ULTRAFILTRATION EXPERIMENTS WITH THE VIRUSES OF LARYNGOTRACHEITIS AND CORYZA OF CHICKENS¹

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Ultrafiltration experiments with laryngotracheitis virus were first reported by Beach (1930), Gibbs (1931) and Beach (1931). These investigators used the porcelain filters of Berkefeld and the fibrous filters of Seitz and agreed that the virus particles passed readily through the Berkefeld V filters, occasionally through the Berkefeld N filters, and were completely retained by the Berkefeld W filters using water pressure systems. There was some disagreement over the Seitz filtration experiments. Beach did not find sufficient virus in his ultrafiltrates to infect susceptible chickens, while Gibbs thought these filters superior to Berkefeld N filters, but not as consistent in results as the Berkefeld V filters.

Later Gibbs (1933) introduced hormone broth as a menstruum for the suspension of laryngotracheitis virus particles, according to the technique of Ward and Tang (1929), Ward (1929) and Tang (1930), and found some improvement in filtration, but the results were far from satisfactory because the mucous and tissue cells in the exudate were not removed. During centrifugalization some of the virus particles were apparently thrown down with the cellular débris, while finer particles still remained in suspension to plug the pores of the filters.

The purification of suspensions of vaccine virus by adding dilute solutions of organic acids until the iso-electric point is

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reached, and titrating back to the neutral point with dilute sodium carbonate, as suggested by Behrens and Morgan (1932) and Behrens and Nielsen (1935) suggested a new approach to these filtration studies of infectious laryngotracheitis virus, to which was added later a similar study of coryza.

The iso-electric point of laryngotracheitis exudate was readily determined by adding a 0.04 molar solution of citric acid, drop by drop, to a triturated suspension in distilled water. When the iso-electric point was reached, the cellular débris flocculated and settled to the bottom of the tube, leaving a faintly turbid supernatant suspension of the virus. The settling of the cellular material may be aided by slow centrifugalization, and the supernatant fluid pipetted off and neutralized with a 1/25 molar solution of sodium carbonate, using 0.04 per cent brom-thymol-blue as an indicator. This neutralized supernatant fluid will be referred to in this paper as the purified virus suspension.

Furthermore, it was found that the purified virus suspension passed through the collodion membranes of Cox and Hyde (1932) and Allisbaugh and Hyde (1935 with comparatively little absorption or decrease in virulence. This method of ultrafiltration is a marked improvement over the technique that has been used hitherto for the study of laryngotracheitis virus.

THE ETIOLOGY OF INFECTIOUS CORYZA

Recently Gibbs (1935) has used this method of ultrafiltration in the study of the etiology of infectious coryza or epidemic colds in chickens and found that six widely separated outbreaks in Massachusetts were due to filterable agents. Secondary bacterial infection was found in exudates from four of these outbreaks that were somewhat virulent to susceptible fowls and chickens, but the microorganisms belonged to three different bacterial families and could not have been the causative agent involved in this disease.

The relation of these secondary microorganisms to coryza and laryngotracheitis have been studied in this laboratory for five years, and considerable evidence has been secured to indicate that there are some important secondary invaders in both of these
diseases. The results of these studies on secondary infection in coryza and laryngotracheitis will form the basis of another report. It is sufficient to state in passing that they have been eliminated from these ultrafiltration studies as much as possible.

**ULTRAFILTRATION STUDIES**

A comparative study was made of the ultrafiltering properties of laryngotracheitis and coryza viruses. These studies revealed for the first time that the virus suspensions before purification and after purification, though diluted 100 times with distilled water, were more virulent than the undiluted fresh exudate from the larynx and trachea of diseased chickens. The reason for this increased virulence is unknown, unless it is due to the fact that

| TABLE 1 |
|------------------|---|---|---|
| Virulence of laryngotracheitis virus |
| NUMBER OF CHICKS |
| Died | Recovered | Unaffected |
| Fresh tracheal exudate | 40 | 20 | 20 |
| Suspension before determining iso-electric point | 60 | 16 | 4 |
| Neutral suspension after determining iso-electric point | 59 | 17 | 4 |
| Purified virus suspension | 56 | 18 | 6 |

the virus particles are contained in the nuclei of the cells in the fresh exudate, as stated by Seifried (1930), and many of them are destroyed before entering the living cells of the mucous membrane of the inoculated chickens. When the triturated virus suspensions are inoculated intratracheally into susceptible chickens the virus particles are free to enter directly into the living cells of the mucous membrane of the host.

The virulence of laryngotracheitis virus as found in this experiment is recorded in table 1.

Inspection of table 1 will show that the suspension before determining the iso-electric point and the neutral suspension after determining the iso-electric point were more virulent than either the fresh tracheal exudate or the purified virus suspension, and
furthermore that the purified virus suspension was more virulent than the fresh tracheal exudate.

The virulence of the coryza virus used in this experiment is as shown in table 2.

The results in table 2 show that the coryza virus, freed from secondary microorganisms and inoculated into susceptible chickens reared in the laboratory and likewise free from secondary infection, was not as virulent as the laryngotracheitis virus, although the respiratory tract was affected in both cases. The three coryza suspensions were virulent in the same order as the laryngotracheitis suspensions, but not in the same degree.

<table>
<thead>
<tr>
<th>TABLE 2</th>
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<tr>
<td><strong>Virulence of coryza virus</strong></td>
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</table>

<table>
<thead>
<tr>
<th>NUMBER OF CHICKS</th>
<th>Died</th>
<th>Recovered</th>
<th>Unaffected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh nasal exudate</td>
<td>0</td>
<td>20</td>
<td>60</td>
</tr>
<tr>
<td>Suspension before determining iso-electric point</td>
<td>2</td>
<td>40</td>
<td>38</td>
</tr>
<tr>
<td>Neutral suspension after determining iso-electric point</td>
<td>1</td>
<td>40</td>
<td>39</td>
</tr>
<tr>
<td>Purified virus suspension</td>
<td>0</td>
<td>34</td>
<td>46</td>
</tr>
</tbody>
</table>

THE RELATIVE SIZE OF LARYNGOTRACHEITIS AND CORYZA VIRUS PARTICLES

Next, a comparative study of the relative size of laryngotracheitis and coryza viruses was made by filtering each through a graded series of filters following the technique of Allisbaugh and Hyde (1935) and using the formula of Cox and Hyde (1932) for estimating the pore size. It was found that the coryza virus was completely retained by the 4 per cent filter and the laryngotracheitis virus failed to pass through the 5 per cent membrane. These filtration experiments have been repeated ten times for each series of filters, and the results appear to be quite uniform. However, it may not be wise to conclude from these results that the laryngotracheitis virus particle is larger than the coryza virus particle, in view of the greater virulence of the former. It may
be that both viruses passed through the 4 per cent filters in small quantities, and that laryngotracheitis being the more virulent was manifested in the inoculated chickens while the coryza virus had no effect.

Within certain limits it was possible to check the results of the ultrafiltrations by filtering colloidal particles of known dimensions through a duplicate series of collodion membranes. A small unnamed bacterium isolated from the tracheal exudate of a chicken in 1933, and a 0.1 per cent solution of methylene blue, Allisbaugh and Hyde (1935), were selected for these tests. The bacterium was chosen because it represented a small form of bacterial life, 0.5μ by 0.1μ, and its presence in the ultrafiltrates could be easily determined by plating in chicken-infusion agar. The methylene blue, 0.03μ to 0.01μ, was selected because it possesses a smaller colloidal particle, and furthermore it aided in detecting bubbles that may have been on the collodion membranes. Its presence in the ultrafiltrates was indicated by its color.

The formula derived by Cox and Hyde (1932) for estimating the pore size of the capillaries in collodion membranes was used for calculating the size of the pores in the graded series of filters directly as the suspensions of laryngotracheitis virus, coryza virus,
chicken bacterium and methylene blue were filtered. The results of these ultrafiltrations are as shown in table 3.

According to the results reported in table 3, the virus particles of laryngotracheitis are less than 0.082μ in diameter, the coryza particles less than 0.135μ, the chicken bacterium less than 0.584μ, and some of the methylene blue colloidal particles less than 0.020μ in diameter. In general these findings are in line with the investigations of Elford (1931), Bauer and Hughes (1934), who state that the particulate size of filterable viruses includes the range of laryngotracheitis and coryza virus particles as estimated in this experiment.

SUMMARY

An improved technique for the ultra-filtration of laryngotracheitis and coryza viruses is described, in which the respective tracheal and nasal exudates are triturated in distilled water with powdered Pyrex glass until the mass is worked into a smooth emulsion. Then, 0.04 molar solution of citric acid is added, drop by drop, until the iso-electric point is reached, and the cellular débris flocculates and begins to settle to the bottom of the tube, leaving an almost clear supernatant suspension of the virus above. The settling of the flocculent material may be hastened by centrifugalization. The supernatant fluid is either poured or pipetted off and neutralized with a 1/25 molar solution of sodium carbonate, using brom thymol blue as an indicator.

The neutralized virus suspension is filtered through a graded series of collodion membranes and the size of its particles estimated. This technique shows some improvements over the methods that are commonly employed in ultrafiltration studies and should find extensive use in bacteriological laboratories for the study of filterable viruses in general.

REFERENCES

BEACH, J. R.  1930 Science, 72, 633.
LARYNGOTRACHEITIS AND CORYZA OF CHICKENS

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