THE USE OF GRADED COLLODION MEMBRANES FOR THE
CONCENTRATION OF A BACTERIAL ENZYME CAPABLE
OF DECOMPOSING THE CAPSULAR POLYSACCHARIDE
OF TYPE III PNEUMOCOCCUS

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The bacterial enzyme which decomposes the capsular polysaccharide
of Type III Pneumococcus is an endocellular substance which is
released from the specific bacterial cells by autolysis (1, 2) or by freezing
and thawing (3).

The crude enzyme preparation is obtained as a cell-free filtrate
which contains, in addition to the constituents of the medium, the
metabolic products of the bacterial growth, and those products of
autolysis which are capable of passing through Berkefeld and Seitz
filters. The toxicity of this crude material has already been described
(2), as well as earlier methods for its purification and concentration (2).

It has been found possible, for instance, to concentrate the enzyme
to dryness, without appreciable loss of activity, by evaporation under
reduced pressure and by precipitation in the cold with acetone, alcohol,
and ammonium sulfate. As shown in a previous publication (2) it is
also possible to concentrate the enzyme by ultrafiltration, using
membranes of cellulose acetate which give an inactive ultrafiltrate
because they hold back the active principle on their surface. This
method combines in the same operation, concentration of activity,
and some measure of purification by the elimination in the filtrate of
much of the irrelevant material.

To give the best possible results, the method requires that mem-
branes should be used with pores of the largest possible diameter
compatible with recovery of the enzyme in the residue; this would
increase the rate of filtration, and allow the removal of a greater
variety of extraneous substances, leaving the active principle itself in a purer state.

In recent years a method has been described by Elford (4) for the preparation of graded collodion membranes, the pores of which are remarkably uniform in size. The method has been used extensively for work with filterable viruses. However, since it is possible to prepare membranes covering a very wide range of pore size, the technique also permits of the separation of certain proteins in mixed solution. For instance ovalbumin can be separated from a mixture with hemoglobin by selective filtration through a membrane of proper pore diameter (7 mμ). Under these conditions ovalbumin passes into the ultrafiltrate, whereas hemoglobin is held back on the membrane (5).

It has been observed that the bacterial enzyme which decomposes the capsular polysaccharide of Type III Pneumococcus is rapidly inactivated by crystalline trypsin and by commercial papain, at hydrogen ion concentrations at which the bacterial enzyme is very stable in the absence of the proteolytic enzymes. This observation suggests that the activity is associated with a protein. An attempt was therefore made to subject the enzyme preparation to a process of selective ultrafiltration, in the hope that membranes would be found which would allow the passage of the autolytic products into the filtrate, but hold back on their surface the active enzyme-protein complex.

Methods

The Enzyme.—The enzyme was prepared and titrated by the methods described in a previous paper (3). The method of preparation leaves the enzyme at an alkaline reaction which proves to be most favorable for filtration through the collodion membranes. The filtration experiments were carried out in the presence of chinosel, or preferably chloroform, antiseptics which do not exert any unfavorable action on the activity of the enzyme or on the properties of the membrane.

The Filtration Equipment.—The membranes were prepared and calibrated according to the description given by Bauer and Hughes (5); the filters used were those described by these same authors.

1 Unpublished observations. The preparation of crystalline trypsin was generously supplied by Dr. John Northrop, to whom the authors extend their heartiest thanks.
EXPERIMENTAL

Filtration End-Point.—By filtration end-point is meant the smallest pore diameter which permits the test material to pass through the membrane and to appear in the filtrate.

Experiment 1.—In a first series of exploratory tests, the enzyme solution was filtered through a series of membranes with pore diameters ranging from 6.2 μm to 115 μm. Lots of 25 cc. of enzyme were subjected to ultrafiltration at 50 pounds pressure, and the filtration was interrupted after 20 cc. of filtrate had been collected in each case. Both the filtrates and residues were titrated for enzymatic activity. The average pore diameter of the membranes (A.P.D.) and the enzymatic activities of the filtrates and residues, expressed in minimum amount required for the decomposition of 0.01 mg. of specific capsular polysaccharide, are presented in Table I.

The results of Experiment 1 show that the enzyme passed readily through pores of 115 μm diameter but was held back by membranes of smaller pore size. Contrary to expectations, however, titrations of activity carried out on the residue left on all the membranes of large pore diameter revealed that no concentration had taken place but rather a loss of enzyme. Membrane 143, with the smallest pore size (6.2 μm), showed on the contrary a marked concentration of enzyme in the residue.

TABLE I

<table>
<thead>
<tr>
<th>Membrane No.</th>
<th>A.P.D. μm</th>
<th>Residue cc.</th>
<th>Filtrate cc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>182</td>
<td>115</td>
<td>0.0025</td>
<td>0.0025</td>
</tr>
<tr>
<td>185</td>
<td>72</td>
<td>0.005</td>
<td>More than 0.1 cc.*</td>
</tr>
<tr>
<td>150</td>
<td>45</td>
<td>0.005</td>
<td>&quot; &quot; 0.1 &quot; &quot; *</td>
</tr>
<tr>
<td>121</td>
<td>20</td>
<td>0.003</td>
<td>&quot; &quot; 0.1 &quot; &quot; *</td>
</tr>
<tr>
<td>211</td>
<td>15</td>
<td>0.003</td>
<td>&quot; &quot; 0.1 &quot; &quot; *</td>
</tr>
<tr>
<td>210</td>
<td>10.6</td>
<td>0.003</td>
<td>&quot; &quot; 0.1 &quot; &quot; *</td>
</tr>
<tr>
<td>125</td>
<td>8.0</td>
<td>0.002</td>
<td>&quot; &quot; 0.1 &quot; &quot; *</td>
</tr>
<tr>
<td>143</td>
<td>6.2</td>
<td>0.001</td>
<td>&quot; &quot; 0.1 &quot; &quot; *</td>
</tr>
</tbody>
</table>

Control solution of enzyme 0.002 cc.

* This was the largest amount tested.
These observations suggested that adsorption of the enzyme had taken place in those membranes with pores of a diameter sufficient to allow the enzyme to penetrate, but not to pass through.

Elford (6) has recommended the use of meat infusion nutrient broth to reduce adsorption by the membrane during ultrafiltration. Although Bauer and Hughes (5) have observed that a preliminary passage of broth through the membranes alters the filtration end-point, it was decided to use this technique for studying the passage of the enzyme through membranes of different pore size.

<table>
<thead>
<tr>
<th>Membrane No.</th>
<th>A.P.D.</th>
<th>Filtrate</th>
<th>Residue (resuspended in original volume of saline)</th>
</tr>
</thead>
<tbody>
<tr>
<td>240</td>
<td>28</td>
<td>0.002</td>
<td>More than 0.1 cc.*</td>
</tr>
<tr>
<td>129</td>
<td>24.4</td>
<td>0.002</td>
<td>&quot;    &quot; 0.1 cc.*</td>
</tr>
<tr>
<td>121</td>
<td>20.4</td>
<td>0.002</td>
<td>&quot;    &quot; 0.1 cc.*</td>
</tr>
<tr>
<td>229</td>
<td>17.6</td>
<td>0.002</td>
<td>&quot;    &quot; 0.1 cc.*</td>
</tr>
<tr>
<td>178</td>
<td>14.8</td>
<td>0.002</td>
<td>&quot;    &quot; 0.1 cc.*</td>
</tr>
<tr>
<td>235</td>
<td>12.8</td>
<td>0.002</td>
<td>&quot;    &quot; 0.1 cc.*</td>
</tr>
<tr>
<td>210</td>
<td>10.6</td>
<td>0.005</td>
<td>0.005</td>
</tr>
<tr>
<td>232</td>
<td>8.2</td>
<td>More than 0.1 cc.*</td>
<td>0.002</td>
</tr>
<tr>
<td>143</td>
<td>6.2</td>
<td>&quot;  &quot; 0.1 cc.*</td>
<td>0.002</td>
</tr>
<tr>
<td>144</td>
<td>4.6</td>
<td>&quot;  &quot; 0.1 cc.*</td>
<td>0.002</td>
</tr>
<tr>
<td>Control enzyme solution</td>
<td></td>
<td>0.002</td>
<td></td>
</tr>
</tbody>
</table>

* This was the largest amount tested.

Experiment 2.—50 cc. of nutrient meat infusion-peptone broth were added to 250 cc. of enzyme solution. Filters were fitted with membranes of pore diameters ranging from 4.6–28 μ, and the whole apparatus carefully washed with broth. To each of these ultrafilters were added 25 cc. of broth-enzyme mixture and the filtration, carried out at 50 pounds pressure, was continued until all the fluid had been forced through the membranes. The membranes were then placed in 25 cc. saline (the original volume used) in an attempt to release any enzyme concentrated on the surface; both residues and filtrates were then titrated for activity (Table II).

This experiment confirms the finding that the presence of broth prevents or minimizes the adsorption of the enzyme on the collodion
membranes. With this technique, the enzyme passes completely through membranes with pores of 12.8 m\(\mu\) diameter or larger, and is completely held back by pores smaller than 8.2 m\(\mu\). In the case of the latter membranes, the whole enzymatic activity can be recovered by merely redissolving the residue in saline or distilled water.

**Rate of Filtration of the Enzyme through a Collodion Membrane, and Example of Concentration**

In all the previous experiments, the filtration was limited to small amounts of enzyme solution; it was of interest to determine whether the accumulation of concentrated material on the surface of the membrane would inhibit further filtration.

**TABLE III**

<table>
<thead>
<tr>
<th>Time (hr.)</th>
<th>Total amount of filtrate (cc.)</th>
<th>Rate per hr. (cc.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-3</td>
<td>25.1</td>
<td>8.4</td>
</tr>
<tr>
<td>3-6</td>
<td>39.8</td>
<td>4.9</td>
</tr>
<tr>
<td>6-28</td>
<td>113.8</td>
<td>4.7</td>
</tr>
<tr>
<td>28-52</td>
<td>210.8</td>
<td>3.3</td>
</tr>
<tr>
<td>52-75</td>
<td>270.1</td>
<td>2.6</td>
</tr>
<tr>
<td>75-97</td>
<td>328.8</td>
<td>2.6</td>
</tr>
<tr>
<td>97-126</td>
<td>397.5</td>
<td>2.3</td>
</tr>
<tr>
<td>126-170</td>
<td>491.5</td>
<td>2.2</td>
</tr>
</tbody>
</table>

**Experiment 3.**—A filter was used of 100 cc. capacity, fitted with a membrane of 2.5 cm. diameter; the membrane (No. 119) had an average pore diameter of 6.52 m\(\mu\). Filter and membrane were washed with nutrient broth and the filtration carried out at 50 pounds pressure. The filter was refilled several times and filtration continued for 170 hours. The rate of filtration is described in Table III.

The results presented in Table III show that, following a sharp initial drop in the rate of flow within the first 28 hours, the subsequent rate of filtration changes only slowly allowing the passage of fairly large volumes of fluid through the same membrane.

The pore size of the membrane was such that, according to previous results, the active enzyme could not pass into the filtrate. In an
attempt to recover the enzyme in solution, the membrane was washed three times with small amounts of distilled water, giving a final volume of 12 cc.; this corresponded to a 42-fold concentration (in volume) when compared with the original 491.5 cc. of enzyme solution.

The control enzyme solution, the ultrafiltrate, and the concentrate were titrated for enzymatic activity, and the results of the titration are given in Table IV.

Under the conditions of the experiment, it appears that ultrafiltration with a membrane of 6.2 m\(\mu\) average pore diameter gives a non-filterable residue, the activity of which is increased in direct proportion with the concentration in volume; the method is therefore available for the concentration of the crude enzyme preparations under practical conditions.

**Practical Aspects of the Method of Concentration by Ultrafiltration**

It has been shown that concentration of the enzyme has been obtained by the use of membranes of average pore diameter smaller than 8 m\(\mu\); in practice we have used membranes with pore diameters ranging from 5.5 to 7.5 m\(\mu\) without any loss of enzymatic activity.

For the concentration of amounts of enzyme larger than those required in the experimental work previously described, it was found necessary to increase the filtration area by using membranes of 6 cm. diameter. For that purpose, larger filters (750 cc. capacity), made of stainless steel, were used. Filtration was carried out at a pressure of 50 pounds obtained by the use of a reducing valve on the compressed air line. To minimize the possibility of contamination by air bacteria and particles of dust, the air was filtered through Seitz asbestos pads inserted between the air line and each individual filter. A number of technical difficulties were encountered, of which only two will be mentioned.

### Table IV

**Comparative Enzymatic Activity of the Ultrafiltrate and of a Solution of the Residue Representing a 42-Fold Concentration of Original Volume**

<table>
<thead>
<tr>
<th>Material</th>
<th>Minimal amount of material required for the decomposition of 0.01 mg. of capsular polysaccharide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original enzyme solution</td>
<td>0.005</td>
</tr>
<tr>
<td>Ultrafiltrate</td>
<td>More than 1 cc.</td>
</tr>
<tr>
<td>Concentrate</td>
<td>0.00015</td>
</tr>
</tbody>
</table>
Although the body of the filter was made of stainless steel, the application of air pressure for filtration brought about an electrolytic corrosion at points of juncture of the different parts of the filters. This was eliminated by modifying the design so that the inside of the filter offered only a continuous, unbroken surface to the enzyme solution.

As stated before, chloroform is used as an antiseptic during filtration; it was therefore impossible to use as washers in the body of the filters any material such as rubber or leather containing substances soluble in chloroform. Washers made of heavy parchment paper proved very satisfactory as they are stable in the presence of chloroform and produce effective air-tight joints.

Under the conditions described, it took 5 days to filter to dryness 600 cc. of enzyme solution (previously saturated with chloroform). At the end of filtration, the membranes were washed several times in distilled water and all the activity thus recovered in solution. After filtration through Berkefeld V candles the enzyme preparation was frozen and in this state evaporated to dryness in vacuum (7, 8). This final product was redissolved and tested in animals to determine both its toxicity and activity in vivo. The results of these tests will be reported later.

DISCUSSION

The data presented in this paper indicate that, when proper precautions are taken to prevent adsorption of the active principle on the collodion membranes, the bacterial enzyme which decomposes the capsular polysaccharide of Type III Pneumococcus passes through pores of 10.6 μm average diameter, but is held back by pores of 8.2 μm diameter. When the enzyme thus held back on the membrane is placed again in the presence of water, it immediately goes into solution, indicating that the enzyme particles are not adsorbed in the pores, but are merely concentrated on the surface.

The sharpness of the filtration end-point, however, is not sufficient evidence for a determination of the actual size of the enzyme particle, since the shape of the particle and the play of electrical and other forces affect the possibility of its passage through pores of a definite diameter. It can only be stated that the property to decompose the polysaccharide of Type III Pneumococcus is carried by a protein molecule of not unusual dimensions.

In this respect it may be pointed out that Grabar and Riegert (9)
have subjected urease solutions to selective ultrafiltration through graded collodion membranes; they found that the enzyme is completely held back by membranes the pores of which are smaller than 30 m\(\mu\). Working with invertase prepared from the intestinal juice of the dog, Grabar (10) observed also that this enzyme is retained by pores of 10 m\(\mu\) diameter while most of it passes through pores of 13 m\(\mu\).

Finally, it should be remarked that the membranes used for concentration have a pore diameter (5.5–7.5 m\(\mu\)) large enough to allow the passage of a certain number of proteins, such as egg albumin, for instance. It is therefore to be expected that the process of ultrafiltration separates from the active enzyme itself many of the split products of proteins and other autolytic and metabolic products of the cells. In a subsequent publication evidence of the purification will be shown in the decreased toxicity of the preparations thus obtained.

### SUMMARY

1. The enzyme which decomposes the capsular polysaccharide of Type III Pneumococcus is associated with a protein which under optimal conditions of filtration passes through membranes with an average pore size of 10.6 m\(\mu\) but is held back by pores having a diameter of 8.2 m\(\mu\).

2. When enzyme solutions are filtered to dryness through membranes of such porosity as to hold back the active principle, and when proper precautions are taken to prevent or minimize adsorption, the enzyme can be completely recovered in solution by immersing the membrane in distilled water or physiological salt solution.

3. These results are discussed with reference to the dimensions of the enzyme particle, and to the purification obtained in the course of ultrafiltration.

4. A practical method is described for the concentration and purification of the crude enzyme preparation by the use of graded collodion membranes.

### BIBLIOGRAPHY