STUDIES ON CAPSULE FORMATION

II. THE INFLUENCE OF ELECTROLYTES ON CAPSULE FORMATION BY KLEBSIELLA PNEUMONIAE

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INTRODUCTION

It was shown in a previous publication (Hoogerheide 1939) that Klebsiella pneumoniae (Friedländer's bacterium) is able to develop large capsules when allowed to grow in suitable media, and that, due to this abundant encapsulation, such cultures become very viscous.

From a colloid-chemical point of view such an encapsulated culture may be considered as a suspension of small hydrophilic gel particles of polysaccharide nature, in a medium which itself is very poor in colloids. It seems, therefore, reasonable to assume that rules which have been found to be valid for hydrophilic sols and gels will be valid also, to a certain extent, for suspensions of encapsulated bacteria.

In general, hydrophilic colloids maintain their stability due to the combined action of two factors: (a) the particles usually possess an electric charge (positive or negative), (b) the particles are more or less hydrated and surrounded by layers of water molecules which act as buffers in preventing union of two particles. Both factors not only determine the stability of such a hydrophilic colloid but also contribute to its viscosity.

It is well known that the addition of small amounts of NaCl or other electrolytes to solutions of starch, agar-agar, casein or gelatin markedly decreases the viscosity of these hydrophilic colloids. Kruyt and co-workers (Kruyt-de Jong 1922, Bungen-
berg de Jong 1933) have been able to show that this decrease in viscosity caused by the addition of small amounts of electrolytes is due to a neutralization of the charge of the sol particles. No appreciable change in hydration is observed. The decrease in viscosity is determined by the valency and concentration of the anion when the particles are positively charged and by the valency and concentration of the cation when the charge is negative. No "lyotropic series" is observed by addition of small amounts of electrolytes; all monovalent ions behave similarly, but differently from the bivalent ones, the latter having a much stronger action.

When, however, high concentrations of electrolyte are used, the effects of all monovalent cations are no longer identical, and the "lyotropic series" appears, which means an increasing action of cations in equimolar concentrations in the direction: \( \text{Li}^+ < \text{Na}^+ < \text{K}^+ \) and \( \text{Mg}^{++} < \text{Ca}^{++} < \text{Sr}^{++} < \text{Ba}^{++} \). The same is true in case of a positively charged sol for the actions of different anions in the direction: \( \frac{1}{2} \text{SO}_4^{2-} < \text{Cl}^- < \text{Br}^- < \text{NO}_3^- < \text{I}^- < \text{CNS}^- \). Electrolytes in these high concentrations directly affect hydration.

Since bacteriological media usually contain electrolytes it was decided to see to how great an extent the presence of electrolytes would influence the viscosity observed in growing cultures of Friedländer's bacterium. When investigating this problem we found that the effects of electrolyte additions to growing cultures of Friedländer's bacterium were far too great to be explained as the result of a neutralization of the electric charge of the cells. Evidence will be given that certain electrolytes can bring about an almost complete inhibition of capsule formation, without any influence whatsoever on growth.

**EXPERIMENTAL**

**A. The influence of electrolyte addition on the viscosity of a full-grown culture of Friedländer's bacterium**

Six-hour-old cultures of Friedländer's bacterium in 4 per cent neopeptone-1 per cent glucose, which are usually very viscous in nature, were used. Sufficient formalin was added to stop growth.
STUDIES ON CAPSULE FORMATION

Five-milliliter amounts of such cultures were placed into each of several Ostwald viscosity tubes, the tubes being placed in a water bath at 37°C. After viscosity measurement readings had become constant, a known amount of electrolyte was added to each tube. As soon as the electrolyte was dissolved another reading was made. A marked decrease in viscosity was observed almost instantly after the added electrolyte was dissolved, reaching the maximum value in a few minutes. The decrease in viscosity depends on the kind and the amount of the electrolyte used. As may be observed from figure 1 the drop in viscosity becomes greater the more NaCl is added; a 1 per cent NaCl concentration causes a decrease amounting to 30 per cent of the original viscosity increase. Higher NaCl concentrations up to 2½ per cent have hardly any additional effect, however.

Other (monovalent) alkali-chlorides were studied in order to determine whether they would behave similarly. It was found that equimolar concentrations of LiCl, NaCl, KCl and NH₄Cl
have almost identical effects on the viscosity of full-grown cultures of Friedländer's bacterium. The upper line in figure 1, therefore, represents also the results obtained by addition of LiCl, KCl and NH₄Cl.

When, however, CaCl₂ was added a distinctly different curve was obtained; small concentrations caused a much more pronounced effect than was obtained with equimolar concentrations of the alkali-chlorides. In general, the level where viscosity does not further decrease upon addition of more electrolyte is reached with about half the concentration necessary with the alkali-chlorides.

Equimolar concentrations of MgCl₂, CaCl₂, SrCl₂ and BaCl₂ have again identical effects and the level ultimately reached is approximately the same as that obtained with the alkali-chlorides (lower line in fig. 1).

Finally, other inorganic salts, e.g., nitrate, sulphate, sulphocyanate, added in equimolar concentrations produce effects identical with those obtained with chlorides. Consequently, there is no evidence of the existence of a lyotropic series, and therefore it may be assumed that these electrolytes, in the concentrations used, have no effect on hydration.

These facts undoubtedly indicate that the decrease in viscosity observed in full grown cultures of Friedländer's bacterium caused by addition of electrolytes, is due to a neutralization of the charge of the encapsulated bacteria and that no significant dehydration takes place with the concentrations of electrolytes used.

B. The influence of electrolyte addition to the medium on growth and encapsulation of Friedländer's bacterium

(a) Effect on growth. In general it may be stated that the growth of Friedländer's bacterium in 4 per cent neopeptone-1 per cent glucose is only slightly, or not at all, affected by the addition of electrolytes to the medium, even in rather high concentrations. NaCl, KCl and NH₄Cl may be added in concentrations of 3 per cent or even higher without any inhibition of growth. 1 per cent solutions of these salts markedly stimulate growth. LiCl, on the other hand, in concentrations of 1 per cent
and higher inhibits growth; there appear involution forms, long thread-like organisms, often swollen and irregularly bent. This is in accordance with the fact that this salt is often added to culture media in order to obtain rough variants.

MgCl₂, CaCl₂, SrCl₂ and BaCl₂ may be added to the medium in concentrations up to 2 per cent without any appreciable inhibition of growth. As in the case with the alkali chlorides, in 1 per cent concentrations these salts markedly enhance growth. The same is true for addition of Na₂SO₄, NaNO₃, NaBr and NaI.

**TABLE 1**

*Growth inhibiting doses of several inorganic salts, when added to the culture medium (4 per cent neopeptone-1 per cent glucose)*

Inhibition of growth was detected by making counts and comparing these with counts made from cultures in the same broth without inhibitor

<table>
<thead>
<tr>
<th>ELECTROLYTE ADDED</th>
<th>PARTIAL TO COMPLETE INHIBITORY DOSES (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RbCl</td>
<td>0.3 - 2.0</td>
</tr>
<tr>
<td>CsCl</td>
<td>0.3 - 1.0</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>0.2 - 0.6</td>
</tr>
<tr>
<td>NaNO₂</td>
<td>0.1 - 0.5</td>
</tr>
<tr>
<td>NaCNS</td>
<td>0.05 - 0.8</td>
</tr>
<tr>
<td>NaF</td>
<td>0.05 - 0.2</td>
</tr>
<tr>
<td>Na₂CrO₄</td>
<td>0.01 - 0.03</td>
</tr>
<tr>
<td>NaCN</td>
<td>0.001 - 0.04</td>
</tr>
<tr>
<td>NaClO₃</td>
<td>0.002 - 0.003</td>
</tr>
</tbody>
</table>

An interesting phenomenon was observed when NaNO₃ or NaNO₂ was added to the medium. While abundant gas production was observed during growth in glucose-containing broth, such gas production did not take place in the presence of NaNO₃ or of NaNO₂; even 0.1 per cent of these salts is sufficient to suppress all gas formation. Growth and acid formation, however, are not affected. Nitrate is actively reduced during growth of Friedländer’s bacterium and appreciable amounts of nitrite are formed.

Table 1 gives the inhibitory doses for several inorganic salts when added to 4 per cent neopeptone-1 per cent glucose medium. Higher concentrations prevent growth of Friedländer’s bacterium almost completely.
Among the compounds listed in table 1 the addition of CsCl and of Na₂CrO₄ causes the appearance of a great many involution forms when concentrations are applied which partially inhibit growth.

(b) Effect on encapsulation. As has been stated previously, the results, as far as viscosity is concerned, obtained by electrolyte addition to the medium in which Friedländer's bacterium is growing, differ considerably from those obtained by electrolyte addition to full-grown cultures. Since growth is, in most cases, not inhibited and often even stimulated, one would expect only a slight effect on the increase in viscosity during growth and no effect on capsule size.

TECHNIQUE

Increasing amounts of different electrolytes were added to a solution of 4 per cent neopectone-1 per cent glucose of pH 7.5, and 5 ml. of each concentration were transferred to Ostwald viscosity tubes, inoculated and incubated at 37°C. together with a "blank" of the same medium without electrolyte addition. Half-hourly viscosity readings were made and at the end of the experiment (6 to 8 hours after inoculation) counts were made from each tube. A dilution curve in which the viscosity observed was plotted against the number of bacteria present in each dilution was made for each tube. As a diluent, broth of the same composition as that in which growth had occurred was used.

Figure 2 gives the results of such an experiment in which increasing amounts of KCl were added to the medium.

As may be seen from figure 2 the viscosity for the same number of bacteria is considerably smaller in the presence of 0.4 per cent KCl than it is in the absence of KCl. A culture containing 2 per cent KCl gave an increase in viscosity which is only 14 per cent of the increase shown by the blank for the same number of bacteria. Such a low viscosity can not be explained merely as the result of the elimination of the negative charge of the encapsulated bacteria as was shown for a full-grown culture. Also, since no dehydration will take place with the KCl concentrations applied, the only reasonable explanation left is
that encapsulation is strongly inhibited by addition of KCl. This indeed is what happens; considerably less capsular polysaccharide is present in cultures of Friedländer's bacterium grown in the presence of 1 per cent electrolyte than in its absence, as was shown by a serological technique described in an earlier paper (Hoogerheide 1939). In order to obtain this marked inhibitory effect of KCl, it must be added to the medium before or only very shortly after incubation. When added 4 hours after inoculation, the bacteria are already protected by large capsules; even 2 per cent KCl no longer has the slightest influence and the capsule size is perfectly normal.

Similar experiments were performed with the addition of LiCl, NaCl and NH₄Cl. It was found that these salts also strongly inhibit encapsulation, not all, however, to the same degree. In general LiCl produces less inhibition than does NaCl which in turn produces less effect than KCl or NH₄Cl.

Figure 3 shows the results of the effects of increasing amounts of the alkali-chlorides on encapsulation. For each concentration

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**Fig. 2.** 2a, increase in relative viscosity of cultures of Friedländer's bacterium in 4 per cent neopeptone—2 per cent glucose medium to which varying amounts of KCl have been added. 2b, viscosities of dilutions of the same cultures taken at the close of the experiment (diluent: original nutrient broth).
used the viscosity observed was compared with that of the blank for the same amount of bacteria.

Contrary to the results of electrolyte addition to full-grown cultures of Friedländer's bacterium, it appears from figure 3 that the lyotropic series: Li < Na < K is valid.

Similar results were obtained when the chlorides of the alkaline-earth metals were added to the culture medium. Encapsulation was strongly inhibited, and with these compounds also the lyotropic series: Mg < Ca < Sr < Ba was observed, as can be seen from figure 3.

Valency apparently does not play any rôle in the inhibition of encapsulation by electrolytes.

The effect is by no means restricted to chlorides of the alkaline and alkaline-earth metals but was found also upon addition of sulfates, bromides, iodides, thiocyanates, nitrates and nitrites.

Figure 4 shows the results obtained upon the addition of the Na salts of these inorganic acids. As may be seen, inhibition by
different anions occurs also in the order of the lyotropic series: 
\(\frac{1}{2} \text{SO}_4^{2-} < \text{Cl}^- < \text{CNS}^-\).

Nitrate forms an exception, inasmuch as extremely small concentrations strongly suppress encapsulation. Its true place is between Cl\(^-\) and CNS\(^-\). The reduction of nitrate to nitrite by the bacteria, however, makes it impossible to attribute this action to the nitrate alone.

![Graph showing inhibitory effect of different anions on capsule formation](http://jb.asm.org/)

**Fig. 4. The Inhibitory Effect of Different Anions on Encapsulation of Friedländer's Bacterium, When the Electrolytes Are Added to the Culture Medium**

Viscosity increases observed in the presence of electrolyte are expressed as percentage of the viscosity increase obtained for an equal number of bacteria grown under the same conditions without electrolyte addition.

It seems, therefore, an almost general rule that all inorganic electrolytes will inhibit encapsulation of Friedländer's bacterium, although to different degrees.

Salts of organic acids such as Na-acetate, Na-pyruvate, Na-succinate, Na-formate, Na-citrate in 1 per cent solutions in general inhibit much less than the salts of inorganic acids; with some of these organic salts there is hardly any inhibition. Oxalate, however, is an exception, inhibiting encapsulation almost as much as do the inorganic salts.
DISCUSSION

From the results of these experiments it is evident that inhibition of encapsulation of Friedländer's bacterium can be brought about by a great variety of electrolytes. Since we are dealing with such a non-specific inhibition it is worthwhile to see whether or not there is a common factor which might explain this inhibitory effect.

In this regard it has been shown that the adsorption of electrolytes on surfaces, in general, follows the lyotropic series and increases in the direction Li < Na < K, etc. (v.d. Hoeve, 1930).

Inasmuch as the inhibition of encapsulation also follows these lyotropic series, it might be possible that physical adsorption of an electrolyte on the bacterial cell, and perhaps on the enzymatic systems which are responsible for polysaccharide synthesis, is sufficient to inhibit these enzymes.

SUMMARY

1. Addition of electrolyte to full grown cultures of encapsulated Friedländer's bacterium eliminates the negative charge of the bacteria thus causing a slight decrease in the relative viscosity.

2. Addition of electrolyte to growing cultures of this organism strongly inhibits capsule formation.

3. The appearance of the lyotropic series in these inhibitions suggests that probably adsorption of electrolyte is sufficient to inhibit the enzymes responsible for the synthesis of bacterial polysaccharide.

REFERENCES


