# STUDIES ON CAPSULE FORMATION

## III. Inhibition of Capsule Formation of Klebsiella pneumoniae (Friedländer's Bacterium) by an Agent Produced by a Soil Bacillus

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### INTRODUCTION

The conditions under which *Klebsiella pneumoniae* forms its largest capsules were reported in an earlier publication (Hoogerheide, 1939). The underlying principle of that study was the hypothesis that capsular polysaccharide synthesis is brought about by the action of an enzyme system in the cell, the activity of which can be studied in the same way as that of other metabolic enzyme systems. In a second paper (Hoogerheide, 1940) the influence of electrolytes on this enzyme system was studied, and it was found that electrolytes, when added to the culture medium, bring about a marked inhibition in the production of capsular polysaccharide.

While engaged in attempts to isolate from bacterial sources possible enzymes capable of hydrolyzing the capsular substance of Friedländer's bacterium (similar to the enzymes described by Dubos and Avery (1931) and by Sickles and Shaw (1934, 1935), which digest the polysaccharides of *Pneumococcus* types I, II, III and VIII), we isolated several strains of a bacillus which appeared to have the remarkable property of excreting a soluble agent. By means of this agent, the bacillus is able to prevent Friedländer's bacterium from forming capsules. The present paper describes the isolation of this soil bacillus and the preparation of extracts containing the active principle.

## EXPERIMENTAL

### *Isolation of a sporulating bacillus from soil, capable of preventing capsule formation of Friedländer's bacterium*

Small Erlenmeyer flasks containing a solution of 0.1 per cent $K_2HPO_4$, 0.1 per cent $NH_4Cl$, 0.05 per cent $Mg_2SO_4$, and 0.01 per cent (type B) Friedländer polysaccharide in tap water, were inoculated with small amounts of soil samples from different locations around Philadelphia and incubated at 37°C. After 1 or 2 days' incubation, growth could be observed. Several weekly transplants in sterile media of the same composition were made, after which the cultures were plated on peptone agar. Bacteria which had formed colonies of various appearances on these plates were isolated in pure culture and tested for their ability to grow in the polysaccharide medium. By this method, 24 strains of bacteria, belonging to several genera, were isolated from soil samples. These bacteria were able to multiply in a synthetic medium in which Friedländer polysaccharide, type B, was the only source of carbon. Although breakdown of this polysaccharide must have occurred, it was found that none of these strains would elaborate an enzyme which readily hydrolyzed the polysaccharide. Even after prolonged incubation (3 weeks), it was still possible by serological technique to demonstrate apparently unaltered polysaccharide.

With little hope of success, it was decided to determine whether or not the soil bacteria digest the natural capsules of Friedländer's bacterium. All of the 24 strains of soil bacteria were therefore inoculated into 4 per cent neopeptone-1 per cent glucose broth and incubated overnight. When growth had proceeded sufficiently the cultures were inoculated with Friedländer's bacterium. After 24 hours the presence or absence of capsules around Friedländer's bacterium was investigated microscopically using Burri's India ink method.

Of the 24 strains tested, there were three (which later proved to be identical) that appeared to inhibit encapsulation of Friedländer's bacteria very strongly, since practically no capsules could be detected; the remaining 21 strains did not inhibit the

capsule formation of Friedländer's bacterium. Since there was no indication that the growth of the Friedländer's bacterium had been retarded, to any extent, by the presence of the three inhibiting strains, it was decided to study more extensively this phenomenon of inhibition of encapsulation.

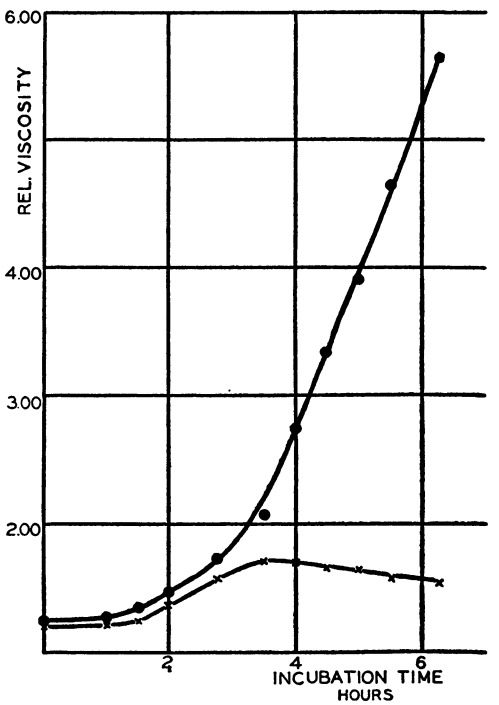


FIG. 1. INCREASE IN RELATIVE VISCOSITY OF A CULTURE OF FRIEDLÄNDER'S BACTERIUM DURING 6 HOURS' INCUBATION AT 37°C. (INITIAL pH 7.5).
o——o in 4 per cent neopeptone-1 per cent glucose.
x——x in the same medium, but after previous 24-hour growth of 11M3.

The technique used to study the effect on average capsule size was the same as that described in the earlier papers of this series, namely, measuring the increase in relative viscosity during the growth of Friedländer's bacterium.

Figure 1 gives the results of an experiment in which the relative viscosity was measured during growth of Friedländer's bacterium in 4 per cent neopeptone-1 per cent glucose broth as

well as during growth in the same medium, inoculated 24 hours previously with a strain of the soil bacillus (11M3).[1] As may be seen from this figure, the curve representing the relative viscosity when soil bacilli are present is quite different from that representing the control culture. Three hours after inoculation, at which time the great increase in viscosity begins to manifest itself in the control culture, no similar increase was observed in the culture in which 11M3 had grown. A considerable decrease in viscosity occurred, notwithstanding vigorous growth of and fermentation by the Friedländer's bacterium. At the close of the experiment practically no capsules could be observed.

Similar results were obtained whether the growth of 11M3 had proceeded for 24 hours or for one month. The medium, after being readjusted to the proper pH, apparently contained a factor very unfavorable for capsule formation by Friedländer's bacterium.

The results obtained could not be attributed to an inhibition of growth due to exhaustion of the medium during the previous growth of 11M3 since such a culture, when filtered through a Seitz or Berkefeld filter, behaved as did the control. Apparently, filtration through Seitz EK pads or Berkefeld N candles removed the principle responsible for the inhibition of encapsulation.

When a culture of 11M3 in 4 per cent neopeptone-2 per cent glucose was centrifuged at high speed, a supernatant fluid was obtained which was practically free of bacteria (no growth was obtained when with a bent platinum needle a drop was streaked on peptone agar plates). Even when this supernatant fluid was incubated in a viscosity tube for 6 hours, under the same conditions observed in all of these experiments, no growth of 11M3 was observed. Nevertheless, such a culture medium, practically free of living cells, was very active in preventing capsule formation. Furthermore, it could be pasteurized, or even boiled, without loss of activity. Adsorption of this medium with Norit (Pfanstiehl), however, readily removed the active principle. We are probably dealing therefore with an excretion product of a soil bacillus which agent is heat-stable and readily adsorbable on

[1] 11M3 is the laboratory designation of one of the three strains of isolated soil bacteria which inhibit encapsulation.

Norit and other adsorbents. By saturating the cell-free supernatant liquid of a culture of 11M3 in peptone-water with $(NH_4)_2SO_4$ a scum was formed, containing practically all of the active principle. By dialysis of this precipitate a purified, concentrated solution could be obtained.

The active principle is present not only in the supernatant fluid of the culture medium, but can be isolated from the washed precipitate also. When a suspension of 11M3 in distilled water was subjected to prolonged sonic vibration in a super-sonic oscillator, followed by high speed centrifugation, a cell-free solution of colloidal appearance was obtained. This solution contained the active principle which, when added to culture medium, would prevent capsule formation by Friedländer's bacterium. This colloidal solution appeared to be very stable and could be stored for months in the refrigerator without any loss of activity. However, when adjusted to pH 4.0 to 5.0 with acetic acid a precipitate was formed, containing all of the active principle. The precipitate redissolved when the pH was shifted either toward the alkaline side (pH 6.0) or to the acid side (pH 3.0). Such a precipitation can be repeated *ad libitum* without loss of activity. The active principle does not pass a cellulose dialyzing membrane; upon dialysis, however, a precipitate is formed, containing most of the active principle. Besides precipitation at pH 5.0, the active principle can be precipitated from colloidal solution by adding electrolytes. It can also be removed by treatment with Norit or colloidal iron (Merck).

The active principle is elaborated by the soil bacilli not only in the medium described but also during growth on any kind of medium, e.g., a 1 per cent gelatin solution containing the necessary inorganic salts. This indicates that the isolation on a medium containing Friedländer polysaccharide as the only source of carbon may have been just a coincidence. The three strains have been transplanted now for more than two years on the usual laboratory culture media without any sign of loss in activity. Furthermore, the active principle isolated from these soil bacteria not only prevents encapsulation of Friedländer's bacterium, type B, but also of strains of Friedländer's bacterium, type A.

The inhibitory principle is not an enzyme which destroys

capsular polysaccharide. When solutions of the active principle and of capsular polysaccharide are mixed, no breakdown occurs even after a prolonged incubation time. There is also no decrease in viscosity when the active principle is mixed with a heat-killed suspension of well-encapsulated Friedländer's bacterium. The active principle, therefore, presumably acts directly on the enzyme system responsible for the synthesis of capsular polysaccharide by inhibiting or even inactivating the enzyme. The amount of active principle necessary to inhibit encapsulation is very small: 0.005 mgm. of purified material per milliliter of culture medium is sufficient to cause an appreciable inhibition; 0.02 mgm. will almost completely prevent an increase in viscosity during growth. As was shown by making bacterial counts, the growth of Friedländer's bacterium is not affected, even when using 10 mgm. of active principle per milliliter of culture medium. Work is now in progress toward further purification and identification of the active material and will be published shortly.

*Description of the organisms isolated*

*Colonies by growth on yeast-extract agar plates.* Colonies are circular, 1 to 2 mm. diameter after 24 hours' incubation at 37°C., flat, smooth, entire edges, white, pearl-like glistening, sometimes becoming slightly yellowish-brown on further incubation. Mesophylic, strictly aerobic.

*Morphology.* Actively motile, gram-negative rods, single or in pairs, central spores, rods swollen during sporulation, endospores: ellipsoids.

*Growth in nutrient broth.* Moderate clouding, slight pellicle as well as sediment.

*Carbohydrates.* No fermentation.

*Litmus milk.* After 2 days: reduction of litmus, milk clotted, gradual peptonization, alkalinization.

*Nitrate reduction.* Active reduction and nitrite formation.

*Gelatin liquefaction.* Complete liquefaction of peptone-gelatin with considerable $NH_3$ formation.

*Starch.* No appreciable hydrolysis of starch.

*Indole.* No indole formation in tryptophane broth.

*$H_2S$.* After 1 week: growth on Pb acetate agar slightly brownish.

*Catalase.* Positive.

*Isolation of the organism from soil*

A soil suspension is made from about 1 gram soil in 10 ml. of water. This suspension is pasteurized for 10 minutes at 80°C. After sedimentation of the heavier soil particles 1 ml. of the supernatant fluid is used for inoculation, in a small Erlenmeyer flask, of 10 ml. of a sterile solution of 0.1 per cent $K_2HPO_4$, 0.1 per cent $(NH_4)_2SO_4$ and 0.1 per cent Friedländer polysaccharide, type B, in tap water.

After incubation for 3 days at 37°C. one loopful is transplanted to the same medium and this procedure repeated several times. After 3 or more transplants, plates are made on 0.1 per cent yeast-extract agar and the colonies, corresponding to the description above, isolated and tested for their ability to inhibit encapsulation of Friedländer's bacterium.

Because of the extreme complexity of the family Bacillaceae no extended attempt has been made to identify the isolated organism.

## SUMMARY

Several strains of a gram-positive, spore-forming, aerobic bacillus have been isolated from soil. These organisms when growing on liquid media release a soluble agent capable of preventing Friedländer's bacterium, types A and B, from forming capsules.

A cell-free solution of the active principle was prepared from the supernatant fluid of a broth culture of this soil bacillus by adding $(NH_4)_2SO_4$, redissolving the scum and then dialyzing the solution. A similar solution was prepared from the centrifuged and washed bacteria by the sonic vibration of a bacterial suspension.

The active principle is heat-stable and is readily absorbed on Norit or colloidal iron. It has its minimum solubility at pH 5.0 and does not pass a dialyzing membrane.

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