Quantitative Studies of Oncornaviruses in Thin Sections

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SUMMARY

A thin sectioning procedure was used to detect and enumerate oncornavirus particles in tumour-cell culture fluids, tumour homogenates, mouse blood plasma, mouse and human milk specimens, and density gradient fractions of these specimens. Oncornaviruses studied included mouse mammary tumour, murine sarcoma, ESP-I, and RD-II viruses. In this procedure particles were sedimented on to small membrane filter discs in the ultracentrifuge using inexpensive commercially available adapters and tubes. Particles in cross sections of the disc were counted and their total number determined by relating the effective surface area of a field to the surface area of the entire membrane disc. Particles may be reliably identified and counted in preparations containing predominately cellular debris. Linear dose response plots were obtained in serial dilution experiments utilising vaccinia virus, adenovirus type 2, and murine sarcoma virus, demonstrating the reliability of the procedure and its wide applicability. The counts obtained for adenovirus and vaccinia virus preparations were comparable to counts obtained for the same preparations in other laboratories by established methods. Statistically reliable counts have been obtained using sample vol. of 0.01 ml or less.

INTRODUCTION

The most commonly used procedures for counting virus particles in the electron microscope are essentially modifications of either the spray droplet method of Williams & Backus (1949) or agar sedimentation method of Sharp (1949). These methods, reviewed by Sharp (1965), involve the counting of negatively stained (Watson, 1962; Smith & Melnick, 1962) or metal shadowed particles which have been suitably deposited on an electron transparent film. Although negative staining or shadowing aids in virus identification, it has become increasingly evident to many investigators that reliable recognition of some virus particles may be accomplished only in thin-sectioned specimens where characteristic virus internal structures may be seen. This is particularly true of specimens containing few particles in relation to non-virus components. For example, attempts to determine if RNA tumour viruses are secreted in human milk have led to a complex and possibly erroneous classification of particulate components observed following negative staining (Sarkar & Moore, 1972; Calafat & Hageman, 1973). According to these investigators, it is not certain that any of these particles are really oncornaviruses. The potential role of viruses in the genesis of human breast cancer is not likely to be determined until more reliable and sensitive morphological methods are available for their detection.

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Recently, Gehle & Smith (1970) reported a method for counting virus particles in thin sections of high-speed pellets. The problem of recognizing virus particles was overcome, but the technique has some drawbacks which limit its applicability. Enough particles or particles combined with cellular debris are needed so that a visible pellet can be obtained and its vol. measured. The embedded pellet must be cut out and re-embedded to achieve appropriate orientation prior to sectioning. Moreover, impure samples of particles are difficult to quantitate since they tend to form stratified pellets.

The procedure described in the present report combines the advantages of thin sectioning for particle counting with the use of membrane filters for support of sedimented virus particles (McCombs, Benyesh-Melnick & Brunschwig, 1968). The procedure permits quantitation of virus particles in impure samples containing as few as $10^6$ particles. It has been found sufficiently sensitive to detect virus-like particles in human milk specimens containing less than $10^6$ particles.

**METHODS**

*Specimen preparation.* Millipore membrane filter discs (Type VF, 25 nm) of 4 mm diam. were punched from larger filters supported on a small rubber mat using a length of sharpened stainless-steel tubing of 4 mm inside diam. Discs were positioned glossy side up on top of reusable flattened supports made of Epon embedding media in 3/8 x 1 1/8 in. nitrocellulose centrifuge tubes (Fig. 1). The upper portion of a disposable 1 ml glass pipette conveniently serves as a plunger for loading tubes with supports and membranes. Virus in a known vol. was then sedimented on to membrane discs in a Spinco SW 39 L, SW 50 L, or SW 50.1 rotor (Centrifuge tubes and adapters to hold them may be purchased from Beckman Instruments, Palo Alto, California, U.S.A.). After centrifuging, the supernatant fluid was removed and the sample was fixed in phosphate buffered 3 % glutaraldehyde for 1 h at 4 °C. The centrifuge tube was slit open with a razor blade and the membrane disc with sedimented virus was removed and washed in phosphate buffer. Specimens were post-fixed in buffered osmium tetroxide, dehydrated in a graded series of isopropl alcohols, cleared in toluene, and flat embedded in an Epon–Araldite mixture. The use of isopropanol and toluene is recommended in preference to ethanol and propylene oxide. Swelling and curling of membranes occurs if the latter are used (Friedman, Blais & Shaffer, 1968).

After polymerization, membranes with sedimented virus were orientated in the microtome so that sections could be cut perpendicular to the plane of the membrane. Sections of membranes were then mounted perpendicularly to the grid wires of 200- or 300-mesh parallel wire grids (Fig. 2), double stained with uranyl acetate and lead citrate, stabilised with carbon, and examined in an electron microscope.

*Calculations.* In all methods involving counting of sedimented particles, virus concentration was calculated by relating the vol. counted to the total vol. of virus particle suspension. In the present method counts were determined in a manner similar to that used by Gehle & Smith (1970). However, the membrane surface area rather than pellet vol. was used and the portion of a particle which must be present in a given section to enable reliable recognition was also taken into consideration. For example, in order for a particle to be recognized and counted, it has been estimated that approximately half of an adenovirus particle, a third of an oncornavirus particle, and a quarter of a vaccinia virus particle must lie within the section examined.

Thin sections of virus will invariably include some particles which lie only partially in the section. Therefore, a given thin section effectively samples a portion of the specimen somewhat thicker than the section itself. The manner in which this ‘effective section thickness’
is determined may be explained with the aid of Fig. 3. The diagram depicts particles having a diam. approximately equal to the section thickness and is analogous to the relationship of oncornavirus diam. to section thickness. If it is assumed that a third or more of the particle diam. must extend into the section for reliable recognition to be made, it can be seen that particles whose centres lie outside the dotted lines would not be recognized or counted. Conversely, all particles whose centres lie between the dotted lines would be counted. The effective section thickness, $T'$, may be determined from the equation

$$T' = T + D_p - 2fD_p,$$

where $T$ is the actual section thickness, $D_p$ is the particle diam., and $f$ is the estimated fraction of a particle required for accurate recognition. In the present study effective section thicknesses, $T'$, were 0.09 $\mu$m for adenovirus, 0.123 $\mu$m for oncornavirus, and 0.215 $\mu$m for vaccinia virus. Vaccinia virus particles were treated as if they were spherical particles with a diam. of 0.25 $\mu$m in calculations.

The actual section thickness may be controlled as follows. When selecting thin sections of sedimented virus for particle counting, particular care must be exercised so that only sections of consistent thickness are utilized. In the present study only sections of pale gold colour were collected. According to Peachey (1958) this interference colour corresponds to a section
thickness of approximately 900 Å. The error in estimates of section thickness is minimized by averaging particle counts of fields selected from several different pale gold sections.

The average area of fields in which virus particles are counted is determined from the effective section thickness, \( T' \), and the length of the field examined, \( L \). Field length is limited by the lowest magnification consistent with virus particle recognition and may be measured against a standard diffraction grating replica. Most thin-sectioned particles photographed at a magnification of 1500 to 2000 x may easily be recognized and counted if negatives are projected onto a screen or in a device such as an optical comparator. Superposition of a grid of fine lines on projected negatives simplifies virus particle enumeration.

The total number of particles sedimented on to a membrane disc \( (V_m) \) may be calculated from the total surface area of the disc and the average effective area of a field \( (LT') \). Accordingly,

\[
V_m = \frac{\pi(\frac{1}{2}D)^2 V_f}{LT'}
\]

where \( D \) is the diam. of the membrane disc and \( V_f \) is the average number of particles counted in fields of length \( L \) and thickness \( T' \). By substituting the appropriate values in this equation, the number of particles in the sample is calculated and then expressed per unit vol., per unit weight, or on any other basis desired.

Viruses. Crude tissue culture fluid containing vaccinia virus which had been counted by the agar sedimentation method (Sharp & Beard, 1952) was kindly provided by Dr D. G. Sharp (University of North Carolina School of Medicine, Chapel Hill, North Carolina, U.S.A.). Celite filtered tissue culture fluid containing adenovirus type 2 which had been counted by a modified agar sedimentation method (Smith & Melnick, 1962) was kindly provided by Dr K. O. Smith (University of Texas Medical School, San Antonio, Texas, U.S.A.). Preparations of Soehner-Dmochowski strain murine sarcoma virus (MSV-SD, Soehner-Dmochowski, 1969) and other RNA tumour viruses were produced in our own laboratories. Murine mammary tumour virus (MMTV) in milk and tumour tissues was
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Table 1. Precision obtained by the thin sectioning procedure

<table>
<thead>
<tr>
<th>Vol. per membrane (µl)</th>
<th>Mean count (particles/field*)</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Particles/field % of mean</td>
</tr>
<tr>
<td>Vaccinia virus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>57</td>
<td>6.5</td>
</tr>
<tr>
<td>50</td>
<td>153</td>
<td>3.8</td>
</tr>
<tr>
<td>150</td>
<td>484</td>
<td>11.4</td>
</tr>
<tr>
<td>Adenovirus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>119</td>
<td>15.4</td>
</tr>
<tr>
<td>150</td>
<td>300</td>
<td>26.1</td>
</tr>
<tr>
<td>500</td>
<td>1357</td>
<td>51.9</td>
</tr>
<tr>
<td>MSV-SD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.75</td>
<td>120</td>
<td>11.3</td>
</tr>
<tr>
<td>2.5</td>
<td>309</td>
<td>17.2</td>
</tr>
<tr>
<td>7.5</td>
<td>798</td>
<td>30.4</td>
</tr>
</tbody>
</table>

* Field length = 50 µm.

obtained from RIII/Dm strain mice. Human milk specimens used in this study were kindly provided by Dr M. Brennan and associates of the Michigan Cancer Foundation (Detroit, Michigan, U.S.A.).

RESULTS

The reliability of this particle enumeration method has been evaluated in two ways: (i) by analysis of dose–response data obtained by varying the amount of virus per membrane disc, and (ii) by comparing the counts obtained in the thin section procedure with those derived in two other laboratories by standard methods.

Crude tissue culture fluid containing vaccinia virus, Celite-filtered culture fluid containing adenovirus type 2, and polyethylene glycol concentrated MSV-SD from the 1.16 g/ml region of a sucrose gradient were diluted in PBS. Virus particles in 0.5 ml vol. of fluid were sedimented on to membrane discs by centrifuging for 90 min at 50,000 rev/min in a Spinco SW 50.1 rotor. Vaccinia virus particles may be seen in Fig. 4(a) and (b), adenovirus particles in Fig. 4(c) and (d), and MSV-SD particles in Fig. 6(d). All preparations contained cellular debris, but particles could easily be identified and counted.

The precision obtained by the thin section counting procedure is demonstrated in Table 1. For each dilution of the above virus preparations four fields were randomly selected from different thin sections. Average counts per field are listed along with the corresponding standard deviations (s.d.) which ranged from 2.4 to 12.9 % of mean values. In each case the relative s.d. was smallest in the group of fields containing the greatest number of particles.

Regression plots of the average number of particles per field against vol. added per membrane are shown in Fig. 5 for vaccinia virus, adenovirus, and MSV-SD. In each case a linear relationship was obtained. These data indicate that precise counts may be computed using specimens containing 50 to 1500 particles per 50 µm long field. Virus counts could be determined with as little as 15 µl of unconcentrated vaccinia virus supernatant culture fluid, or less than 1 µl of purified MSV-SD suspension. While the number of particles needed is similar to that required for agar sedimentation methods, it is substantially less than that needed to examine virus pellets by other thin section methods. The use of a membrane for supporting the particles during processing is the chief factor which permits the use of small quantities of virus.

From the values given in Table 1, concentrations of vaccinia and adenovirus particles were calculated and compared to the concentrations derived by the agar sedimentation method (Table 2). Vaccinia virus concentrations of $3.8 \times 10^9$ and $4.5 \times 10^9$ particles/ml were obtained.
Fig. 4. (a) Unpurified vaccinia virus particles sedimented on to a membrane disc (M) for counting. (b) Vaccinia virus particles, easily distinguished among cellular debris, are shown at higher magnification. (c) Adenovirus type 2 particles; only particles in which internal structures are evident were counted. (d) Adenovirus type 2 particles resting on a membrane disc (M) for particle counting.

by the thin section and agar sedimentation procedures, respectively. Adenovirus concentrations of $7.6 \times 10^9$ and $1.1 \times 10^{10}$ particles/ml were obtained by the two respective techniques. These results clearly show that counts derived by the thin section method are comparable to counts obtained by established methods.
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Fig. 5. Regression plots showing numbers of particles counted per field against vol. added per membrane disc for adenovirus type 2, MSV-SD and vaccinia virus. Data were plotted by method of least squares.

Table 2. Comparison of virus particle counts obtained by the thin section and the agar sedimentation procedures

<table>
<thead>
<tr>
<th>Virus Type</th>
<th>Thin Section</th>
<th>Agar Sedimentation</th>
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</thead>
<tbody>
<tr>
<td>Vaccinia virus</td>
<td>3.8 x 10^9</td>
<td>4.5 x 10^8 *</td>
</tr>
<tr>
<td>Adenovirus type 2</td>
<td>7.6 x 10^9</td>
<td>1.1 x 10^10 †</td>
</tr>
</tbody>
</table>

* Counted in the laboratory of Dr D. G. Sharp.
† Counted in the laboratory of Dr K. O. Smith.

Wide application of the thin section procedure has been found in studies of RNA tumour viruses. For example, virus yields in the culture fluid of several continuous cell lines chronically infected with oncornaviruses have been evaluated. MSV-SD, produced by a cell line derived from a rat bone lesion occurs at titres of 1 to 3 x 10^8 particles/ml in the supernatant fluid of confluent cell cultures. RD-114 virus, a candidate human oncornavirus (McAllister et al. 1972) has been found to grow to a concentration of 1.1 x 10^8 particles/ml. The thin section counting procedure has also been applied to culture fluid from the ESP-1 cell line which produces ESP-1 virus, another candidate human oncornavirus (Priori et al. 1970). Although this culture is contaminated with mycoplasma whose elementary particles are similar in size and shape to oncornavirus particles and outnumber the ESP-1 particles by a wide margin, quantitation of the particles has been possible. Comparatively low counts of 10^5 to 10^6 particles/ml have been found.

Besides studies of virus particles in tissue culture fluids, the thin section procedure has also been applied to viruses from other sources. One application in which this procedure has proven useful is in the quantitation of particles in tumour tissues. Fig. 6(c) shows type B particles extracted from the mammary tumour of an RIII/Dm mouse. This tumour contained 6.7 x 10^11 virus particles per g of tissue. Note that virus particles may be distinguished easily from cellular debris of diverse configurations.

Extensive use of the technique has also been made in studies of murine mammary tumour
Fig. 6. (a) Virus particles partially purified from RIII/Dm strain mouse milk resting on a membrane disc (M). (b) Type B particles from RIII/Dm strain milk; Particles are well preserved and easy to distinguish among debris. (c) Virus extract from an RIII/Dm strain mouse mammary tumour; Particles are more dispersed than in (b) due to large amount of debris. (d) MSV-SD particles resting on a membrane disc (M) for particle counting.
Fig. 7. Virus-like particles detected in human milk specimens by the thin section method. (a-g) Particles resembling type C oncornaviruses. (h-p) Particles resembling type B oncornaviruses.

virus from mouse milk. An example of this application is illustrated in Fig. 6(a). Partially purified type B virus particles from the milk of RIII/Dm strain mice are shown resting on a membrane disc. Virus from clarified milk (Lyons & Moore, 1965) was partially purified by sedimentation through a Ficoll gradient. Fig. 6(b) shows a portion of the RIII milk virus preparation at higher magnification. Virus particles were morphologically well preserved and easily distinguished from debris. This milk specimen contained $5.7 \times 10^8$ particles/ml
assuming no loss during purification. Particle counts determined over a period of time have allowed for precise monitoring of virus particles in milk produced by a mouse colony. The technique has also been used to evaluate procedures for purifying human milk virus particles.

In addition to the usefulness of this procedure for particle counting, the membrane-sedimentation principle can also be applied to studies in which the goal is to detect rather than quantitate virus particles. Only a few minutes are required to examine each field and a sample containing $2 \times 10^6$ oncornavirus particles would result in the observation of approximately 1 virus particle in a field 50 $\mu$m long. This calculated sensitivity and the uniform deposition of particles on membranes permits detection of fewer than $2 \times 10^6$ virus particles by examining many fields in a given specimen. As a result of this sensitivity it has been possible to detect particles in the blood plasma of mice of RIII/Dm and C3H strains in which the counts were less than $10^6$ particles/ml.

Particles resembling murine mammary tumour virus have been observed in thin sections of human mammary tumour tissues (Dmochowski et al. 1968) and in human milk specimens by negative staining (Sarkar & Moore, 1972) and by thin sectioning (Feller & Chopra, 1971). Definite recognition of virus particles in impure negatively stained milk specimens has been difficult and there has been little correlation obtained between thin sectioning and negative staining methods (Sarkar & Moore, 1972; Calafat & Hageman, 1973).

Because of its demonstrated reliability and calculated sensitivity, the thin section technique described here has been applied to several human milk specimens. Milk from 19 normal women with family medical histories of breast cancer, other types of cancer, or no cancer at all were partially purified and concentrated in discontinuous Ficoll gradients and sedimented onto membrane discs. Fig. 7 shows examples of virus-like structures detected in 9 of these milk specimens. Particles depicted in Fig. 7(a–g) resemble type C oncornavirus particles and those in Fig. 7(h–p) resemble type B oncornavirus particles. Particles were few in number and present in the milk of women with and without family medical histories of cancer. These data are part of a continuing study and no conclusions have yet been reached relevant to the breast cancer disease. The application of this technique should enable the electron microscopic evaluation of human milk specimens with more confidence than has previously been possible.

**DISCUSSION**

The procedure described here offers a simple means for determining virus particle counts in samples containing as few as $10^6$ virus particles and for detecting virus in specimens containing less than $10^6$ particles. It yields morphologically well preserved specimens in which virus particles can readily be distinguished from cellular debris and is the only thin section particle counting method available in which samples of concentrated virus suspensions as small as 1 $\mu$l may be enumerated. Consequently, valuable virus preparations can be conserved for additional biological, biochemical or immunological experimentation.

The only disadvantage is that the preparation time takes longer than some other procedures. However, sample preparation is less complicated than in a previously reported thin section method (Gehle & Smith, 1970) and abbreviated dehydration and embedding schedules may be used since sedimented preparations are usually quite thin. If desired, virus suspensions may be counted within 24 to 36 h by this procedure.

This procedure has been successfully applied to preparations of virus purified in sucrose, glycerol, and Ficoll gradients. Ficoll has been used preferentially since the osmotic pressure exerted by Ficoll solutions is much lower than that exerted by sucrose or glycerol (Lyons
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& Moore, 1965). Virus particles are subjected to less osmotic stress and morphological preservation may be superior. No particular problems with loss of samples due to lack of adherence to membranes were experienced. It seems probable, however, that some highly purified samples containing little or no cellular debris might lack coherence and tend to become dislodged from membranes during handling. Should this be a problem, erythrocytes can be sedimented on top of virus particles to hold them in position (Gehle & Smith, 1970).

The shape of centrifuge cells in which particles are sedimented on to a flat surface affects the uniformity of distribution of particles. Ideally, sector shaped cells should be used in which the walls conform to the radii of the centrifuge rotor (Sharp, 1949). However, a tendency for sedimented virus particles to be concentrated at the periphery of a cylindrical tube decreases as the ratio between tube diam. and rotor radius approaches zero. In the present method the error resulting from the use of a cylindrical tube has been considered negligible since the inside diam. of tubes was only 4 mm while the radius is 98 mm or greater. The small standard deviations obtained upon counting fields from several different thin sections indicate that error due to uneven distribution is quite small.

During preliminary experimentation, Millipore membrane filters of a wide variety of pore sizes as well as polycarbonate membranes (General Electric Co., Nucleopore & Filtration Products, Pleasanton, California, U.S.A.) were employed. Although virus particles in partially purified preparations did not penetrate 0.22 μm Millipore membranes, filters with a pore size of 50 nm or less were preferred since their glossy surfaces were less irregular. Polycarbonate membranes, although desirably thinner, had a tendency to curl during dehydration. They became transparent and, in general, were difficult to handle.

There is no doubt that virus particles of types other than those reported here could be quantitated by this procedure. The effective section thickness, T', would have to be adjusted in accordance with virus particle diam., actual section thickness, and the fraction of a particle required in a section for recognition. The actual section thickness rather than effective section thickness could be used in calculations for small virus particles.

Preliminary results obtained through the use of this technique were presented at a meeting of the Electron Microscopy Society of America (Miller et al. 1972). We thank Dr D. G. Sharp and Dr K. O. Smith for providing and enumerating virus suspensions used in this study. We thank Dr M. Brennan, Dr S. Albert and Dr M. Rich for providing us with human milk specimens. We are also indebted to Dr J. Purifoy and Dr C. Walter for helpful advice in deriving formulae. The excellent technical assistance of J. Chesner, R. Collins, D. C. Hixson, and W. C. Williams is gratefully acknowledged.

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