ESTIMATION OF THE PURITY OF PREPARATIONS OF ELEMENTARY BODIES OF VACCINIA

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Investigations on the nature of viruses were hampered for many years by the impurities of virus suspensions. This difficulty was surmounted in the case of vaccine virus by Craigie's introduction of a technique (1) for obtaining large amounts of relatively pure elementary bodies, the minute structures that are now considered to constitute the virus of vaccinia. In order to establish the real value of certain studies of the chemical nature of vaccine virus it is essential to have a simple method for the estimation of the purity of the virus preparations. Knowledge in this field has advanced to the stage where we believe that such an estimation can be made by the determination of the ratio of the number of infective units in a given preparation to the total number of the virus particles or elementary bodies. For instance, it has been shown (2) that the number of elementary bodies of vaccinia, determined by enumeration, can be correlated with the infective titer of a suspension of virus. Furthermore, Parker (3) recently published evidence which strongly suggests that a single active particle of the virus is capable of producing infection under the proper circumstances. It remained to be determined, however, what proportion of the virus particles is active, i.e., the minimal number of washed elementary bodies that must be present in a sample in order to insure the inclusion of a single infective unit. The present report throws some light on this question.

Materials and Methods

Preparation of Suspensions of Elementary Bodies.—The C.L. strain of virus, obtained from Dr. Craigie a number of years ago and maintained in this laboratory by continuous rabbit dermal passage, was used throughout the experiments. The technique used by Craigie (1) and by Parker and Rivers (4) for the preparation of elementary bodies from the skin of infected rabbits was followed. Other workers (5) have modified this technique in such a manner as to increase the relative amount of impurities in the final preparation. Although the modification may increase the yield of virus, we have not employed it in the present studies since highly purified preparations were desired. In
fact, the importance of discarding the sedimented material obtained by horizontal centrifugation in the stages before and after the process of washing the virus in the angle centrifuge should be emphasized. Accordingly, in the method used by us the material from four rabbits, following the third sedimentation in the angle centrifuge, was resuspended in 160 cc. of dilute citric acid-disodium phosphate buffer solution, pH 7.2, and distributed in four 50 cc. centrifuge tubes. The tubes were run in a horizontal centrifuge at 3000 R.P.M. for 40 minutes. In order to avoid agitation of the sediment at the end of the run, the head of the centrifuge was permitted to coast to a stop; this was accomplished by moving the brake lever just far enough to disengage the brushes from contact with the armature. The supernatant fluid containing the monodispersed washed elementary bodies was carefully removed with a 50 cc. volumetric pipette. Several cc. of fluid were left in each tube above the 0.3 to 0.4 cc. of white sediment made up of agglutinated elementary bodies, bacteria, and debris; the sediment was usually saved for other purposes. 5 cc. of ethyl ether were added to the flask containing the suspension of washed elementary bodies before it was stored in the ice box at 3°C. for a period of several days to several weeks.

Additional Treatment of the Elementary Bodies.—The accumulated lots of washed elementary bodies in suspension were pooled at intervals of 2 to 4 weeks. The elementary bodies in this pool were thrown down in the concentration centrifuge of Bauer and Pickels (6) at 15,000 R.P.M. for 12 minutes. This treatment was sufficient to sediment completely the elementary bodies. The entire amount of virus was then resuspended in 50 cc. of dilute citric acid-disodium phosphate buffer, pH 6.0, and again sedimented in the ultracentrifuge. In a similar manner the virus was washed two additional times, once with 50 cc. of dilute buffer, pH 8.0, and once with 50 cc. of distilled water. The concentration and washing described above required an entire day. At this point the virus was again taken up in 50 cc. of distilled water and stored in the ice box overnight. The following morning the material was sedimented once more. Sufficient distilled water, approximately 8 cc., was added to the sediment in order to facilitate its transfer to a single lusteroid centrifuge tube. After another centrifugation the supernatant fluid was removed, and the tube containing the packed virus was placed in CO₂ ice for a few minutes after which the virus was dried in vacuo from the frozen state. In order to insure adequate dehydration, the dried virus was then placed under vacuum over phosphorus pentoxide in an incubator at 50°C. for a day, after which it was stored in a cold room, under vacuum in the same desiccator for from several weeks to a month before it was weighed on a microbalance.

Titration of Virus.—The infective titer of each lot of virus, i.e., each 160 cc. lot from four rabbits, was determined. Usually this was carried out at one time with all the lots immediately before they were put into a given pool. Tenfold dilutions of each lot were prepared and duplicate,—in a few instances quadruplicate,—intracutaneous injections of 0.25 cc. of the dilutions 10⁻⁶ to 10⁻¹⁰ were made in rabbits. No significant variation in the titer of different lots was noted. Hence each lot was considered an aliquot of the pool and the results of the several titrations were grouped. The number of infective units in the pool was then calculated on the basis of the 50 per cent end point method (2, 3).

Determination of Number of Bacteria in Suspensions of Washed Elementary Bodies.—The number of bacteria in each lot of elementary bodies was determined by plating on blood agar 0.25 cc. of the suspension before ether had been added. The number of
bacterial colonies on each plate varied from none to several hundred, but usually ranged between 5 and 15. The vast majority of the organisms were staphylococci of the \emph{aureus} and \emph{albus} groups. Most of the bacteria were no longer viable several weeks after ether had been added to the suspension. This was not invariably true, however, for a Gram-negative bacillus with most of the characteristics of \emph{Eberthella xenopa} has contaminated certain of the preparations during several periods in the last two years. This organism was rarely detectable in the original cultures, but increased profusely during storage. For this reason each lot of virus was again tested by culture prior to its addition to a pool; if \emph{Eberthella xenopa} was encountered the lot was discarded. The total number of bacteria in a given pool was estimated on the basis of the maximal counts for each lot of virus.

A rough estimate of the dry weight of the bacteria mixed with the pooled virus was made in order to know the relative proportion of bacteria and virus. Dr. René Dubos supplied the following data on pneumococci: One liter of culture contains approximately \(3 \times 10^{11}\) viable bacteria which when washed and dried weigh about 150 mg. Hence \(5.0 \times 10^{-13}\) gm. was considered as the weight of a dried pneumococcus and this value was used to estimate the weight of the dry bacteria in each elementary body preparation.

**Theory and Equations**

In previous experiments (7) elementary bodies of vaccinia suspended in different media were subjected to ultracentrifugation. The density of the particles, when suspended in dilute buffer solution, pH 7.2, was found to be 1.16 gm. per cc. and their minimal diameter in the same medium was calculated at \(236 \times 10^{-7}\) cm. On the basis of this minimal value, the volume of a single elementary body, which is approximately spherical, is \(6.88 \times 10^{-15}\) cc. Elementary bodies were shown earlier (8) to contain enough nitrogen to account for 83 per cent of their dry weight in the form of protein. The chief remaining constituents were fat, 8.5 per cent, and residual moisture, 5.5 per cent; this amount of water was not extracted in the course of the desiccation employed in the present experiments. 0.72 per cent of ash and about 2 per cent of undetermined material accounted for only a small portion of the dried elementary bodies; therefore, the density of the dried bodies may be computed without sensible error without a consideration of these materials. The densities of protein, fat, and residual moisture may be taken respectively as 1.325, 0.92, and 1.0 gm. per cc. The average density of the dried elementary body, \(D_d\), is of course the combined masses of the constituents divided by their combined volumes, and since the volume of each is its mass divided by its density, we have:

\[
D_d = \frac{83 + 8.5 + 5.5}{83 + 8.5 + 5.5} = \frac{97}{62.6 + 9.3 + 5.5} = \frac{97}{77.4} = 1.26 \text{ gm. per cc.}
\]

If the presence of residual moisture in the dehydrated elementary body is neglected entirely then its density would be 1.27 gm. per cc.

A comparison of the volume of a dried elementary body with that of a hydrated particle, \emph{i.e.}, suspended in buffer solution, may be arrived at in the following manner: The volume of the dried particle is equal to the volume of the hydrated particle minus the volume of the extracted water. An analogous relation exists between the masses.
Thus, \( V_d = V_h - V_w \) and \( M_d = M_h - M_w \). Therefore, \( M_d = M_h - V_w \) since the density of water = 1 gm. per cc. The last relation shows that \( V_w \) is equivalent to \( (M_h - M_d) \) and when this value is substituted into the first of these equations, we have:

\[
V_d = V_h - M_d = V_h - V_h D_h + V_d D_d \ 	ext{ since } D_h = \frac{M_h}{V_h} \text{ and } D_w = \frac{M_w}{V_w}.
\]

Therefore,

\[
V_d = V_h \left(\frac{D_h - 1}{D_h - 1}\right).
\]

Substituting the above values of \( V_h, D_h, \) and \( D_d \):

\[
V_d = 6.88 \times 10^{-15} \times (0.16) = 6.88 \times 10^{-15} \times 0.615 = 4.24 \times 10^{-15} \text{ cc.}
\]

The masses of both the hydrated and dried elementary bodies are readily obtained by the multiplication of their volumes by the respective average densities; the values are \( 8.00 \times 10^{-15} \) gm. for an elementary body suspended in dilute buffer solution and \( 5.34 \times 10^{-15} \) gm. for an elementary body dehydrated by the method employed in the present work.

It is interesting that the above calculations indicate that the maximal amount of water which can be extracted by desiccation at 50°C. is approximately 38.5 per cent of the particle’s volume. This is in line with earlier experiments (7) in which it was found that the amount of water extracted from elementary bodies when placed in a 53 per cent solution of sucrose corresponds to at least a third of the original volume of the particle.

RESULTS

Elementary bodies of vaccinia were prepared as described above during a period of 3½ months, and the lots of virus were placed in consecutive pools. The number of infective units in each pool was determined from the volume of the pool and its infecting titer. The number of dry elementary bodies was estimated by dividing the calculated mass of a single dehydrated elementary body, i.e., \( 5.34 \times 10^{-15} \) gm., into the dry weight of the virus material. Finally, the ratio of the experimentally determined number of infective units to the estimated number of elementary bodies was then obtained. The data on seven consecutive pools of virus are summarized in Table I.

The number of infective units in 0.25 cc. of the pooled suspensions of virus ranged between \( 10^9 \) and \( 10^{9.5} \). The yield of dry virus material per rabbit varied in the different pools between 6.5 and 9.4 mg. and averaged 8.0 mg.; this factor bore no relationship to the ratio of infective units to elementary bodies. The number of bacteria in each pool was small and their dry weight was insignificant. The highest ratio of the number of infective units to elementary bodies, 1:2.4, was obtained with pool 1; the lowest, 1:9.2, was observed in pool 7. An average value for the ratios of the seven pools was 1:4.2.
A comparison was made between the number of infective units of vaccine virus and the number of elementary bodies in seven preparations. The former value was obtained from the results of titrations of infectivity and the latter by estimations of the number of elementary bodies from the dry weight of the preparations. This estimation was based on a calculated weight for a single desiccated elementary body, viz., $5.34 \times 10^{-15}$ gm., and on the assumption that the preparations contained only elementary bodies.

**TABLE I**

Summary of Data on Seven Preparations of Elementary Bodies of Vaccinia

<table>
<thead>
<tr>
<th>No. of rabbits</th>
<th>E.B. Pool</th>
<th>Volume</th>
<th>Infective units per ml</th>
<th>Total infective units</th>
<th>Total dry weight per ml</th>
<th>Calculated number of E.B.</th>
<th>Total bacteria in pool</th>
<th>Estimated weight of dry bacteria</th>
<th>Ratio of infective units to E.B.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20</td>
<td>200</td>
<td>$10^{8.05}$</td>
<td>$10.11 \times 10^{12}$</td>
<td>131.0.65</td>
<td>$24.5 \times 10^{4}$</td>
<td>3.0 $\times 10^{4}$</td>
<td>0.000015</td>
<td>1:2.4</td>
</tr>
<tr>
<td>2</td>
<td>23</td>
<td>920</td>
<td>$10^{8.16}$</td>
<td>$9.72 \times 10^{12}$</td>
<td>151.0.66</td>
<td>$28.3 \times 10^{4}$</td>
<td>2.2 $\times 10^{4}$</td>
<td>0.000010</td>
<td>1:2.9</td>
</tr>
<tr>
<td>3</td>
<td>284150</td>
<td>109.84</td>
<td>$10.48 \times 10^{12}$</td>
<td>263.3.94</td>
<td>49.2 $\times 10^{4}$</td>
<td>7.0 $\times 10^{4}$</td>
<td>0.000035</td>
<td>1:4.7</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>241000</td>
<td>109.42</td>
<td>$10.76 \times 10^{12}$</td>
<td>212.1.81</td>
<td>39.7 $\times 10^{4}$</td>
<td>4.2 $\times 10^{4}$</td>
<td>0.000021</td>
<td>1:3.7</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>16400</td>
<td>109.84</td>
<td>$8.09 \times 10^{12}$</td>
<td>129.8.81</td>
<td>24.2 $\times 10^{4}$</td>
<td>2.6 $\times 10^{4}$</td>
<td>0.000013</td>
<td>1:3.0</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>16405</td>
<td>109.84</td>
<td>$7.58 \times 10^{12}$</td>
<td>151.0.94</td>
<td>28.3 $\times 10^{4}$</td>
<td>6.0 $\times 10^{4}$</td>
<td>0.0003</td>
<td>1:3.7</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>281100</td>
<td>109.80</td>
<td>$4.40 \times 10^{12}$</td>
<td>217.9.7.8</td>
<td>40.6 $\times 10^{4}$</td>
<td>2.6 $\times 10^{4}$</td>
<td>0.000013</td>
<td>1:9.2</td>
<td></td>
</tr>
</tbody>
</table>

Infective titers were determined by the 50 per cent end point method. A single dry elementary body was considered to have a weight of $5.34 \times 10^{-15}$ gm. and a single dry bacterium $5.0 \times 10^{-13}$ gm.

* 20/20 inoculations of the $10^{-9}$ dilutions were positive but since $10^{-10}$ dilutions were not tested, the titer was considered as $10^{-9.5}$.

† Elementary bodies in this pool were washed in the angle centrifuge but additional washing in the ultracentrifuge was omitted.

The average value in seven experiments for the ratio of infective units to elementary bodies was 1:4.2. We were surprised to find such a close relationship between these two factors. Of course, under ideal circumstances each elementary body might be expected to produce infection (3), but these conditions are not likely to be attained in experiments of this type. Errors in methods of titration of infectivity are well recognized, even when the best available techniques are employed. Aggregation of particles would decrease the titer of a given material but would not affect the calculated number of elementary bodies. The fact that varying proportions of elementary bodies in different suspensions may be in a state of aggregation, even though agglutination is not evident macroscopically, was in-
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dicated in previous experiments (7). Furthermore, a certain amount of error might be expected to be introduced from the use of the calculated value for the dry weight of a single elementary body; for in each instance where a choice of values was possible, the figure taken was the one which would give the largest estimated number of elementary bodies in the dry material. It is also worth noting that the general result is not seriously altered when appreciably different values are assigned to the physical constants of the elementary body. For instance, if the average density of a dried particle is taken as 1.325 gm. per cc., (the same as that of protein), instead of 1.26 gm. per cc., the average ratio of infective units to elementary bodies is computed to be 1:4.9 instead of 1:4.2. Finally, the virus of vaccinia, although relatively stable, does become inactivated when stored. Therefore, a certain number of elementary bodies undoubtedly had lost their infectivity during manipulation and storage. In view of conditions just cited, we are inclined to regard the preparations investigated as being composed almost entirely of active and inactive elementary bodies; that is, we believe that only relatively small amounts of non-viral material were present.

The data on correlation of infective units and elementary bodies, obtained by Parker and Rivers (2) on suspensions of elementary bodies of vaccinia, are not of the type which can be compared with the present results. These authors state: "... the portion of the curve which has been defined is not sufficiently long, nor is its probable error sufficiently small to warrant assumption as to its true form. For this reason it is not justifiable to produce the curve to its base and to state that an infectious unit of virus contains, on the average, 41.9 elementary bodies." The present experiments do, however, lend strong weight to Parker's (3) results which tend to show that a single active particle of virus is capable of producing infection.

An application of the method of calculation described above to earlier data recorded from these laboratories (8) for suspensions of elementary bodies of vaccinia prepared by the same technique, but titered for infectivity without the benefit of the 50 per cent end point method, gives a ratio of 1:31. On the other hand, data on suspensions of elementary bodies of vaccinia which MacFarlane and Salaman (5) obtained by a somewhat different technique give entirely different results. These workers suspended the washed material from each rabbit in 10 cc. of fluid and characterized the suspensions as follows: "They contain, as a rule, from 20 to 30 mg. of dry matter per rabbit, but as much as 60 mg. have been obtained... Their infective titters, as judged by the intracutaneous injection of 0.20 cc. of serial tenfold dilutions into the rabbit, vary between $1 \times 10^4$ and $1 \times 10^8$."


If one uses the values 10 cc., $10^8$ units in 0.2 cc., and 25 mg. to represent the volume, titer, and dry weight of a lot of virus and employs these in a calculation of the type applied to the present experiments, one obtains a ratio of the order of 1:1000. Such a ratio, we believe, indicates the presence of an excessive amount of impurities in the preparations. Moreover, it is obvious that such preparations should not be used for studies of the nature of the virus of vaccinia.

**SUMMARY AND CONCLUSIONS**

A method of estimating the purity of preparations of elementary bodies of vaccinia is described. It depends on the comparison of the number of infective units of virus in a given material with the number of elementary bodies. The latter figure is estimated from the dry weight of the preparation by means of a calculated value for the weight of a single dehydrated elementary body.

Values for the ratio of infective units of vaccine virus to elementary bodies varied between 1:2.4 and 1:9.2 in seven consecutive experiments; the average was 1:4.2. These ratios indicate a high degree of purity of the preparation. Moreover, they indicate that a relatively high percentage of the elementary bodies in the preparations was infective.

**BIBLIOGRAPHY**