ANALYTICAL DIFFUSION OF INFLUENZA VIRUS AND MOUSE ENCEPHALOMYELITIS VIRUS

By JAQUES BOURDILLON
(From the Eldridge R. Johnson Foundation for Medical Physics, University of Pennsylvania, Philadelphia)
(Received for publication, June 21, 1941)

In a recent publication, (1) a method was described for the analytical study of the diffusion of biologically active material. The present paper reports an attempt to apply this method to the study of two animal viruses, epidemic influenza A and mouse encephalomyelitis.¹

Method

In brief the technique (1) consists in placing a layer of solvent above a layer of solution containing the virus, allowing the latter to diffuse upwards for a few days, sampling at various heights, and determining the virus titer in the samples. The original virus concentration, expressed in lethal doses, or minimal infective doses, per inoculum, is $C_0$, and $C_x$ is the concentration $x$ centimeters above the initial boundary, at the end of the experiment. In order to determine the diffusion constant $D$, $\log \frac{C_x}{C_0}$ is plotted against $x$, and the theoretical curve is drawn which gives the most satisfactory fit. For $x = 0$, $\log \frac{C_x}{C_0}$ is always equal to $-0.3$. (This point, therefore, does not have to be determined by experiment; it is indicated on the charts as a half circle.) The diameter $d$ of the virus particles may then be derived from the diffusion constant, the assumption being made that the diffusing particles are approximately spherical.

In the experiments reported here, the virus concentration at the bottom of the diffusion cell was taken as $C_0$. Sampling was done up to 1.2 or 1.3 cm. above the initial boundary; above this level the fluid usually still contained a trace of virus, but too little for quantitative titration.

Diffusion of Influenza A Virus from Mouse Lung

Material and Titration

The stock material was a suspension of mouse lung tissue infected with the PR8 strain, prepared by grinding infected lungs with alundum and salt solution (usually 0.15 M NaCl or 0.04 M Na borate) so as to make a 10 per cent suspension, centrifuging at 2500 R.P.M. for 30 minutes, and discarding the sediment. This yielded a reddish turbid fluid, containing about 1 per cent protein in solution; much

¹ Part of the experiments reported in this paper were performed at the laboratories of the International Health Division of The Rockefeller Foundation, New York.
of the latter was hemoglobin and blood serum proteins; some was lung tissue protein, characterized by irreversible precipitation below pH 6. The limiting lethal dilution of the suspension for mice was about $10^{-5}$, when inoculated intranasally in 0.05 cc. amounts. In one experiment, (No. 5) the material used was more concentrated: The suspension was made of 10 gm. of lungs ground with 4 cc. of mouse serum and 6 cc. of 0.85 per cent NaCl. The titer was about $10^{-6}$. The drop in titer of these preparations at 4° was about 0.3 log after 2 to 4 days.

For titration, serial decimal dilutions of the diffusion samples were made in 10 per cent horse serum broth; 4 to 6 young albino mice were inoculated intranasally, under ether anesthesia, with 0.05 cc. of each dilution. The 50 per cent mortality end-point was calculated by the method of Reed and Muench (2) and based on the number of dead and surviving mice 10 days after inoculation; the virus concentrations were expressed in lethal units per 0.05 cc.

**EXPERIMENTAL**

In order to determine whether the results obtained were in any way dependent on the conditions of diffusion, the latter were varied as much as possible. They have been summarized in Table I.

The approximate composition of the buffers is given in the second column; they were all alkaline, since the virus is less stable in acid medium. In Experiment 1, a small amount of stock borate buffer (pH about 9.8) was added to the original lung suspension; in Experiment 2, borate buffer and solid NaCl were added; in Experiment 3, the material was first dialyzed against the buffer used, before diffusion. Experiment 4 was prepared as follows: 10 cc. of infected lung suspension in 0.04 M borate was dialyzed for 3 days in the cold against 10 cc. of normal mouse lung suspension in 0.05 M Na phosphate; the

**TABLE I**

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Buffer Composition</th>
<th>pH</th>
<th>molality</th>
<th>Total protein concentration</th>
<th>Control virus titer M.L.D.</th>
<th>Diffused into</th>
<th>Diffusion time (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.12 M NaCl; 0.02 M Na borate</td>
<td>9.5</td>
<td>0.14</td>
<td>1</td>
<td>5.3</td>
<td>Water</td>
<td>188,000</td>
</tr>
<tr>
<td>2</td>
<td>0.55 M NaCl; 0.02 M Na borate</td>
<td>9.5</td>
<td>0.57</td>
<td>1</td>
<td>5.5</td>
<td>Water</td>
<td>153,000</td>
</tr>
<tr>
<td>3</td>
<td>0.04 M NaCl; 0.02 M Na borate</td>
<td>9.7</td>
<td>0.06</td>
<td>1</td>
<td>4.5</td>
<td>Same buffer</td>
<td>243,000</td>
</tr>
<tr>
<td>4</td>
<td>0.025 M Na phosphate; 0.02 M Na borate</td>
<td>8.8</td>
<td>0.045</td>
<td>1</td>
<td>4.7</td>
<td>Normal lung solution in same buffer</td>
<td>430,000</td>
</tr>
<tr>
<td>5</td>
<td>0.15 M NaCl</td>
<td>7.4</td>
<td>0.15</td>
<td>6</td>
<td>6.0</td>
<td>Water</td>
<td>256,000</td>
</tr>
</tbody>
</table>
resulting pH was 8.8. In this manner the salt composition and salt concentration must have become the same in both solutions; furthermore the protein concentration too must have become identical on both sides, due to the osmotic pressure; it is also probable that the protein composition was about the same in the two fluids. The coarser particles were removed from the normal lung suspension by filtration through asbestos, whereas the infected suspension was left unfiltered. Great care was used in dialysis to avoid contamination of the normal fluid, which was the fluid into which diffusion was to take place. Finally, for Experiment 5, a more concentrated virus preparation was used, as described above.

Thus, the salt molarity of the diffusing solutions ranged in these experiments from 0.045 to 0.57; the pH from 7.4 to 9.7. The total protein concentration was in general 1 per cent, in one case 6 per cent. Diffusion was allowed to take place into pure water (Experiments 1, 2, and 5); into buffer (Experiment 3); into buffered normal lung suspension (Experiment 4). Diffusion time was varied from 2 to 5 days.

RESULTS

The results have been plotted in Fig. 1. The ordinates give the difference between the titer of the control (the titer at the bottom of the diffusion cell) and the titer at various levels above the initial boundary; the abscissae measure the distance above the initial boundary.

Examination of Fig. 1 reveals two facts: One is that the results from all five experiments were quite similar in spite of great differences in the conditions of diffusion; the other, that the values obtained obviously did not yield ideal diffusion curves (which should have been convex toward the upper right hand corner of the chart), but broken curves composed of two fairly distinct limbs. Since the results appeared to be completely independent of such experimental conditions as salt concentration, pH, presence or absence of a simultaneously diffusing salt or protein, one may be allowed to draw the conclusion that they were dependent only on the particle size of the virus in the preparation; and since they did not yield ideal diffusion curves, one may conclude that the virus was not homogeneous.

Whereas an attempt to fit the observed values with one curve would have been quite unsatisfactory, much better agreement was found when two curves were used, to express the simultaneous but independent diffusion of two sets of particles. This is shown in Fig. 1. The curves chosen for the purpose were: (A) a curve expressing the diffusion of particles 200 m\(\mu\) in diameter (diffusion constant \(D = 0.13 \times 10^{-7}\)), starting from the axis of ordinates at \(\log C_x/C_0 = 0.3\); (B) a curve expressing the diffusion of particles 6 m\(\mu\) in diameter (\(D = 4.3 \times 10^{-7}\)) and fitting the values above \(x = 0.2\) approximately, thus intersecting the axis of ordinates about \(-2\) or \(-3\).
The curve chosen to express the diffusion of the small fraction of the virus is thus displaced downwards by an amount equal to the proportion of the small fraction in the total virus. This is simply because the reference concentration $C_0$ is that of the total virus and not that of the small fraction only. A correct representation of the simultaneous diffusion of two sets of particles would obviously be a single compound curve; but, as can be shown graphically, if the two sets are of very different sizes and in very different amounts, two curves drawn independently will closely resemble the correct compound curve. Indeed, it is apparent that below $x = 0.3$ approximately, there can be too little of the small fraction to affect appreciably curve (A), and, above $x = 0.3$, too little of the large fraction to affect appreciably curve (B).

Because of the inaccuracy of the analytical procedure it is impossible to say whether the distribution in two distinct fractions was as clearcut as has been assumed, and evidently the choice which was made of diameters of 200 and 6 m\(\mu\) respectively can only be a rough approximation. But it is probable that the particle diameter of the large fraction must have been about 20 or 30 times that of the small fraction, which means a molecular weight ratio of the order of 10,000 to 1. In Experiments 1 to 4, since curve (B) intercepts the axis of ordinates about $-2$, the proportion of the small virus fraction in the total virus
must have been about 1:100; in Experiment 5, about 1:1000. As mentioned in the beginning, the lung suspension in Experiment 5 was made in mouse serum and was more concentrated, which may account for the difference.

Thus it appears that in the influenza preparation investigated about 1 per cent, or 0.1 per cent, of the total infectivity was present in particles about the size of normal serum albumin or globulin molecules. The rest of the virus, that is, the bulk of it, appeared to be present in particles of the order of magnitude which has been found by ultrafiltration or ultracentrifugation (3, 4), or about 100 or 200 mμ.

Other diffusion experiments with mouse lung virus (not reported) suggested that the fast diffusing fraction was less stable than the rest of the virus. Thus in one case the samples removed after diffusion, placed in celluloid tubes and forming a shallow layer at the bottom of the tubes, were kept 24 days at −76° before titration. A general drop in titer was then found but much more marked in the samples coming from the upper part of the diffusion cell. The same phenomenon was observed when, before diffusion, the suspension was rocked for 2 hours in a shallow trough at 28°, exposed to light and air.

**Diffusion of Influenza A Virus from Chick Extra-Embryonic Fluids**

**Material and Methods**

The material has been investigated by Henle and Chambers (5). It was obtained from 11 to 12 day old embryos infected with the virus. In the normal embryo at this stage the volume of the allantoic fluid is about 6 cc., that of the amniotic fluid 2 cc. They contain about the same salts as a serum ultrafiltrate and are both nearly protein-free until the 12th day; the allantoic fluid receives from the embryo increasing amounts of uric acid. About the 12th day the amnion opens into the albumin sac, thus acquiring suddenly a high protein concentration (6). The infected fluids used were almost clear and their protein content was insignificant. We found that both normal and infected fluids contained an appreciable amount of small particles, the size of which was about at the limit of ordinary microscopic visibility (ca. 0.2μ in diameter) and which appeared to be identical with the mitochondria from normal chorioallantoic cells. A few red cells and cellular debris in the fluid were removed by low speed centrifugation. For each diffusion experiment a different batch of fluid was used.

Chambers and Henle (7) have made a detailed study of the behavior of this fluid in the angle centrifuge. They found that the titer of the virus in the supernatant after 30 minutes at 25,000 r.p.m. in the ultracentrifuge of Rawson, Scherp, and Lindquist (8) remained quite high (only 1 or 2 log below the original titer); they suggested that an appreciable fraction of the virus must have a

---

2 We are much indebted to Dr. W. Henle and Dr. L. A. Chambers for supplying us with the material and for performing the titrations.
particle diameter of 10 μm or less. For three of the diffusion experiments reported here the fluid was first diluted 1:1 with buffered saline (0.15 M NaCl, 0.01 M PO₄, pH 7.0), then ultracentrifuged as shown in Table II. In Experiment 4, the supernatant, after a first run at 25,000 r.p.m. for 30 minutes, was centrifuged again, and the second supernatant used for diffusion. The fluid from the top of the tubes was pipetted off with care each time, to avoid possible stirring of the sediment.

Before diffusion, enough NaCl solution was added to the fluid to make the total salt concentration about 2 per cent. Diffusion took place into distilled water, for 1 or 2 days. The technique was as described for mouse lung, except that the cell was placed in a mixture of ice and water at 0°, instead of a constant temperature bath at 4°.

### TABLE II

**Diffusion of Influenza A Virus from Chick Extra-Embryonic Fluids**

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Diffusion time</th>
<th>Virus titer of control M.I.D.</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>153,000</td>
<td>5.8</td>
<td>Not centrifuged</td>
</tr>
<tr>
<td>2</td>
<td>87,000</td>
<td>4.9</td>
<td>Supernatant after ½ hr. centrifugation at 25,000 r.p.m.</td>
</tr>
<tr>
<td>3</td>
<td>86,400</td>
<td>3.8</td>
<td>&quot;</td>
</tr>
<tr>
<td>4</td>
<td>149,000</td>
<td>4.2</td>
<td>Supernatant recentrifuged ½ hr. at 25,000 r.p.m.</td>
</tr>
</tbody>
</table>

The titration of the diffusion samples was essentially as described for lung virus, except that the minimum infective dose (the dose just sufficient to induce one pulmonary lesion in a mouse) was chosen for the end-point. The M.I.D. titer thus obtained was about 2 log above the M.I.D. titer and was found more convenient since titrations could be made over a wider range of dilutions. The titer of the controls given in Table II was that of the virus at the bottom of the cell after diffusion. Except in Experiment 4, the material was not fresh and had been stored at −15° or −75° for from 1 to 5 weeks; this usually caused an appreciable drop in titer from that of the fresh material.

### RESULTS

They are shown in Fig. 2. They do not differ essentially from the mouse lung experiments, since they all reveal the presence of a fast diffusing fraction, which a curve drawn for the theoretical diffusion of particles 6 μm in diameter fits approximately. In Experiment 1, the values obtained did not warrant the drawing of two curves, as was done for mouse lung virus. In Experiment 2, ultracentrifugation did not achieve a clearcut separation of the fast diffusing...
fraction, which, on theoretical grounds, might be expected only of a mixture containing two sets of particles of very different size. In Experiments 3 and 4, centrifugation was more effective, since the curves obtained are almost smooth curves; here the intercept of the curves with the y axis indicates that the ratio of the fast diffusing fraction to the rest of the virus must have been of the order of 1 to 3. Too little is still known of the nature of the particulate components of the fluid investigated, and of the state and stability of the virus, to warrant further speculation at this point.

An attempt to rediffuse the diffusate from a first experiment proved futile, the virus having completely lost its infectivity during the second diffusion. This confirmed the impression gathered from mouse lung preparations that the virus is particularly labile in its more disperse state.

It may be concluded from these experiments that influenza A virus in chick extra-embryonic fluids was in an inhomogeneous condition and that a small fraction of it appeared, from diffusion measurements, to have a particle diameter as small as 6 mμ.

**Diffusion of Mouse Encephalomyelitis Virus**

The virus isolated by Theiler and recently studied in detail by Theiler and Gard (9) was used. It is fairly resistant and, thanks to Gard’s method (10) it
can be titered with sufficient accuracy. Ultrafiltration by Elford's method (4) gave Theiler and Gard a value of 9–13 mµ for the diameter of the virus particles.

**Material and Titration**

A 20 per cent suspension in pneumococcus broth of ground mouse brains infected with strain GD VII was prepared; it was centrifuged at 2500 R.P.M. for a short time and the sediment discarded. Titration of the diffusion samples was carried out as described by Gard (10), using the intracerebral inoculation route.

The values obtained by this titration method could be read directly as the logarithms of the relative concentrations of the diffusion samples.³

**RESULTS**

In Experiment 1, the 20 per cent brain suspension in broth was directly diffused against water for 2 days (158,000 sec.). In Experiment 2, the suspension was first dialyzed against 0.16 m NaCl and Na phosphate buffer, pH 7.3, then diffused against another portion of the same buffer for 3 days (237,000 sec.). Fig. 3 shows that the results from both experiments are closely

³ We are much indebted to Dr. M. Theiler for supplying us with the material and for performing the inoculations.
comparable. In the sample withdrawn at $x = 1.14$ cm. no virus was detected; the small circle drawn on the chart at this point gives the minimum relative concentration at which virus would have been detectable by Gard's method; in other words, any theoretical curve drawn to fit the other points should pass below approximately $5.5 \log$ at $x = 1.14$.

The same remarks made about influenza apply to these results as regards the choice of curves. Evidently the virus was not homogeneous, and an attempt to fit all the points with one curve would have been unsatisfactory; on the other hand, the results were not such as to warrant the drawing of two separate curves, as was done with mouse lung influenza. One can nonetheless obtain an idea of the diameter which must have been that of at least some of the particles by drawing a curve through the values for $x = 0.33$ and $x = 0.68$. This has been done on the chart, and yields a diameter of 15 m\(\mu\); the intercept of the curve with the axis of ordinates indicates that this fraction represented roughly 10 per cent of the total virus. The value found by diffusion for the particle diameter of an appreciable fraction of the virus is therefore in fair agreement with ultrafiltration data.

**DISCUSSION**

The results reported above from mouse encephalomyelitis virus diffusion require little comment, since they agreed rather satisfactorily with ultrafiltration data; they suggested, however, that the preparation was not homogeneous, and that only about 10 per cent of the activity was present in particles 15 m\(\mu\) in diameter; whether these were the smallest units could not be ascertained; it appeared only that smaller virus units could not have been present in considerable quantity.

The experiments with influenza virus revealed the presence of a fast diffusing fraction which the methods thus far employed had not been able to detect. Experience gained from the diffusion of catalase (1), and the fact that great variations in the conditions of diffusion did not influence the type of curves obtained, warranted the conclusion that the results were not due to experimental error. The persistence of infectivity in the supernatant after ultracentrifugation of extra-embryonic fluid offered additional evidence that part of the virus must have existed in a highly disperse state.

The apparent contradiction between our value of 6 m\(\mu\) and that of about 100 m\(\mu\) derived from ultrafiltration and ultracentrifugation of mouse lung virus by Elford and collaborators (3, 4) can be easily explained if one bears in mind that the fast diffusing fraction of our preparations represented only a small percentage of the total, and therefore could be detected only by a method of adequate sensitivity. Ultrafiltration and ultracentrifugation, as well as ultramicroscopy, as they have been applied thus far to the study of animal viruses, can be expected to give information only on the state in which the bulk
of virus infectivity is present. Thus when mouse lung virus is filtered it appears now that the end-point obtained is determined by the size of the larger particles, the diffusion of which gave, in our experiments, the first part of the curves shown in Fig. 1; it should not seem surprising that a small, highly disperse, and probably easily adsorbed fraction of the virus should not be able to pass a membrane filter to an appreciable degree.

If the ultimate virus units are comparatively small molecules, the larger particles become relatively unimportant. Similar particles have been observed in preparations of numerous viruses, and because they carry most of the infectivity they have usually been assumed to represent the actual virus units. But in recent years the specificity of these particles has become very doubtful. Claude (11) first observed that some normal tissue preparations contained non-active particles which in size and chemical composition were exactly similar to the supposed agent of chicken tumor I. Other investigations have since been carried out by numerous authors (reviewed by Henle and Chambers (12)) on material from various sources, leading to the general conclusion that particles 0.1 to 0.3μ in diameter, or material sedimentable only by ultracentrifugation, can be obtained from a great variety of normal tissues. Henle and Chambers (12) have recently observed that such particles exist in preparations of normal mouse lungs. We have found, as mentioned above, that particles of about the same size are present in the extra-embryonic fluids of the chick, both normal and infected, and appear to be identical with the mitochondria of normal cells. It now seems probable that in influenza preparations such particles or mitochondria act as virus carriers, the actual virus units being of a much smaller order of magnitude. Preliminary diffusion experiments have suggested to us that this is also true of vaccinia. Bronfenbrenner and collaborators (13) have reached the same conclusion on the nature of bacteriophage. The ultimate units of these viruses appear to consist of comparatively small molecules adsorbed on non-specific colloidal carriers.

**SUMMARY**

Analytical diffusion has been applied to a study of influenza A virus in mouse lung, influenza A virus in the extra-embryonic fluids of the chick, and mouse encephalomyelitis virus in mouse brain.

The results from influenza in mouse lung suggested that about 99 per cent of the infectivity was present in particles 200 μ in diameter, and 1 per cent in particles 6 μ in diameter.

The results from influenza in extra-embryonic fluids indicated that the preparation was inhomogeneous and that the smallest virus units were about 6 μ in diameter.

The results from mouse encephalomyelitis virus indicated that the prepara-
tion was also inhomogeneous, with 10 per cent of the infectivity in particles about 15 μ in diameter.

It has been suggested that in virus preparations normal colloidal particles can act as carriers of much smaller virus units.

REFERENCES