-two hours completely inactivated the virus.

Results of the experiments presented in this chapter indicate that small samples of milk from BALB/cfC3H/Crgl mice contain MTV of sufficient quantity and stability to permit further and more detailed biophysical studies. In the following chapter, characterization of MTV milk-samples in several density gradient solutions is described.
TABLE 2.1  STABILITY of MILK-BORNE MTV BIOACTIVITY to EXPOSURE to pH 3<sup>a</sup> or TREATMENT with CHLOROFORM

<table>
<thead>
<tr>
<th>Treatment at 4°C for 30 minutes</th>
<th>Total No. Mice with HAN&lt;sup&gt;b&lt;/sup&gt;</th>
<th>% Mice with HAN</th>
</tr>
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<tr>
<td>pH 3</td>
<td>10/12</td>
<td>83</td>
</tr>
<tr>
<td>Chloroform</td>
<td>0/11</td>
<td>0</td>
</tr>
<tr>
<td>None (Control)</td>
<td>7/14</td>
<td>50</td>
</tr>
</tbody>
</table>

<sup>a</sup>See Materials and Methods for details

<sup>b</sup>HAN = hyperplastic alveolar nodule
TABLE 2.2  STABILITY of MILK-BORNE MTV BIOACTIVITY to TREATMENT at SEVERAL TEMPERATURES\(^a\)

<table>
<thead>
<tr>
<th>TEMPERATURE °C</th>
<th>PERIOD OF INCUBATION</th>
<th>NO. OF MICE</th>
<th>% OF MICE WITH HAN(^b)</th>
</tr>
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<tbody>
<tr>
<td>25</td>
<td>0 hours</td>
<td>12</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>12 hours</td>
<td>11</td>
<td>72</td>
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<tr>
<td></td>
<td>24 hours</td>
<td>15</td>
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<tr>
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<td>72 hours</td>
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<tr>
<td>37</td>
<td>0 hours</td>
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<tr>
<td></td>
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<td>5</td>
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<tr>
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<td>96 hours</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>50</td>
<td>0 minutes</td>
<td>5</td>
<td>80</td>
</tr>
<tr>
<td></td>
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<td>4</td>
<td>50</td>
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<tr>
<td></td>
<td>30 minutes</td>
<td>7</td>
<td>72</td>
</tr>
</tbody>
</table>

\(^a\)See Materials and Methods for details  \(^b\)HAN = hyperplastic alveolar nodule
TABLE 2.3  EFFECT of NON-VIRAL MILK COMPONENTS on the STABILITY of VSV\textsuperscript{a} INFECTIVITY to EXPOSURE to pH 3 or 50°C\textsuperscript{b}

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>MILK ADDED</th>
<th>INFECTIVITY TITER \textsuperscript{c}</th>
<th>INACTIVATION\textsuperscript{d}</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 3</td>
<td>-</td>
<td>2.9 x 10\textsuperscript{5}</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2.1 x 10\textsuperscript{7}</td>
<td>-</td>
</tr>
<tr>
<td>50°C</td>
<td>-</td>
<td>5.3 x 10\textsuperscript{4}</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2.5 x 10\textsuperscript{5}</td>
<td>+</td>
</tr>
<tr>
<td>None</td>
<td>-</td>
<td>1.5 x 10\textsuperscript{8}</td>
<td></td>
</tr>
<tr>
<td>(Control)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a}VSV = vesicular stomatitis virus  \hspace{1cm} \textsuperscript{c}PFU = plaque forming units

\textsuperscript{b}See Materials and Methods for details  \hspace{1cm} \textsuperscript{d}Inactivation relative to control sample; reduction of \geq 10\textsuperscript{2} or greater is significant
Figure 2.1. a) Apparatus used for obtaining milk from lactating mice. The vaccine vial cap with the silicone teat cup attached is fitted into the 2 dram vial prior to milking. A vacuum is drawn through the bent needle and the milk collected into the vial.

b) Actual milking of a lactating mouse using the milking apparatus described.
Figure 2.2. Infectivity-titration of BALB/cF3H/Crgl MTV milk using the hyperplastic alveolar nodule (HAN) assay for MTV. Number of mice tested at a given dilution is shown alongside each datum point. ( ) Primaparous whole-milk serially diluted in PBS. ( ) Multiparous skim-milk serially diluted in PBS. ( ) Multiparous skim-milk serially diluted in sterile saline. See text for methods of milk preparation and virus assay (Sections B, 2 and 5). Note that the maximum infectivity does not occur at the highest virus concentration.
Fig. 2.2
Figure 2.3. Effect of EDTA on infectivity of MTV-skim milk tested by induction of hyperplastic alveolar nodules (HAN) in test mice. Number of mice tested at a given dilution is shown alongside each datum point. ( ) Treated with 0.08 M EDTA. ( ) Control (no EDTA treatment). Both test and control samples were serially diluted with sterile saline. See text for details of treatment (Section B. 7). Differences in the infectivity of EDTA treated samples and control samples are not statistically significant.
Fig. 2.3

Percent of mice with HAN

Dilution
CHAPTER III

Isopycnic-zonal Centrifugation and Characterization of MTV

A. Introduction

B. Material and Methods
1. Source of virus
2. Solutions used
3. Centrifugation of MTV
4. Buoyant-density determination and gradient fractionation
5. Immunological detection of MTV
6. U.V. spectrophotometry
7. Electron microscopy

C. Results
1. MTV banding characteristics
2. Correlation between U.V. absorption and MTV-B antigenicity
3. Electron microscopy of negatively stained milk preparations

D. Discussion
CHAPTER III
Isopycnic-zonal Centrifugation and Characterization of MTV

A. Introduction

Several buoyant densities have been reported for the mouse mammary tumor virus (MTV) based on isopycnic centrifugation in different gradient solutions: 1.22 gm/ml in RbCl (Lyons and Moore, 1962), 1.19 gm/ml in potassium tartrate (Sykes et al., 1964), 1.10 to 1.14 gm/ml in Ficoll (Lyons and Moore, 1965; Hall and Feller, 1967), 1.22 gm/ml in sucrose-D$_2$O (Duesberg and Blair, 1966). It has long been known that buoyant densities of other virus particles in various solutions are related to gradient composition (Schachman and Lauffer, 1949; Lauffer and Bendet, 1954; Schachman, 1959; Anderson, 1966), and this may explain the variations in MTV isodensity among the above reported values. However, previous MTV buoyant density studies are difficult to interpret since differing preparative methods and centrifugation conditions and several different sources of virus were used. (For the purpose of this thesis, no distinction is made between the terms buoyant density, isodensity and isopycnic density.)

I have made a comparison of the isodensity characteristics of MTV in several gradient solutions using largely a single pool of MTV-infected BALB/cfC3H/Crgl milk as the source of the virus. Identical
milk samples were isopycnically centrifuged in continuous gradients of sucrose, sucrose-D₂O, potassium tartrate, CsCl and RbCl. The gradients were then analyzed for banding characteristics, optical density profile, distribution of viral antigens and particle morphology.

B. Material and Methods

1. Source of virus. Milk was obtained from MTV-infected BALB/cfC3H/Crgl and MTV-free BALB/cCrgl mice by means of a pulsating breast pump. The MTV-positive milk, largely from a single pool, was divided into one ml aliquots and stored at -70°C until needed. In some instances, as noted, infective samples from other milk pools were used.

2. Solutions used. The diluent used for preparing both milk samples and gradient reagents was Tris-buffered EDTA-saline (TES), pH 7.4 (0.01 M Tris HCl, 0.1 M NaCl, and 0.001 M EDTA). The presence of EDTA in buffered saline has been shown to stabilize the activity of Rous sarcoma virus (Pitkannen, 1965; Robinson et al., 1965) and not to reduce the infectivity titer of MTV-skim milk preparations (Chapter II, C). All preparations were maintained at 2 to 5°C unless otherwise stated.

Stock solutions used to form the gradients were prepared by dissolving the requisite amount of sucrose, potassium tartrate, CsCl or RbCl in TES to produce densities of 1.08 gm/ml and 1.25 gm/ml. Sucrose-D₂O of density 1.25 gm/ml was prepared by dissolving 65 gm of sucrose in 100 ml of twenty per cent D₂O; the solution of density 1.08 gm/ml was made by dissolving 16 gm of sucrose in 100 ml of buffered twenty per cent D₂O.

3. Centrifugation of MTV. Figure 3.1 schematically illustrates
the centrifugation procedure used. Aliquots of frozen milk were rapidly, thawed and diluted with 5 ml of TES. Milk was depleted of fat and rapidly sedimenting material by centrifugation at 10,000 RPM for ten minutes in a Spinco 40 rotor. Cleared milk was drawn off and the fat pellicle and pellet discarded. The loss of MTV due to differential centrifugation was estimated to be 15 to 20% of that in the initial whole milk sample. (Estimated loss of MTV was based on values given in Spinco Manual, Memo II-B 2.0 corrected for particle density and fluid volume centrifuged.) The cleared milk was adjusted to 0.025 M EDTA and allowed to stand at room temperature for fifteen minutes to enhance disruption of residual casein micelles (the calcium-dependent stability of these micelles was discussed in Chapter II, B, 4).

Discontinuous step-gradients were formed in 5.4 ml nitrocellulose tubes by layering 0.5 ml of a given 1.08 gm/ml solution over an equal volume of a 1.25 gm/ml solution of the same composition. The remaining volumes were then filled with the cleared milk and the tubes placed in a Spinco SW50L rotor and centrifuged at 50,000 RPM for fifteen minutes. The intense light-scattering band which formed at the 1.08/1.25 gm/ml density interface was collected in approximately 0.25 ml through a hole puncture in the bottom of the tube, then diluted to 1 ml with TES.

Continuous density gradients, 1.08 to 1.25 gm/ml, were formed using a device similar to that described by Martin and Ames (1961), and the same stock solutions as those used to form the discontinuous gradients. The diluted density interface material was layered onto the preformed gradients and centrifuged in a Spinco SW50L rotor at 50,000 RPM
for sixty minutes.

4. Buoyant-density determination and gradient fractionation. Light-scattering patterns formed as a result of isopycnic centrifugation were photographed and the densities of the bands determined from their positions relative to buoyant density markers (Sondell Scientific Instruments, Palo Alto, California). The isodensity markers were checked against solution densities determined by pycnometric and refractive index methods and found to be correct to the third decimal place. Gradients were fractionated by piercing the bottom of the tube with a No. 23 needle and collecting four drop fractions using an apparatus similar to that described by Schaffer and Fromhagen (1965).

5. Immunological detection of MTV. Gradient fractions were assayed for the presence of MTV B-particle (MTV-B) and normal (non-viral milk) antigens, using immunoprecipitin methods (Blair, 1965, 1966). Rabbit antiserum to be used to precipitate MTV (furnished by Dr. Phyllis B. Blair, University of California, Berkeley, Ca.) was obtained from an animal immunized with MTV partially purified by differential centrifugation from a homogenate of BALB/cF3H/Crgl mammary tumor tissue. MTV-free, defatted, mouse milk was added to the rabbit anti-MTV antiserum before use to agglutinate antibodies against normal milk components. Antiserum used to detect normal (non-viral) milk antigens was obtained from rabbits injected with milk from MTV-free mice.

Ochterlony immunodiffusion was performed using a gel which consisted of 0.65% Noble agar, 0.85% NaCl and 1:10,000 merthiolate in distilled water, pH 7. Antisera and undiluted aliquots of isodensity grad-
lent fractions, 0.02 ml, were placed in their respective wells simultaneously. The wells were not subsequently refilled. Distinct precipitation lines were easily observable in the gel following incubation at 37°C for sixteen hours; at this time the plates were scored for the presence or absence of MTV-B antigens and non-viral milk antigens. (Agar plates incubated at room temperature produced identical results after thirty to forty-eight hours.)

6. **U.V. spectrophotometry.** Following the removal of aliquots for immunologic analysis, gradient fractions (approximately 0.25 ml) were diluted to a volume of 1 ml with TES. Absorbance at 260 μm was measured at room temperature using a Beckman DU Spectrophotometer.

7. **Electron microscopy.** Light-scattering band material from isopycnic sucrose density gradients of both MTV-positive and MTV-negative milk preparations were fixed with 0.12% glutaraldehyde in PBS at pH 6.9, for one to two hours. The fixed material was then sedimented onto formvar coated, carbon-backed 400 mesh electron microscope grids by centrifugation in a Sorval S rotor at 17,000 RPM for thirty minutes. Light-scattering band fractions of MTV-positive milk samples centrifuged in sucrose-D₂O gradients were prepared similarly. Grids were then negatively stained with 2% phosphotungstic acid (PTA) at pH 6.7, and examined with a Siemens 1A electron microscope by Dr. A. J. Hackett.

C. **Results**

1. **MTV banding characteristics.** Isopycnic centrifugation of MTV-positive milk preparations resulted in the formation of one or two discrete
light-scattering bands. The number and position of the bands were dependent upon the composition of the density gradient (Figure 3.2). Two characteristic light-scattering zones were observed following banding in continuous gradients of sucrose, sucrose-D₂O or potassium tartrate (Figures 3.2a and 3.2b). In contrast, identical MTV preparations centrifuged in gradients preformed with the heavy salts, CsCl or RbCl, resulted in the development of a single light-scattering band (Figure 3.2c). Reproducible banding patterns were obtained, and no significant changes in light-scattering characteristics were noted following centrifugation for longer periods of time. As seen in Figure 3.2d, no distinct light-scattering band or bands were observed when similar preparations of MTV-free milk were centrifuged in sucrose density gradients.

Buoyant densities of the bands in different gradient solutions, as presented in Table 3.1, were found to be as follows: sucrose, 1.163 and 1.170 g/ml; sucrose-D₂O, 1.188 and 1.201 g/ml; potassium tartrate, 1.163 and 1.172 g/ml; CsCl, 1.179 g/ml; RbCl, 1.180 g/ml.

2. Correlation between U.V. absorbance and MTV-B antigenicity.

Optical density profiles of MTV-positive milk samples banded in several gradient solutions are shown in Figure 3.3. Optical densities were not corrected for light-scattering (the turbidity of the diluted gradient fractions was negligible). In all cases, the fractions having maximum absorbance at 260 μm corresponded with those in which MTV-B antigenicity was detected. MTV-B precipitin lines developed similarly to those previously shown to contain MTV-B particles (Blair et al., 1966, 1968). A lower degree of absorbance and a broader distribution of viral antigens was associated with fractions from the heavy salt gradient (Figure 3.3d).
Immunoprecipitin lines from heavy-salt gradient fractions were also less intense and more diffuse than the lines associated with fractions from sucrose or potassium tartrate gradients. In these MTV-positive light-scattering band gradient fractions, normal milk-component antigens were also often detected.

Isopycnically banded MTV-free milk samples showed little absorbance (Figure 3.3e). As expected, no MTV-B antigenicity was detected in any fraction from gradients of MTV-free milk samples.

3. Electron microscopy of negatively stained milk preparations. MTV-positive and MTV-free milk preparations from light-scattering regions of sucrose and sucrose-D_2O density gradients were examined with the electron microscope. Electron micrographs of particles characteristic of the upper and lower light-scattering bands from MTV-positive milk samples are shown in Figure 3.4. Material from the lower band (1.170 gm/ml) appeared relatively homogeneous with respect to both size and shape; these particles in negatively stained preparations had an average diameter of about 105 μm.

Particles from the upper band of the sucrose gradient (1.163 gm/ml) were quite polydisperse and pleomorphic. Almost all particles observed from both upper and lower bands possessed spike-like projections on their outer membrane surfaces. Examination of isopycnically centrifuged MTV-free milk samples revealed particles of diverse size and shape (Figure 3.5) but never showed spike-like projections.
D. Discussion

Results of the studies presented here establish that the buoyant density of MTV is similar to that of other RNA tumor viruses centrifuged under somewhat similar conditions. (For reasons noted above in Section I, no comparative study with the MTV values previously reported is attempted here.) Table 3.1 provides a comparison of the isopycnic values of MTV from this investigation with values reported in the literature for other RNA tumor viruses in several gradient solutions. One sees quantitatively that the buoyant densities of these viruses are all similar in a given gradient solution but that they do depend upon the gradient composition.

Variations in the sedimentation properties of viruses in different gradient solutions can result from a number of factors including (a) the extent of the virus hydration mantel (Schachman and Lauffer, 1949), (b) the existence of an internal water compartment (Sharp et al., 1946), and (c) the extent of electrostatic interactions between the virus and ions in solution (Lauffer et al., 1952). The higher buoyant density of MTV in sucrose-D₂O gradients could be accounted for by the exchange of H₂O for D₂O in the hydration mantle and/or in the internal water compartment. With respect to electrostatic effects, given the negative charge of the virus at this pH (Moore and Lyons, 1963), the buoyant density of MTV in heavy salt solutions would very likely be increased due to some selective adsorption of positively charged heavy metal ions. This is consistent with the results in Table 3.1.

A compilation of MTV light-scattering band positions from a number of experiments using different milk pools (Figure 3.6) shows
clearly that where two bands were obtained they were completely separated.

The pleiomorphic particles of the upper MTV light-scattering band bear considerable morphological similarity to incomplete forms of influenza virus. It has been shown (Barry and Waterson, 1962) that incomplete influenza virus particles are more heterogeneous in size and shape than the complete virus and that both complete and incomplete particles possess identical spike-like surface projections. This description of influenza particles also applies to the particles I have isolated from MTV-infected milk samples (Figures 4a and 4b). The diameter of what appears to be complete MTV is 105 m, which correlates well with that reported for MTV particles isolated from tumor tissues by Calafat and Hageman (1968).

The buoyant density of the polydisperse, pleiomorphic particles separated from MTV-positive preparations was consistently seen to be lower than that for the more uniform MTV particles. Lyons and Moore (1965) reported that a particle band with a density less than that of their MTV band had a slightly higher percentage of lipid and a lower percentage of RNA than did their MTV band. They suggested that the particles constituting the band were membrane fragments and incomplete virus particles. Unfortunately the detail shown in their electron micrographs is insufficient to make a meaningful comparison with those shown in Figure 3.4b. Uhler and Gard (1954) showed that incomplete influenza virus particles contain more lipid than the complete viruses. If the same is true for the pleiomorphic particles, here suggested to be incomplete MTV, its banding at a lower density position would be expected. Further studies are in progress to characterize more fully the "incomplete" particles in MTV-infected mouse milk.
Results of the studies presented in this chapter demonstrate that the purification of milk-borne MTV using isopycnic centrifugation methods alone is limited since the total elimination of precipitable, non-viral antigens was impossible. This purification procedure is apparently insufficient to yield a viral product of the purity required for detailed immunological and biophysical characterization of the virus. In the following chapter, I will describe a separation method which results in a highly purified MTV preparation.
### TABLE 3.1 BUOYANT DENSITY of MOUSE MAMMARY TUMOR VIRUS (MTV), ROUS SARCOMA VIRUS (RSV) and RAUSCHER LEUKEMIA VIRUS (RLV) in SEVERAL GRADIENT SOLUTIONS

<table>
<thead>
<tr>
<th>Gradient forming material</th>
<th>Buoyant Density (gm/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MTV Lower band</td>
</tr>
<tr>
<td>Sucrose</td>
<td>1.170</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Sucrose-D&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>1.201</td>
</tr>
<tr>
<td>Potassium tartrate</td>
<td>1.172</td>
</tr>
<tr>
<td>Cesium chloride</td>
<td>1.179</td>
</tr>
<tr>
<td></td>
<td>1.179</td>
</tr>
<tr>
<td>Rubidium chloride</td>
<td>1.180</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>O'Connor et al., (1964)  
<sup>b</sup>Robinson et al., (1965)  
<sup>c</sup>Duesberg and Robinson (1966)  
<sup>d</sup>Crawford (1960)  
<sup>e</sup>Field-formed density gradient
Figure 3.1. Flow diagram of the isopycnic centrifugation procedures used to study the properties of MTV banded in different density gradient solutions.
Whole mouse milk +
5 ml Tris-EDTA saline

Centrifuge, 9038 g, 4°C, 12 min

Cleared milk

Pellet and fat pellicle discarded

DENSITY INTERFACE
Discontinuous gradient, 1.08/1.25 g/ml
Centrifuge, 273,910 g, 4°C, 15 min

Interface band +
Tris-EDTA saline

ISOPYCNIC CENTRIFUGATION
Continuous gradient, 1.08-1.25 g/ml
Centrifuge, 273,910 g, 4°C, 60 min

Photograph light-scattering pattern and fractionate gradient

Fig. 3.1
Figure 3.2. Appearance of light-scattering patterns following isopycnic banding of milk samples. MTV-positive milk banded in (a) sucrose, (b) potassium tartrate, and (c) CsCl gradients and (d) MTV-negative milk sample banded in a sucrose gradient. Note the absence of any discrete band in the gradient of MTV-free material. Density values of the spherical markers used were 1.160 and 1.180 gm/ml respectively.
Figure 3.2
Figure 3.3. Optical density of gradient fractions following isopycnic centrifugation of milk samples. Shown are U.V. absorption profiles of identical MTV-positive milk samples centrifuged through preformed gradients of (a) sucrose, (b) sucrose-D$_2$O, (c) potassium tartrate, and (d) CsCl. Also shown is the absorption profile of a MTV-negative milk sample (e); after centrifugation through a sucrose gradient. Vertical arrows locate the positions of the buoyant density markers and horizontal arrows indicate fractions in which MTV-B particle antigenicity was detected.
Figure 3.4. Electron micrographs of phosphotungstic acid-stained particles from a sucrose density gradient MTV-positive milk preparation. Magnification is 80,000X. (a) lower light scattering band with an isodensity of 1.170 gm/ml. (b) upper light scattering band with an isodensity of 1.163 gm/ml. Note the presence of spike-like projections on particles in both (a) and (b).
Figure 3.4

XBB 6910-6687
Figure 3.5. Electron micrographs of negatively stained particles from the 1.16 - 1.18 gm/ml region of an MTV-free milk preparation isopyc- nically banded in a sucrose density gradient. Note that no spike-like projections appear on the surface of particles from virus- free milk samples. Magnification is 60,000X.
Figure 3.6. Isodensity positions of MTV light scattering bands in several density gradient solutions. Although a number of different MTV milk-pools were used, note that the light scattering band positions do cluster and none of the lower isodensity bands in sucrose or potassium tartrate solutions overlap with the bands formed in RbCl or CsCl gradients.
Fig. 3.6
CHAPTER IV

Rate- and Isopycnic-zonal Purification of MTV: Further Characterization of Virus

A. Introduction

B. Materials and Methods

1. Source of virus
2. MTV purification procedure
3. Analysis of purified MTV fractions: electron microscopy, U.V. absorbance, immunodiffusion and radioactivity detection
4. Analytical sedimentation of purified MTV on a 5-20% w/v linear sucrose density gradient

C. Results and Discussion

1. Preparative rate-zonal centrifugation
2. Isopycnic-zonal centrifugation of rate-zonal density-gradient fractions
3. Purity of the rate- and isopycnic-zonal density gradient preparations of MTV
4. Morphology of purified MTV
5. Estimation of the sedimentation coefficient for MTV
6. Bioactivity of purified MTV preparations

D. Summary
CHAPTER IV

Rate- and Isopycnic-zonal Purification of MTV:

Further Characterization of Virus

A. Introduction

Many investigators have attempted to isolate and characterize MTV using ultracentrifugation techniques (see reviews; Dmochowski, 1953; Hall and Feller, 1967; Blair, 1968). Visscher et al., (1942, using lactating mammary-gland extracts) and Bryan et al., (1942, using mammary tumor extracts) independently reported the partial characterization of the then unidentified agent (MTV) following differential centrifugation. Lyons and Moore (1962) described the partial purification of MTV from tumor homogenates using differential and isopycnic density-gradient centrifugation. Recently, Calafat and Hageman (1968) reported that MTV from mammary tumor homogenates could be purified by a multistep process involving, first, centrifugation on a discontinuous gradient followed by two successive rate-zonal density gradient centrifugations.

Working with T₂ and T₃ bacteriophage, Anderson et al., (1966) have shown that a high degree of viral purification can be obtained using a combination of rate-zonal centrifugation (Braake, 1951) and isopycnic-zonal centrifugation (Anderson, 1956). They also suggested that this procedure, which permits the separation of particles first according to size and then
according to buoyant density, may be applicable to the isolation of MTV. The limited success of previous attempts to isolate MTV from virus-infected milk samples using isopycnic methods alone (Chapter III) was apparently due in part, to the similar buoyant density of the virus and of contaminants, such as microsomal particles. In the present investigation, milk-borne MTV was purified by sequential centrifugation on continuous preformed rate- and isopycnic-zonal sucrose density gradients. Viral samples were analyzed at several stages of the purification to determine particle morphology, viral and non-viral antigenicity, U.V. absorbance and concentration of radioactively labeled viral nucleic acid. An aliquot of purified MTV was also used to determine the sedimentation coefficient of the virus.

B. Materials and Methods

1. Source of viruses. Milk obtained from MTV-infected lactating BALB/cfC3H/Crg1 multiparous mice was used as the exclusive source of the virus (Chapter II, C). As a means of radioactively labeling the viral nucleic acid, some mice were injected i.p. with 1 millicurie (mC) of $^3$H-uridine twenty-four hours prior to milking (Duesberg and Blair, 1966). The collection and storage of the milk was as previously described (Chapter II, B. 4).

Poliovirus (LSC strain, Type 1) labeled with $^{32}$P(orthophosphate) was kindly furnished by Dr. Frederick L. Schaffer (Naval Biological Laboratory, University of California, Berkeley, California).
2. **MTV purification procedure.** The methods used to purify milk-borne MTV are schematically illustrated in Figure 4.1. (All procedures were carried out at 2-5°C unless otherwise stated.) Frozen whole milk samples were thawed and divided into 1 ml aliquots. Each aliquot was diluted with 10 ml of TES and centrifuged in a Spinco 40 rotor for twelve minutes at 10,000 RPM. Differential centrifugation under these conditions resulted in an estimated 5% loss in MTV (Spinco Technical Manual, Memo II-B 2.0). Following this centrifugation, the 9 ml of cleared milk was drawn off and was treated with EDTA, then centrifuged onto a 15%/65% w/v sucrose density interface (1.08/1.25 gm/ml) and the interface material collected as previously described (Chapter III, B. 3). The density-interface solution obtained was diluted with a volume of TES sufficient to permit it to float on 15% w/v sucrose.

This diluted interface solution was then centrifuged on a continuous rate-zonal sucrose density gradient (15% - 32.5% w/v sucrose at 50,000 RPM for 12.5 minutes in a Spinco Sw 50.1 rotor). Following centrifugation, a zone of light scattering material was observed to have migrated about two thirds of the length of the tube. The gradient was fractionated dropwise through a hole punctured in the bottom of the tube with a No. 23 needle; the fractions (usually 4 drop) were diluted to 1 ml of TES (Chapter III, B. 4). In certain cases, those fractions containing material from the light scattering band were pooled and diluted, as above, with TES. The diluted-pooled material was then layered onto a continuous 15%-65% w/v (1.08 - 1.25 gm/ml) sucrose density gradient and isopycnically banded during centrifugation at 50,000 RPM for 55 minutes in a Spinco 50.1
rotor. (Centrifugation under these same conditions was shown in Chapter III to result in the banding of MTV.)

3. Analysis of purified MTV fractions: electron microscopy, U.V. absorbance, immunodiffusion and radioactivity detection. Negative stain and thin section electron microscopy were used to study the morphology of particles before and after purification. All fractions from isopycnic gradients were diluted ten-fold with Tris-EDTA (0.01 M Tris-HCl, 0.001 M EDTA) to prevent undesirable osmotic effects. (This procedure reduced the osmolarity from about 1.3 osmol to about 0.130 osmol.) Samples to be negatively stained with PTA were fixed with 0.12% glutaraldehyde in PBS at pH 6.9, for thirty minutes. A microdrop of "fixed" material was placed onto a formvar-coated, carbon-backed 400 mesh electron microscope grid and then negatively stained with PTA. Preparations were examined by Dr. A. J. Hackett, using a Siemens 1A electron microscope.

Samples to be thin sectioned, following dilution as above, were fixed in a 1% glutaraldehyde-parafomaldehyde solution (Appendix) for thirty minutes, and placed into Beem capsules which were in turn placed into 1/2 by 2-1/2 inch nitrocellulose centrifuge tubes. After filling the tubes with cacodylate buffer (Appendix) they were centrifuged at 40,000 RPM for fifteen minutes in a Spinco SW 50.1 rotor. (The excellent suggestion to pellet material directly in the Beem capsule was made by Harriet T. Gagné.) Following centrifugation, the capsules were carefully emptied of fluid. The pellets were then sequentially treated with 1% osmium tetroxide (OsO₄) for thirty minutes and 0.5% uranyl acetate, for fifteen minutes as described by deTkaczevski et al., (1968), followed by
serial dehydration with ethanol and embedding in Epon. Thin sections (≤50 µm) made using a Porter-Blum microtome were post-stained with lead citrate and uranyl acetate, and examined with an RCA EMU electron microscope. (The pellets were handled and examined by Hafriet·T. Gagné.)

The analysis of gradient fractions for both viral and non-viral antigenicity and for U.V. absorbance at 260 µm was similar to that previously described (Chapter III, B. 5 and 6). In this case, however, fractions used for U.V. absorbance alone were diluted with an equal volume of TES (0.25 ml) before monitoring with a Beckman DB Spectrophotometer.

MTV-B particle immunoprecipitin lines were prepared for thin-section electron microscopy by carefully cutting out with a razor blade the desired agar block from the immunodiffusion plate and washing it thoroughly with saline. The agar sections were then treated as described above except that they were not post-fixed with uranyl acetate prior to dehydration. Final preparation of this material and its examination with the RCA EMU electron microscope was performed by Miss Vicki Brauer.

Counting of radioactively labeled material in both rate- and isopycnic-gradient experiments was carried out on three-to-five drop fractions collected directly into 15 ml of BBOT scintillation fluid. (The composition of this fluid is given in the Appendix.) This counting method does not distinguish between total counts and acid precipitable counts. Therefore, some gradients were fractionated by collecting drop-fractions onto pieces of chromatographic paper which were then immersed in cold 5% perchloric acid (PCA). Protein material (including nucleo-proteins) precipitated by the PCA remained in the chromatographic paper.
following removal of the PCA solution and dehydration with ethanol. The paper was then dried and placed into a vial containing 15 ml of scintillation fluid. The radioactivity in each gradient fraction was counted for twenty minutes using a Nuclear Chicago Mark I liquid scintillation counter.

4. **Analytical sedimentation of purified MTV on a 5-20% linear sucrose density gradient.** MTV from tritiated uridine-labeled milk samples, purified as described above, was collected from the isopycnic gradient in a volume of 0.7 ml. Normal rabbit serum, 0.1 ml, was added to this fraction which was then diluted with an equal volume (0.8 ml) of cold saturated (NH₄)₂SO₄ to flocculate the virus (Robinson et al., 1965). This solution was centrifuged at 5,000 RPM for ten minutes in a Spinco SW39 rotor to pellet the flocculated material. After discarding the supernatant the pellet was resuspended in 0.2 ml of TRIS-EDTA (section B.2) to which was added 20μ of ³²P-labeled poliovirus. The viral mixture was then layered onto a continuous 5-20% w/v sucrose gradient and centrifuged at 25,000 RPM for thirty minutes in a Spinco SW 50L rotor. Each four-drop fraction, collected by bottom puncture, was delivered into 15 ml of BBOT scintillation fluid (Appendix). Radioactivity of the ³²P-labeled poliovirus was counted using a Packard Tri-Carb liquid scintillation counter, and that of the ³H-MTV counted as described above.

C. **Results and Discussion**

1. **Preparative rate-zonal centrifugation.** Analysis of rate-zonal density gradient fractions demonstrated that the position of the gradient
tube light scattering band, shown in Figure 4.2, coincided with that containing the fractions having (a) highest U.V. absorbance and precipitable MTV-B antigenicity (Figure 4.3); and (b) highest $^3$H-MTV radioactivity (Figure 4.4). Non-viral antigens were detected in a number of fractions including those showing the presence of viral antigens. The similarity of the U.V. absorbance, MTV-B antigenicity and radioactivity profiles of the gradients indicates that MTV sedimented as a zone and at a faster rate than much of the non-viral contamination.

Further analysis of the rate zonal gradients pointed up the presence of a second component which migrated more slowly than the MTV light-scattering-band (Figures 4.3 and 4.4). Figure 4.4b illustrates that this second radioactively-labeled component can be resolved. In other experiments, using non-labeled preparations, it was found that fractions taken from this "second component" region isopycnically banded at a density similar to that observed for "incomplete MTV" (Chapter III, D). Further studies are required to characterize the particles comprising this region and to establish the viral or cellular nature of the RNA.

The results of the preparative rate-zonal centrifugation of MTV presented above can be more fully understood in terms of the basic physical principles which describe particle migration in centrifugal fields. (For a thorough discussion of the theoretical aspects of ultracentrifugation see: Schachman, 1959; Berman 1966; Schumaker, 1967.) A spherical particle sedimenting through a fluid in a centrifugal field is acted upon by three main forces approximated by (a) the centrifugal force, $F_C (=p_2 \omega^2 x)$, (b) the buoyant force, $F_b (=p_1 \omega^2 x)$ and (c) the frictional or
Stokes force, \( F_t = 6\pi n r \frac{dx}{dt} \), where

\[ \begin{align*}
\rho_1 & = \text{density of the fluid, gm/ml} \\
\rho_2 & = \text{density of the hydrated particle, gm/ml} \\
\omega & = \text{angular velocity of the rotor, radians/sec} \\
\eta & = \text{viscosity of the fluid, poise} \\
x & = \text{distance, axis of rotor and particle, cm} \\
V & = \text{volume of particle, cm}^3 \\
r & = \text{radius of particle, cm} \\
\frac{dx}{dt} & = \text{velocity of particle, cm/sec}
\end{align*} \]

Sedimenting particles almost instantaneously attain a steady state velocity, that is a condition where the net force acting on the particle is zero. Writing this condition of mechanical equilibrium for the above forces, rearranging terms and simplifying, the sedimentation equation may be written as

\[ \frac{dx}{dt} = \frac{2}{9} \frac{r^2 (\rho_2 - \rho_1)}{\eta} \omega^2 x \]  

(1)

This equation shows that the sedimentation rate is dependent upon (a) the square of the particle radius and (b) the difference between the particle and fluid densities. For the following hypothetical case, the velocity of a sedimenting particle is determined primarily by its radius.

Microsomes, thought to be a major contaminant of MTV preparations, are polydisperse and have isodensities similar to that of MTV (Wallach and Kamat, 1964; Anderson et al., 1966; Chapter III, C). Therefore it is of advantage to include in the purification procedure for MTV a method
which separates particles on the basis of size. A simple calculation shows that in water, MTV \((p_2=1.17\, \text{gm/ml} ; r=0.5 \times 10^{-5} \, \text{cm})\) will sediment nearly twice as rapidly as a microsomal contaminant \((p_2=1.15\, \text{gm/ml} ; r=0.4 \times 10^{-5} \, \text{cm})\). In this hypothetical example, a difference in radii of only 100 Å can lead to a significant separation. However, not all sedimentation-rate experiments can be described in terms of particle size; for example, the rate separation of polyribosomes from protein aggregates may be strongly density dependent.

2. Isopycnic-zonal centrifugation of rate-zonal density-gradient fractions. Isopycnic-zonal centrifugation is also easily visualized in terms of Equation (1). Since the term \((p_2-p_1)\) approaches zero as the particle nears its isodensity position in the gradient, the particle velocity as defined by Equation (1) also approaches zero. The condition for isopycnic banding is then simply \((p_2-p_1)=0\). (For an analysis of the approach to density equilibrium in preformed gradients see Baldwin and Shooter, 1963.)

The results of experiments described in this chapter showed that when light-scattering band fractions from rate-zonal gradients were centrifuged isopycnically, single intense bands were observed, as seen in Figure 4.5. The discrete character of the patterns seen in these investigations (Figure 4.5) is in contrast to that observed following isopycnic centrifugation alone (Chapter III, C) and suggests this preparation is of increased purity. Isodensity of the light-scattering band, based on a number of different experiments, (about 1.17 gm/ml) was very close to that previously found for MTV banded by isopycnic centrifugation alone (Chapter III, C).
Isodensity gradient fractions were monitored for U.V. absorbance, viral and non-viral antigenicity or $^3$H-radioactivity with the results given in Figures 4.6 and 4.7. The fractions showing maximum absorbance, highest MTV-B antigenicity and greatest radioactivity coincided with those containing maximum light scattering. Ochterlony immunodiffusion assays (Figure 4.8a) showed that detectable viral antigens produced characteristic MTV-B immunoprecipitin lines. This antigenicity was only found in light-scattering band fractions. Non-viral antigens were not detected in any of the isopycnic gradient fractions. This indicates a greater purification than that previously obtained (Section C.1; Chapter III, C and D). MTV B-particle immunoprecipitin lines (Figure 4.8a) examined with the electron microscope demonstrated the presence of clusters of intact virus particles (Figure 4.8b) similar to those previously found by Blair et al., (1966, 1968).

An estimate of the MTV concentration in the isopycnic gradient fractions can be made based on the U.V. absorbance data shown in Figure 4.6 and the known U.V. absorbance of a suspension containing $10^{12}$ influenza virus particles per milliliter (Anderson, 1967). This estimate indicated that the MTV concentration in Fraction 9 (Figure 4.6) was about $1.5\times10^{12}$ particles/ml. This calculation furnishes only a rough estimate of the actual virus concentration since the 260 m\_ absorbance of influenza virus relative to that of MTV is unknown. Therefore, an independent determination of the number of physical particles per milliliter would be valuable. This information together with infectivity-titration bioassays could establish the number of physical virus particles equivalent to one infectious unit.
3. **Purity of the rate- and isopycnic-zonal density gradient preparations of MTV.** Defining the purity of a virus preparation is difficult, since a preparation which is biologically homogeneous may be physically heterogeneous. Conversely, a sample which migrates as a single peak in the analytical ultracentrifuge and/or Tiselius electrophoresis cell may in fact represent only a part of the total spectrum of infectious particles. (It is recognized that rate- and isopycnic-zonal centrifugation of MTV "select" for certain physical viral characteristics, namely size and density, and this may exclude certain classes of infectious particles.) Nevertheless, it is possible to compare, at least qualitatively, the radioactivity and optical density profiles, as well as the distribution of antigens, in the gradients and to estimate the relative degree of purity of one preparation with respect to another.

I have compared the purity of MTV preparations isolated by combined rate- and isopycnic-zonal centrifugation and by isopycnic-zonal centrifugation alone, on the basis of several criteria: (a) discrete character of the gradient-tube light-scattering band; (b) sharpness of U.V. absorbance profile; (c) distribution of viral antigenicity. A comparison of the present preparation methods made with those described in Chapter III, by Duesberg and Blair (1966) and by Hall and Feller (1967) indicated the following: (a) a much more discrete light-scattering band in Figure 4.5 than in Figure 3.2 or in the photographs of Hall and Feller (1967); (b) a narrower peak in the U.V. absorbance profile in Figure 4.6 than in Figure 3.3 or in the figures shown by Duesberg and Blair (1966); (c) a more compact distribution of MTV-B antigens in Figure
4.6 than in Figure 3.3. In addition, the MTV-radioactivity profiles of isopycnically centrifuged virus preparations, given by Duesberg and Blair (1966) are generally less well defined than those shown in Figure 4.7. By all these criteria, a higher degree of purity was obtained using the combined rate- and isopycnic-zonal centrifugation method presented above than was previously achieved.

Another criterion of purity, often used in virology, is the absence of immunologically detectable impurities (Braake, 1967b). Other workers in describing MTV purification techniques have not reported on the "antigenic purity" of their viral preparations therefore no comparison with the antigenic studies presented here can be made. However, a sample of milk-borne MTV purified using the Ficoll isopycnic method of Lyons and Moore (1965) was kindly made available by Dr. Phyllis B. Blair (University of California, Berkeley, California). This sample when tested in immunodiffusion for the presence of MTV-B and non-viral antigens showed the presence of high concentrations of both virus and contaminants as seen in Figure 4.9. This suggested that while the MTV had been concentrated by isopycnic banding in Ficoll, it had not been highly purified. Also seen in Figure 4.9 is the single immunoprecipitin MTV-B line from a combined rate- and isopycnic-zonal centrifugation of MTV-infected milk. (Non-viral antigens were never detected in the MTV fractions purified as described herein.) The absence of non-viral antigens in this preparation further demonstrates the high degree of viral purity that can be realized using the combined centrifugation procedure.

4. Morphology of purified MTV. The electron micrograph of rate- and isopycnic-zonal density gradient purified MTV, shown in Figure 4.10,
illustrates the degree of morphological purity attained. The virus particles are well defined and show spike-like fringe. A number of particles appear to have a second or an "internal membrane". Note that the virus particles show a high degree of isotropy. (Uniformity of virus size and shape is an important factor in sedimentation rate experiments as discussed later.)

Negatively stained preparations of the purified MTV, Figure 4.11, contain particles with spike-like projections and electron opaque interior regions. These opaque areas are thought to be the viral nucleoids or cores, and seem to be lacking any unique structure. In general, MTV particles purified by rate- and isopycnic-zonal centrifugation appear to be more permeable to PTA than those isolated by isopycnic centrifugation alone. This can be seen by comparing Figure 4.11 with Figure 3.4. This observation suggests that increased "handling" of the virus may result in a slight alteration of the viral envelope.

Some morphological features of MTV substructure can be seen in Figure 4.11b. Various stages of viral disruption are seen in these negatively stained preparations. In Figure 4.11b-1 an MTV particle is seen to have been penetrated by the PTA and to reveal a diffuse core of what is most likely nucleoprotein; some subunit structure of the viral envelope is also visible. This subunit structure is more clearly evident in Figure 4.11b-3 in which a more highly degraded particle shows periodic electron opaque membrane regions which appear to correspond exactly with the position and number of the spikes. This type of viral envelope architecture has recently been described for influenza viruses by Laver and Valentine (1969).
The diffuse nature of the viral nucleoid is also seen in Figures 4.11b-2 and 4.11b-4. In these micrographs the core appears to lack the high degree of morphological order characteristic of the viral envelope. The viral particle shown in Figure 4.11b-2 and 4.11b-4 suggests that the core material is not bound by an "internal membrane". The presence of the "internal membrane" in thin section electron micrographs and its absence in disrupted negatively stained particles is puzzling and suggests that this viral component is extremely labile. Further studies are required to reveal its structure and function.

Electron micrographs of an unpurified MTV milk-preparation, depleted of most fat and casein, are shown in Figures 4.12 and 4.13. Both the thin-sectioned and negatively stained microdrop preparations, in addition to typical MTV particles, show large numbers of other particle aggregates. These complexes of "globular" subunits were readily disrupted with EDTA and are thus probably casein micelles. A cursory review of the dairy science literature indicates the the configuration of casein micelles has not been clearly established. McKenzie (1967) reported that electron microscopic studies of such micelles are difficult, but based on the then available data the complexes were considered to be spherical in shape. In view of the apparent lack of information on the morphology of casein micelles, the electron micrographs of the particles described above may be of considerably interest to those working in the field of milk-protein structure.

5. Estimation of the sedimentation coefficient for MTV. The sedimentation coefficient can be easily derived from the sedimentation
rate equation (Section 1) by normalizing the velocity with respect to
the radial acceleration. This operation permits one to write the
Svedberg equation as

\[ S = \frac{dx}{dt} = \frac{2}{9} \frac{r^2(\rho_2-\rho_1)}{n} \] (2)

where \( S \) is the sedimentation coefficient (sec\(^{-1}\)). For convenience the
coefficient is usually given in terms of Svedbergs (s), i.e., in units
of \( 10^{-13} \) sec. In order to compare the sedimentation rates of various
particles centrifuged under different conditions, the sedimentation co-
efficient is standardized with respect to water at 20°C. For example,
Type 1 poliovirus used in this study has an \( S_{20,w} \) of 160s (Schaffer and
Schwerdt, 1959).

The sedimentation coefficient of MTV can be estimated by compar-
ison of its migration with that of a virus whose coefficient is known (such
as polio virus). (See Braake, 1967a for a concise discussion of this
method.) This estimate is based on the finding by Martin and Ames (1961)
that the ratio of the sedimentation coefficients of two particles of
similar partial specific volume is constant during sedimentation in a
5-20% linear sucrose density gradient.

For this method to be applicable it is important that the partial
specific volumes of the marker and unknown virus be similar. The partial
specific volume of poliovirus has been calculated to be about 0.68 ml/gm
(Schaffer and Schwerdt, 1959). This parameter is not known for MTV,
however, the partial specific volume of influenza virus (similar to MTV in
its composition, size and shape, Schaffer and Schwerdt, 1959; Wildly and
Horne, 1963; Lyons and Moore, 1965) has been reported to be 0.75 ml/gm (Sharp et al., 1950). Assuming the value for MTV to be the same as that for influenza, the ratio of the partial specific volumes, 0.68/0.75, is sufficiently close to unity to permit an estimate of the sedimentation coefficient of MTV based on the $s_{20,w}$ of poliovirus (Schaffer and Schwerdt, 1959).

Figure 4.14 illustrates the sedimentation of $^3$H-MTV in a 5-20% sucrose gradient relative to that of $^{32}$P-poliovirus; MTV migrated as a fairly narrow zone indicating a relatively pure preparation. (This observation is consistent with the electron micrograph of purified MTV shown in Figure 4.10 showing the viral preparation to be quite isotropic.) In addition, the sharp peak of $^{32}$P-poliovirus demonstrates that density instabilities did not arise during the early stages of sedimentation.

Based on the relative sedimentation of the viruses as shown in Figure 4.14, the $s_{20,w}$ of MTV was estimated to be 800-900s. This $s_{20,w}$ value for MTV is significantly higher than that reported for other onco- genic RNA viruses (RSV, 640s, Robinson et al., 1965; RLV, 640s, Mora et al., 1966). However, these latter authors noted that their sucrose density-gradient purified RLV sample was highly anisotropic when observed in the electron microscope. On the basis of this observation they have stated that the 640s reported for the Rauscher virus may represent a minimum value of the $s_{20,w}$. Electron microscopic studies were not included in the sedimentation rate experiments of the other RNA tumor viruses, hence it is not possible to determine if anisotropic factors were operable.
It is theoretically possible to compare the size of the virus particle as determined by electron microscopy with that calculated on the basis of biophysical information (sedimentation coefficient, particle and solution densities and solution viscosity). However, as shown by Sharp et al., (1945) this calculation is not meaningful without knowledge of the hydration and osmotic properties of the virus. Since these properties are not known for MTV, no such comparison is presented. It is important to stress that the estimate of the MTV sedimentation coefficient was based on a single successful experiment. A number of other experiments to determine the sedimentation coefficient failed for differing technical reasons and further work is desired in order to establish an accurate value of the $S_{20,W}$.

6. Bioactivity of purified MTV. The biophysical studies presented above show that combined rate- and isopycnic-zonal centrifugation of MTV results in viral purification significantly better than heretofore realized. This study cannot be considered complete until the bioactivity of the purified preparations has been established. Bioassays to determine the infectivity of the purified virus are planned for the near future.

D. Summary

Milk-borne MTV was purified by combined rate- and isopycnic-zonal centrifugation on sucrose density gradients. An intense light-scattering band, detected following isopycnic centrifugation was shown by electron microscopy to contain, highly purified, morphologically identifiable MTV.
Measurements of U.V. absorption at 260 mp, $^3$H-labeled virus radioactivity and precipitation in agar gel immunodiffusion consistently demonstrated that MTV was localized in the light-scattering band. The biophysical properties of the purified preparations and the absence of detectable non-viral antigens in these samples established the high degree of viral purity attained. Electron micrographs illustrated the homogeneity and isotropy of the viral preparations and indicated a "globular" subunit structure of the viral envelope. These studies also indicated that the nucleoid was not highly structured. The sedimentation coefficient of MTV was estimated to be 800-900s.
Whole mouse milk + 10 ml Tris-EDTA saline

Centrifuge, 9038 g, 4°C, 12 min

Cleared milk

DENSITY INTERFACE
15/65% w/v sucrose
Centrifuge, 273,910 g, 4°C, 15 min

Interface band + Tris-EDTA saline

RATE ZONAL CENTRIFUGATION
15-32.5% w/v sucrose
Centrifuge, 273,910 g, 4°C, 10 min

Fraction with maximum absorption @ 260 m\(\mu\) + Tris-EDTA saline

ISOPYCNIC ZONAL CENTRIFUGATION
15-65% w/v sucrose
Centrifuge, 273,910 g, 4°C, 60 min

Photograph light-scattering band position and fractionate gradient

Figure 4.1. Flow diagram of the rate- and isopycnic-zonal purification of MTV in sucrose density gradients.
Figure 4.2. Light scattering band pattern of MTV-milk preparation following fifteen minutes centrifugation at 50,000 RPM on a 15-32.5% w/v sucrose density gradient; note the distinct light-scattering band.
Figure 4.3. U. V. absorbance profile of MTV rate-zonal density gradient fractionation. Asterisks indicate those fractions containing the material in the light scattering band (Figure 4.2). Arrows indicate the fractions showing detectable MTV-B antigenicity.
Figure 4.4. Rate-zonal fractionation of two $^3$H-labeled MTV milk preparations. Light-scattering band was collected in those fractions noted with an asterisk. (a) Fractionation shows that the rapidly sedimenting labeled material appeared in those fractions containing light-scattering band material; (b) Fractionation shows the coincidence of light-scattering and radioactive peak (see text for a discussion of this observation).
Figure 4.5. Light-scattering band pattern of MTV milk preparation following rate- and isopycnic-zonal centrifugation. Spherical density markers have values of 1.160 and 1.180 gm/ml. Note the presence of only a single intense band at about 1.17 gm/ml.
Figure 4.6. U. V. absorbance profile of an MTV milk preparation centrifuged on isopycnic zonal sucrose density gradient (following rate-zonal separation). Fractionation shows sharp banding of virus. Vertical arrows indicate the positions of the isodensity markers used. Horizontal arrows indicate those fractions containing MTV-B antigens. Asterisks indicate fractions containing material in the light scattering band.
Figure 4.7. $^3$H-MTV radioactivity profile of rate- and isopycnic-zonally purified virus sample. Asterisks indicate the positions of fractions containing light scattering band material. (a) Distribution of radioactivity following the collection of gradient fractions directly into BBOT (see Materials and Methods). (b) Distribution of radioactivity following PCA precipitation of protein material (see Materials and Methods).
Figure 4.8. MTV-B immunoprecipitin reaction in agar gel. (a) Three fractions from the final gradient of MTV purified by the combined rate- and isopycnic-zonal method tested for MTV-B antigens. 
Top left and bottom right wells - anti-MTV antiserum
Middle left well - fraction just prior to light scattering band
Middle center well - light scattering band material
Middle right well - fraction following intense light scattering band
Top right and bottom left wells - empty

Most of the detectable viral antigenicity is associated with fraction containing most of the material from the light scattering band.

(b) Thin section electron micrograph of block cut from MTV-B immunoprecipitin line. Virus particles are clustered and show a fringe of what is probably antibody attached to spikelike projections described in text (section 4). Horizontal bar is equivalent to 100 mµ.
Figure 4.9. Immunodiffusion reactions comparing milk-borne MTV purified by combined rate- and isopycnic-zonal centrifugation with that purified by the Ficoll isopycnic procedure of Lyons and Moore (1965).

- Top left and top right well - anti-non-viral-milk antiserum
- Top center well - anti-MTV antiserum
- Bottom left well - Ficoll density gradient purified MTV
- Bottom right - rate- and isopycnic-zonal sucrose density gradient purified MTV

Note the detection of non-viral antigens in the MTV Ficoll preparation.
Figure 4.10. Electron micrograph of MTV purified by combined rate- and isopycnic-zonal centrifugation. Many MTV particles show spike-like projections on the outer envelope surface. Note the presence of an "inner membrane", associated with many virus particles. This preparation shows a high degree of homogeneity of particle shape. Magnification is 40,000X.
Figure 4.11. Electron micrograph of negatively stained MTV purified by the combined rate- and isopycnic-zonal centrifugation procedure. (a) Micrographs of intact MTV showing spike-like projections and electron translucent interior regions. Magnification is 150,000X. (b) Micrographs of MTV particles which disrupted and were penetrated by PTA. Core material appears diffuse without a distinct limiting "internal membrane". Viral envelope appears to have a periodic subunit structure. Magnification is 80,000X.
Figure 4.12. Thin section electron micrographs of MTV preparation after fat and casein depletion only. A number of MTV particles are clearly visible. Note the presence of many particles, believed to be casein micelles, which appear to be comprised of somewhat similar subunits. Magnification is 40,000X.
Figure 4.13. Electron micrograph of negatively stained particles from a fat and casein depleted MTV-infected milk sample. Characteristic MTV particles are seen amongst particles comprised of subunits. The particle aggregates are thought to be casein micelles. Magnification is 60,000X.
Figure 4. Sedimentation of $^3$H-MTV and $^{32}$P-poliovirus on a 5-20% w/v sucrose density gradient. Note that MTV sedimentation is confined to a narrow zone. The absence of secondary peaks of $^3$H-labeled material suggests that the MTV is sedimenting as single particles and not viral aggregates.
CHAPTER V
Recapitulation and Speculation on Future Work

The results of the biophysical investigations reported in this thesis have significantly extended our knowledge of MTV, in several areas.

In Chapter II, it was shown that high titers of infectious MTV exist in the milk of both primaparous and multiparous BALB/cfC3H/Crgl mice, although the number of physical particles equivalent to one infectious unit has not yet been determined. The high purity of MTV now attainable using combined rate- and isopycnic-zonal centrifugation as described in Chapter IV makes feasible experiments designed to establish the ratio of physical particles to infectious units. Such information would be valuable in further characterizing the host's response in the viral etiology of the mammary adenocarcinoma.

The acid- and thermo-stability of defatted milk-borne MTV bioactivity, as reported in Chapter II, was unexpected. Stability at pH 3 may be due to protective effects of non-viral milk components in the preparation, as was shown to be the case for VSV (Table 2.3). Alternatively, this stability could be due to intrinsic viral factors such as are thought to account for the acid stability of certain NDV strains (Moses et al., 1947). Interestingly, NDV is the only lipoprotein-membraned RNA virus possessing thermoresistance properties like those found for MTV (Table 2.2; Hanson and Brandly, 1958).
NDV and MTV have been shown to have somewhat similar nucleic acid base ratios (Bellet, 1967). Extension of this study to the physical properties of viral envelope components could establish those elements responsible for the physical stability of these viruses.

Isopycnic banding of MTV preparations in several gradient solutions, described in Chapter III, demonstrated the isodensity to be in agreement with known values for other RNA tumor viruses (Table 3.1). Two light-scattering bands appeared following centrifugation of MTV in sucrose or potassium tartrate gradients. The higher isodensity band in sucrose solutions (1.170 gm/ml) was shown to contain intact MTV (Figure 3.4a). Particles from the lower isodensity band (1.163 gm/ml) were quite pleiomorphic (Figure 3.4b) and resembled incomplete influenza virus (Barry and Waterson, 1962). Preliminary MTV-fractionation studies using rate-zonal density-gradient centrifugation, Chapter IV, suggest that these "incomplete" particles may be isolated on the basis of their sedimentation rate. Such an isolation would permit an evaluation of both their biophysical properties and their infectivity relative to that of complete MTV.

A variety of particles from MTV-infected milk were observed to possess spikelike surface projections similar to those previously seen on Myxoviruses and Paramyxoviruses. Particles from the milk of MTV-free mice did not possess such surface projections. These two observations suggest that the detection of "spikes" on surfaces of membraned milk-particles from MTV-infected mice might serve as a rapid assay for MTV bioactivity. Experiments currently in progress should establish the feasibility of such an assay.
Combined rate- and isopycnic-zonal centrifugation of MTV milk-samples on sucrose density gradients (Chapter IV) clearly demonstrated the utility of this procedure for the purification of MTV. The purity of the viral preparation was evaluated on the basis of the (a) U.V. absorption, (b) $^3$H-MTV radioactivity and (c) MTV-B and non-viral antigenicity profiles of density gradients. The MTV samples prepared and studied in this way were seen to be of significantly higher purity than those previously reported. However, bioassays of the purified MTV yet to be completed are necessary to fully evaluate this purification procedure.

Electron microscopic studies (negative stain and/or thin section) of MTV samples were used to analyze particle morphology. In Chapter IV it was noted that the external membrane surrounding the MTV particle appears to be composed of subunits (Figure 4.11b). These elements may be physically analogous to those isolated from the membranes of influenza virus by Laver and Valentine (1969). (Laver and Webster (1966) have shown that three characteristic antigens are associated with the viral envelope of influenza virus.) It should be possible by these methods to determine if similar viral envelope components exist for MTV. Such an analysis may lead to an understanding of the structure-function relationship of MTV-membrane surface projections. A detailed knowledge of the antigenic properties of the viral envelope may also conceivably be of prophylactic value. That is, the surface of MTV-transformed mammary adenocarcinoma cells likely possess antigens common to the virus, since the virus is known to be released from such cells by "budding" from cell membrane (Lyons and Moore, 1965).
Studies of negatively-stained purified MTV preparations suggested that the apparent viral nucleoid of disrupted viruses was not limited by a distinct "inner membrane" (Figure 4.11b), as is often seen in thin section electron micrographs (Figure 4.10 and 4.12). The biophysical and immunological properties of this "membrane" are unknown. However, based on its staining properties as seen in thin section electron micrographs, it is considered unlikely that this component is composed of lipoprotein.

Results of research presented in this thesis make it feasible to study, in detail, the biophysical and immunological properties of both the intact virus and its subunits.
CHAPTER VI

Bibliography


CHAPTER VII
Appendix

Special Reagents

A. BBOT

106.5 gm Naphthalene (purified)
5.56 gm BBOT = 2,5-bis[2-(5-tert-Butylbenzoxazoly)]-Thiophene
100 ml Toluene
665 ml 2-Methoxyethanol

B. Cacodylate buffer

100 ml 0.1 M Na-Cacodylate
7.5 gm Sucrose

C. Paraformaldehyde-Glutaraldehyde

0.5 gm Paraformaldehyde
0.35 ml 0.01 N NaOH
25 ml 0.2 M Na-Cacodylate
2 ml 70% Glutaraldehyde
25 mgm CaCl₂
to 50 ml Distilled water

D. Phosphate buffered saline

8.00 gm NaCl
0.20 gm KCl
2.89 gm Na₂H₂PO₄
100 ml Distilled water
E. Saline-A

800 mgm NaCl
40 mgm KCl
100 mgm Glucose
35 mgm NaHCO₃
0.5 mgm Phenol red
100 ml Distilled water
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