STUDIES ON CAPSULE FORMATION

I. The Conditions under Which Klebsiella pneumoniae (Friedländer's Bacterium) Forms Capsules

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INTRODUCTION

Several genera of bacteria are characterized by the possession of slimy envelopes, usually called capsules. The presence of such capsules may be demonstrated microscopically by certain staining methods or by the India Ink method of Burri (1909). Capsules are particularly well marked in certain species, notably the *Pneumococcus* group, the *Klebsiella* group, the *Salmonella* group, *Bacillus anthracis*, *Clostridium welchii* and *Gaffkya tetragena*. There are serological indications, however, that many more species of pathogens possess envelopes, very similar to capsules, although these envelopes do not have the extreme size which makes them readily demonstrable microscopically.

A close relationship appears to exist between encapsulation and pathogenicity. It need not be stressed, therefore, that a better understanding of the conditions under which certain species form capsules might be of great value in regard to problems concerning the pathogenicity of micro-organisms. Little, if any, work has been done in this field, most of the attention having been concentrated either on the physico-chemical nature of the capsular substances, or on the serological problems related to encapsulation.

To define the conditions under which one species of an encapsulated pathogen (Friedländer's bacterium) forms its capsules has been the aim of this investigation.

METHODS

In preliminary work we attempted to apply the method of Burri to the comparison of the sizes of the capsules of bacteria derived from different cultures. The results were disappointing, the method depending too much on personal judgment, and being influenced greatly by slight irregularities in technique. Only marked differences in capsule size could be demonstrated. This method, therefore, was abandoned.

Growing cultures of Friedländer's bacterium become very viscous when abundant capsule formation takes place, but practically no change in viscosity can be observed when the organism does not form capsules. With this fact in mind the possibility was considered whether measuring the relative viscosity would not provide a more accurate method of comparing average capsule sizes. This, indeed, appeared to be possible.

From a theoretical point of view, one may expect a direct proportionality between relative viscosity and total volume of the suspended particles (in this case the total volume of the encapsulated bacteria) according to the equations of Einstein (1911), von Smoluchowski (1916), and Jeffery (1922). Such equations, from which can be calculated mathematically the size of the capsules, hardly can be expected to be valid in a system so complex as a culture of encapsulated bacteria. Nevertheless, there was evidence that the larger the capsules, the higher the relative viscosity.

Since, however, the relative viscosity of a growing culture is dependent not only on the size of the capsule but also on the number of encapsulated bacteria, a comparison is possible only when the same number of encapsulated bacteria are present. It is necessary, therefore, to make bacterial counts and to plot the relative viscosity observed, against the number of bacteria responsible for this viscosity. From the position of such curves definite conclusions can be drawn as to differences in the average size of the capsules of bacteria derived from different cultures. By using this technique it was possible to demonstrate small differences in average capsule size, which could not, with sufficient accuracy, be demonstrated by the Burri method.

Relative viscosity was measured in the ordinary Ostwald capillary viscosity tubes at 37°C. Since this kind of viscosimeter may be sterilized and handled under sterile conditions, no alterations for bacteriological purposes are necessary. Furthermore, with a single growing culture, as many readings as desired may be made without endangering the sterility of, or sacrificing the culture.

Sterile viscosity tubes were filled with 5 ml. of nutrient broth, previously inoculated with a vigorously growing culture of Friedländer's bacterium and viscosity measurements made at half-hourly intervals after the initial inoculation. Bacterial counts were made using the Levy-Hausser counting chamber and a suitable dilution of the culture.

EXPERIMENTAL

The strain of Friedländer's bacterium used throughout this work was obtained from the American Type Culture Collection (*Klebsiella pneumoniae* No. 4727). The strain was originally isolated and described by Schlossberger and Menk (1930). Several animal passages of the strain were made before this work was begun. Large slimy colonies were formed on solid agar, and in liquid medium extremely high viscosities were obtained under special conditions. An example of such an increase in viscosity during the growth of Friedländer's bacterium, in 4 per cent neopeptone-1 per cent glucose, is shown in figure 1-a.

A few hours after incubation the viscosity begins to increase, until, after 7 hours the relative viscosity has attained a value of almost 6. In this state, large capsules may be demonstrated by the Burri method, and the culture has a slimy consistency which may be seen readily when an inoculating needle is thrust into the tube and withdrawn with a long slimy thread adhering.

It is readily demonstrated that the high viscosity in full-grown cultures of Friedländer's bacterium is due to the presence of intact capsules and is not due to excretion of capsular substance in the medium. It appeared to be possible to disrupt the capsule from the bacteria by a purely mechanical method and to bring the capsular substance into solution without destroying the

bacteria themselves to any appreciable extent. This was done by exposure for about three minutes to sonic vibration in a sonic oscillator wherein a nickel rod is brought into rapid vibration by an alternating magnetic field. The fluid, contained in a glass cylinder surrounding this nickel rod, is also submitted to these

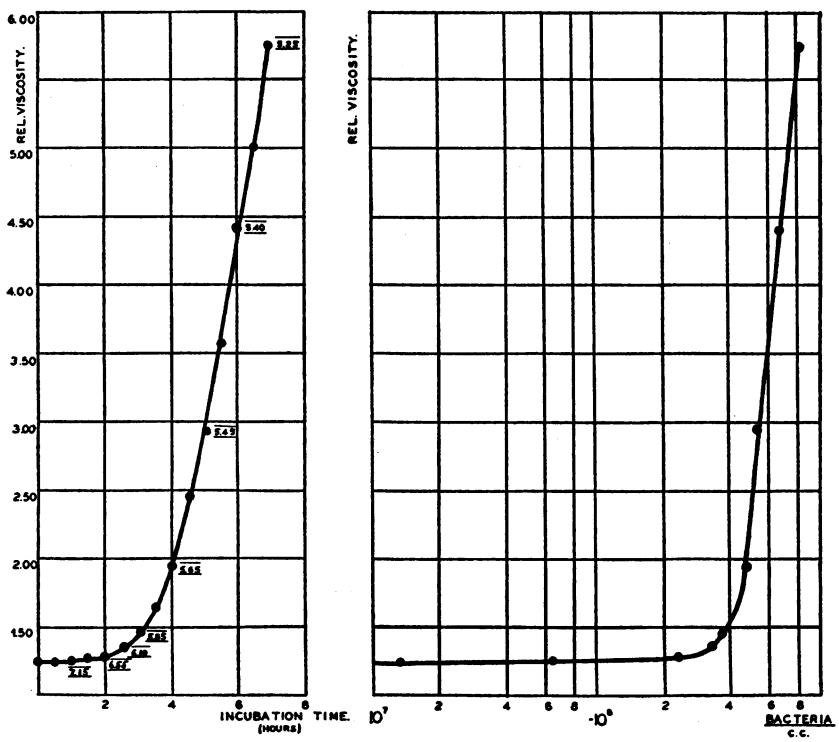

FIG. 1-a FIG. 1-b

FIG. 1-a. Increase in relative viscosity of a culture of Friedländer's bacterium in 4 per cent neopeptone-1 per cent glucose, during 7 hours incubation at 37°C. (initial pH 7.5) (pH changes during growth are shown).

1-b. Relative viscosities of the same culture shown in figure 1-a in relation to the number of bacteria present at varying times during incubation.

vibrations and it appears that encapsulated bacteria present in this fluid quickly lose their capsules.

When a very viscous, full-grown culture of Friedländer's bacterium was submitted to such treatment, it was found that in a very short time the capsules disappeared (Burri method).

At the same time an enormous drop in the viscosity of the culture to almost that of the original broth was observed. Figure 2 shows the result of sonic vibration treatment on the viscosity of a young culture of Friedländer's bacterium in neopeptone-glucose. Dilution series were made from this culture before and after treatment, and also after part of the culture had been heated for 10 minutes in a boiling waterbath. For each dilution, in which the numbers of bacteria were known, relative viscosity was measured. As may be seen from figure 2, high viscosities were observed only

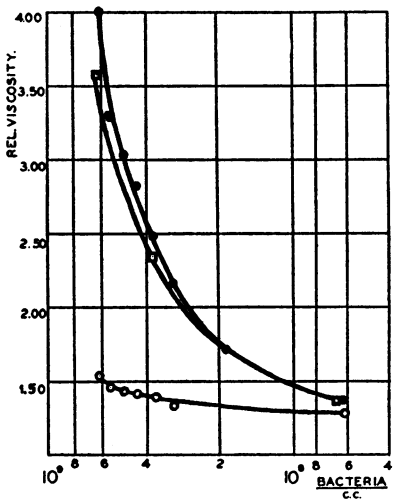

FIG. 2. Relative viscosities of a full grown culture of Friedländer's bacterium in 4 per cent neopeptone-1 per cent glucose and different dilutions of this culture with the original nutrient medium. ●——● untreated. □——□ same culture, 10 minutes boiled at pH 8.8. ○——○ same culture after 2 minutes sonic vibration.

when the structure of the capsule was maintained. The same amount of capsular substance, disrupted from the bacterial cells and dissolved, caused hardly any increase in viscosity. Boiling for 10 minutes did not destroy the capsule, either when the medium was slightly alkaline (pH 8.8), or when it was slightly acid (pH 4.4). A direct correlation therefore appeared to exist between the relative viscosity and the presence of capsules on the bacteria. Figure 2 also gives an example of the interrelation of the relative viscosity and the concentration of encapsulated

bacteria. From a great number of such dilution curves of very viscous cultures it was found that the following equation could be applied:

$$\log \cdot \frac{\eta_s - \eta_0}{C\eta_0} = a + bC$$

In this empirical equation C represents the number of encapsulated bacteria present, $\frac{\eta_s - \eta_0}{\eta_0}$, the relative viscosity for this number of bacteria, and a and b are constants. This formula is identical with the one found to be valid for the interrelation of the relative viscosity and sol concentration of many hydrophilic colloids (Bungenberg de Jong, Kruyt and Lens 1932).

It appears, however, that this equation cannot be applied to the interrelation between relative viscosity and number of bacteria during growth (fig. 1-b). A closer study of figures 1-b and 2 reveals that the curves are decidedly different, thus indicating that the capsule size is not the same during the entire growth period. This was an unexpected result and so was studied more fully. At regular intervals, counts and viscosity measurements were made on a growing culture of Friedländer's bacterium. A curve similar to the one represented in figure 1-b was thereby obtained. In addition, at 3, 4½, 6 and 7½ hour intervals after inoculation, dilution series were made with the same kind of broth in which growth occurred, and viscosities of these dilutions estimated. The results of these experiments are shown in figure 3.

Figure 3 indeed shows that the capsular size in a growing culture of Friedländer's bacterium is not constant. The dilution curves do not coincide as might be expected if capsular size were constant over the whole range of growth. In the logarithmic phase of growth encapsulation is very poor. The capsule size increases rapidly in the later stages of growth.

By measuring the pH during growth, it appeared that encapsulation occurs mainly when the pH is as low as 5.6 to 5.0, therefore, under conditions unfavorable for further multiplication. Such conditions are reached 4 hours after inoculation. After

6 hours, the capsules have attained their optimal size and do not increase further. Seven and one-half hours after inoculation, capsules are the same size as at 6 hours (both dilution curves practically coincide).

The fact that capsules are formed mainly after the logarithmic period of growth was demonstrated clearly, not only with the

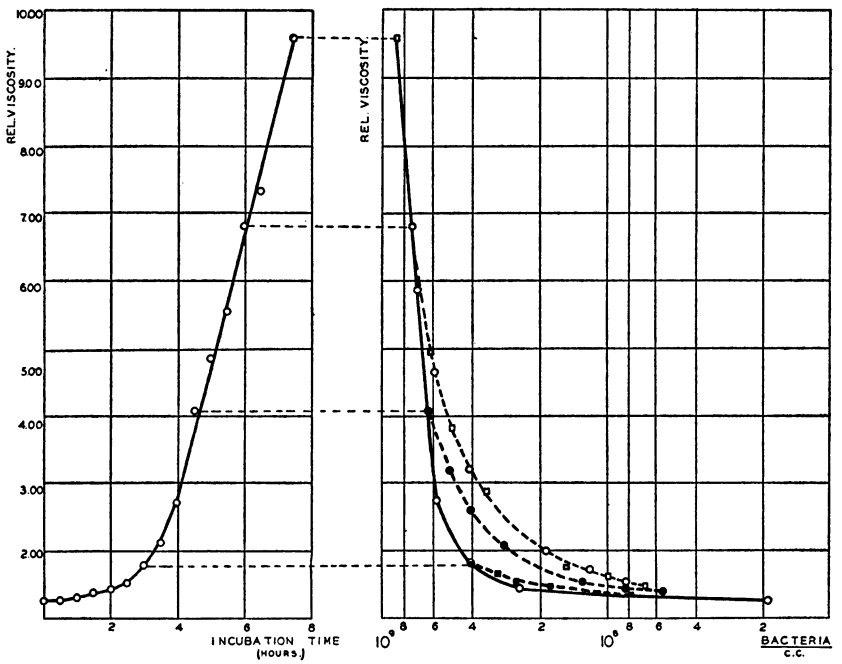

FIG. 3-a FIG. 3-b

FIG. 3-a. Increase in relative viscosity of a culture of Friedländer's bacterium in 4 per cent neopeptone-1 per cent glucose during 7½ hrs. incubation at 37°C. (initial pH 7.5).

3-b. Relative viscosities of the same culture in relation to the number of bacteria present at varying times during incubation (solid line). Viscosities of dilutions of the same culture made with the original nutrient broth at 3, 4½, 6 and 7½ hours incubation (broken lines).

relative viscosity technique, but also with India ink stains, taken at hourly intervals after inoculation (plates 2 and 3).

INFLUENCE OF SUGAR ON CAPSULE FORMATION

Since the capsular substance of Friedländer's bacterium is carbohydrate in nature it seemed of interest to determine whether

the glucose in the culture medium was a factor contributing to the formation of capsules.

It appears that the presence of carbohydrate is not strictly necessary for capsule formation. When cultivated in a 4 per

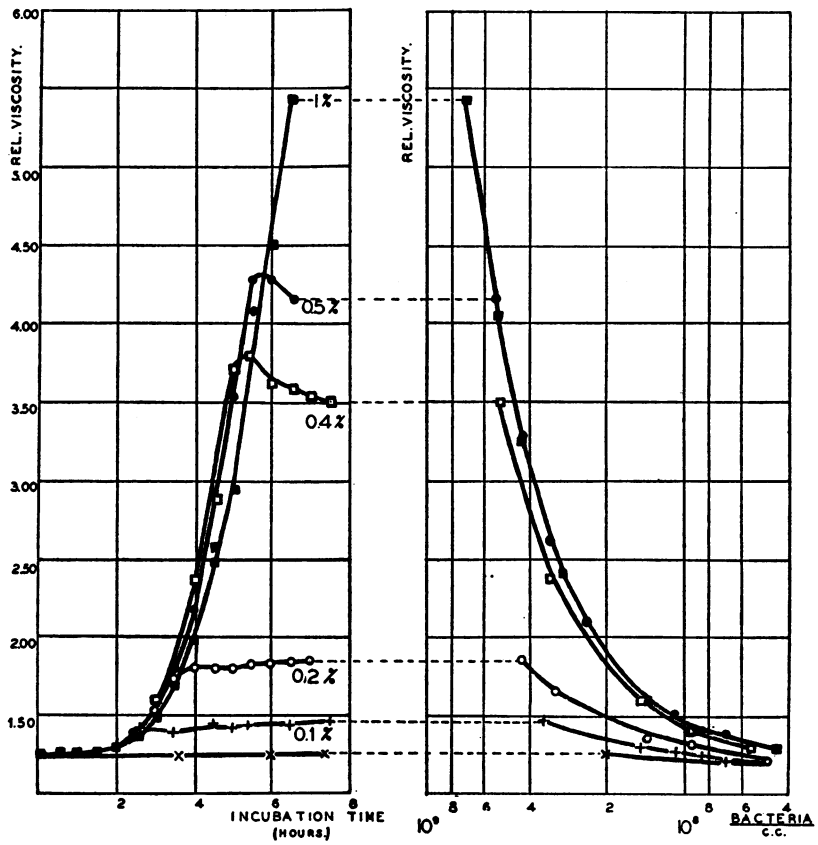

FIG. 4-a FIG. 4-b

FIG. 4-a. Increase in relative viscosity of cultures of Friedländer's bacterium in 4 per cent neopeptone with addition of different amounts of glucose varying from 0 to 1.0 per cent at 37°C. (initial pH 7.5).

4-b. Viscosities of dilutions of the same cultures taken at the conclusion of the experiment (diluent: original nutrient medium).

cent neopeptone solution at pH 7.5 as well as at pH 5.8, the presence of small capsules could be demonstrated by the India ink method after 6 hours of incubation (Plate 1). However, the capsules formed in sugar-free media are far inferior in size to

those obtained in the same medium with added sugar (Plate 3). It also appears that glucose, although not strictly necessary for the formation of capsular substance, plays an important rôle as a component of capsular substance. Figure 4 shows that practically no increase in viscosity is observed when glucose is eliminated from the medium, while the glucose-containing controls show a marked increase. However, this difference is due in part to the very restricted growth in the glucose-free culture, (2.0×10^8 bacteria per ml. compared with 7.0×10^8 bacteria per ml. in the control) at the end of the experiment.

When small amounts of glucose are added (0.1 to 0.5 per cent) (fig. 4) growth and capsule formation occur normally until all of the sugar present has been utilized. If no capsules have been formed during this period, they are not formed thereafter. At least 0.3 per cent glucose is necessary for the production of well-encapsulated bacteria. When all of the sugar has been fermented, multiplication continues, but bacteria developing under these (sugar-free) conditions are very poorly encapsulated. Therefore, a marked drop in viscosity is observed as soon as all of the glucose has disappeared, notwithstanding a slight increase in the bacterial count. The capsule size decreases as soon as the glucose supply is exhausted. Addition of formalin, at the moment when all of the sugar has disappeared, stops further proliferation and therefore no drop in viscosity is observed after further incubation, the capsule size remaining unchanged.

Higher glucose concentrations (up to 10 per cent) very markedly inhibit capsule formation. The greatest capsule size is obtained by adding from $\frac{1}{2}$ to 1 per cent glucose to the culture medium. Lower concentrations are insufficient to obtain the conditions favorable for capsule formation, while higher concentrations are inhibitory.

The utilization of several other fermentable substrates in the synthesis of the capsular polysaccharide was studied. One per cent solutions of different sugars and other fermentable substrates in 4 per cent neopeptone broth were inoculated with Friedländer's bacterium, incubated, and each half hour the relative viscosity

was measured. Figure 5 shows the results of these experiments. As may be seen the most rapid increase in viscosity occurs in the presence of glucose, but considerable increase occurs also in the presence of sucrose, maltose and mannitol. Pyruvic acid, glycerol, rhamnose and salicin give less marked increases. Lac-

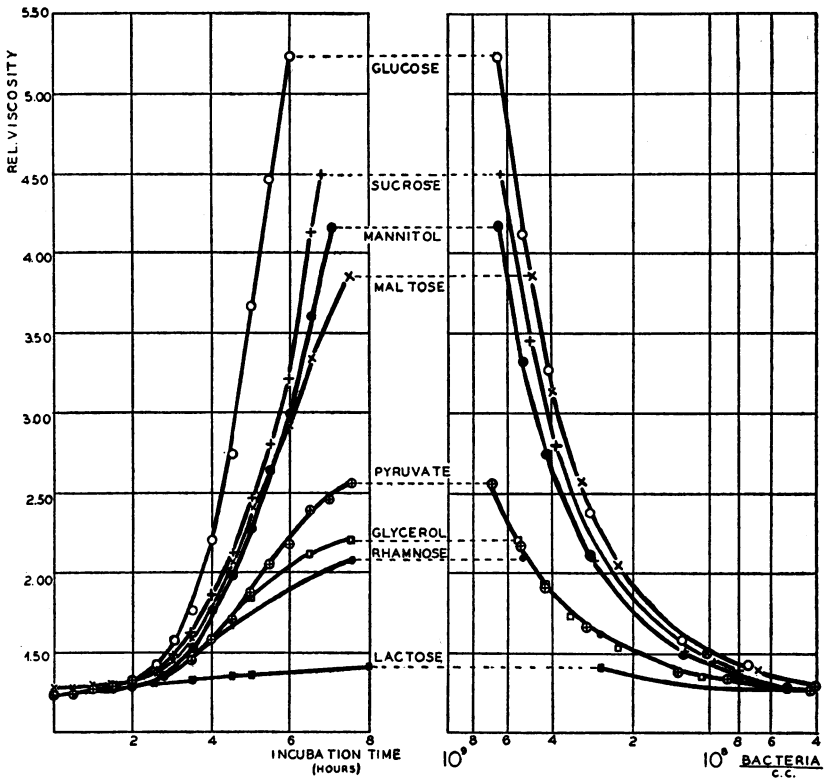

FIG. 5-a FIG. 5-b

FIG. 5-a. Increase in relative viscosity of cultures of Friedländer's bacterium in 4 per cent neopeptone with the addition of 1 per cent of several different fermentable substrates during 8 hours incubation at 37°C. (initial pH 7.5).

5-b. Viscosities of dilutions of the same cultures taken at the conclusion of the experiment (diluent: original nutrient medium).

tose, which is practically non-fermentable by the strain used, produces no increase in viscosity.

In order to compare the average size of the capsules in the presence of the different fermentable substrates, dilution curves were made at the end of the experiment. Relative viscosities

for each of the cultures, in relation to the amounts of encapsulated bacteria present, are represented in figure 6-a. It appears from these curves that capsules in the glucose, sucrose, or maltose medium are practically of the same size. In mannitol medium they are somewhat smaller, and in pyruvate, glycerol or rham-

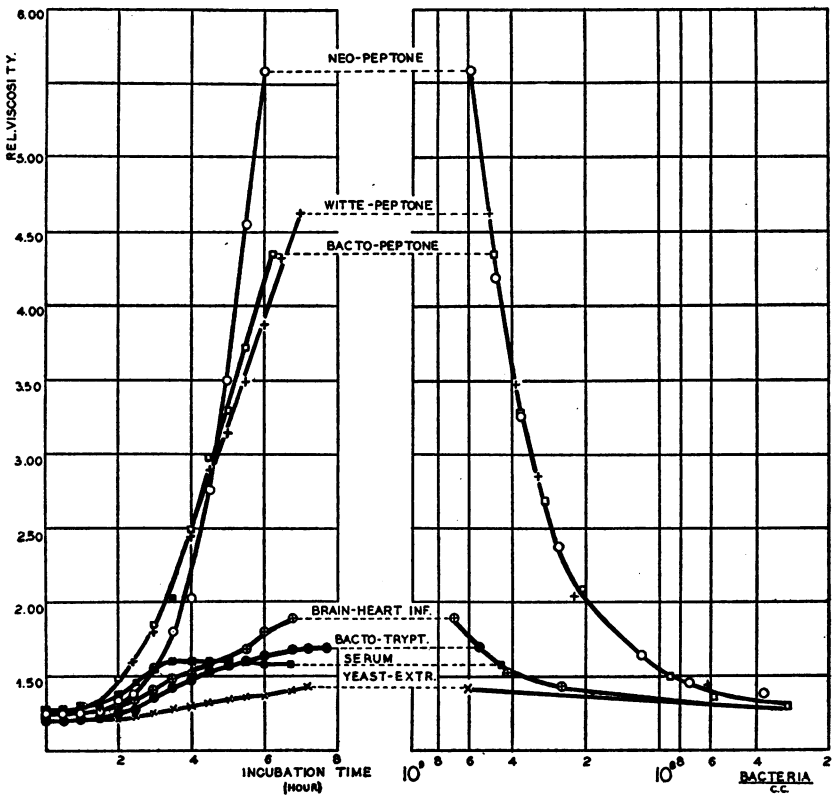

FIG. 6-a FIG. 6-b

FIG. 6-a. Increase in relative viscosity of cultures of Friedländer's bacterium in 4 per cent solutions of different culture media and 1 per cent glucose during 7½ hours incubation at 37°C. (initial pH 7.5).

6-b. Viscosities of dilutions of the same cultures taken at the conclusion of the experiment (diluent: original nutrient medium).

nose medium considerably smaller. It appears also that the capsular substance of Friedländer's bacterium may be synthesized from many different substrates: this is in striking contrast to several non-pathogenic bacteria such as *Betacoccus dextranicus*, which species is able to synthesize its capsules from sucrose only.

INFLUENCE OF THE SOURCE OF PROTEIN ON CAPSULE FORMATION

Higher concentrations of neopeptone favor growth but not capsule formation, inasmuch as capsule size is the same in a ½ per cent as in a 4 per cent neopeptone broth. Other kinds such as (Difco) bactopeptone and Witte peptone give the same result. Growth in these media is somewhat slower, but as may be seen from the dilution curves of figure 6, capsule size in these different media is the same. Unexpectedly, however, many culture media were found in which growth was as good as in neopeptone, but which were completely unsuitable for capsule formation. Four per cent solutions of bactotryptone, yeast extract, Loeffler's blood serum, and brain-heart infusion, all neutralized to pH 7.5, and to which 1 per cent glucose was added, were very favorable culture media for growth, some even better than neopeptone, but capsule formation in these media was extremely poor, as may be seen from the dilution curves in figure 6, and Plate 4. By the addition of increasing amounts of yeast extract to a 2 per cent neopeptone-1 per cent glucose solution, it was found that encapsulation was inhibited; there was greater inhibition with increasing amounts of yeast extract. Apparently yeast extract (and probably also the other unfavorable media) contain some unknown factor which is responsible for this inhibition of encapsulation.

CAPSULE FORMATION IN SYNTHETIC MEDIA

Since Friedländer's bacterium is able to multiply actively in synthetic media of divergent compositions, it was of interest to determine whether capsule formation would occur also in such media. This not only appears to be the case, but capsule size in synthetic media under special conditions may even exceed the size obtained in neopeptone. However, capsule formation in synthetic media is much more delicate than in the ordinary peptone media.

A 2 to 4 per cent solution of asparagin in distilled water containing 1 per cent glucose and neutralized to pH 7.5 does not allow any growth, due to the lack of the required inorganic salts. However, when to this medium are added traces of Na_2HPO_4

(0.0005 to 0.001 per cent) growth becomes possible, but the medium is still far from a favorable one. Bacteria growing under these conditions, however, are surrounded by capsules equal in size or even larger than in the usual neopeptone medium (fig. 7).

When more than traces of Na_2HPO_4 are added (e.g., 0.0012 to 0.0015 per cent or more) growth becomes more pronounced, but encapsulation is very restricted. It also appears that encapsulation in synthetic media takes place only when growth is inhibited by an inadequate amount of phosphate. This fact, of course, is in line with the observation that encapsulation in the 4 per cent

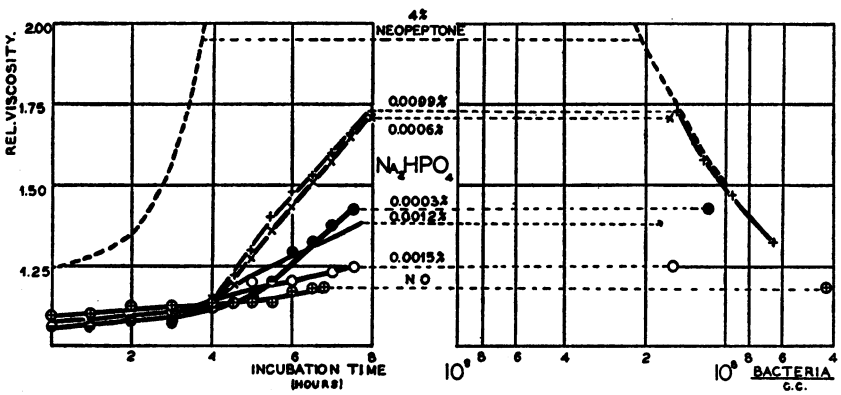

FIG. 7-a FIG. 7-b

FIG. 7-a. Increase in relative viscosity of cultures of Friedländer's bacterium in 3 per cent asparagin-1 per cent glucose solution in distilled water with addition of increasing amounts of Na_2HPO_4 during 8 hours incubation at 37°C. (initial pH 7.5).

7-b. Viscosities of dilutions of the same cultures taken at the conclusion of the experiment (diluent: original nutrient medium).

neopeptone-1 per cent glucose medium takes place after the logarithmic growth phase, when growth is restricted due to unfavorable conditions.

Addition of $MgSO_4$ (0.01 per cent), KCl (0.1 per cent) and a $NaHCO_3$ buffer (1 per cent) added either separately or together, favors growth in the 3 per cent asparagin-1 per cent glucose-0.0005 per cent Na_2HPO_4 medium, but there is still a lack of phosphorus and capsule size remains the same. Due to the increased growth, the relative viscosity is much increased by addition of the inorganic salts.

Similar results are obtained when to a 3 per cent asparagin-1 per cent glucose solution traces of neopeptone are added (0.05 to 0.1 per cent). Growth then becomes possible but the medium is still far from favorable. The bacteria, however, are surrounded by capsules of a size larger than in any other medium tested. Phosphate addition (0.01 per cent) or increase in neopeptone

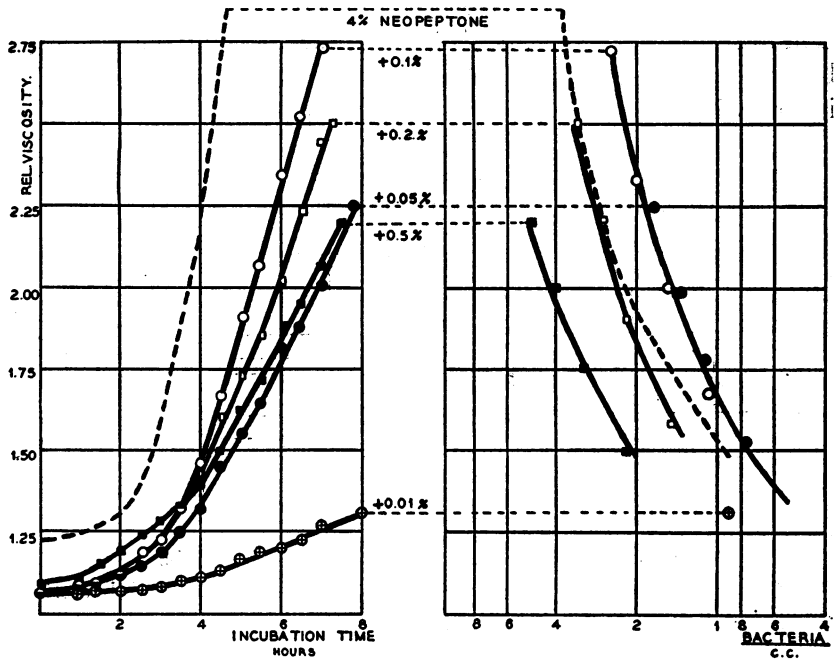

FIG. 8-a FIG. 8-b

FIG. 8-a. Increase in relative viscosity of cultures of Friedländer's bacterium in 3 per cent asparagin-1 per cent glucose solution in distilled water with the addition of increasing small amounts of neopeptone. The same for cultures in 4 per cent neopeptone-1 per cent glucose medium.

8-b. Viscosities of dilutions of the same cultures taken at the conclusion of the experiment (diluent: original nutrient medium).

concentration (0.1 to 0.5 per cent) favors growth, but makes the medium less suitable for encapsulation (fig. 8).

These observations once more stress the fact that the conditions under which capsules are produced are not identical with the conditions under which maximal growth occurs; on the contrary, there seems to exist a contra-relation between growth and en-

capsulation, inasmuch as capsule production seems to occur mainly under conditions unfavorable for growth.

INFLUENCE OF THE pH OF THE MEDIUM AND OF THE INCUBATION TEMPERATURE ON ENCAPSULATION

Friedländer's bacterium is able to grow over a very wide pH range, from about pH 5.0 to pH 9.0, its optimal growth being at

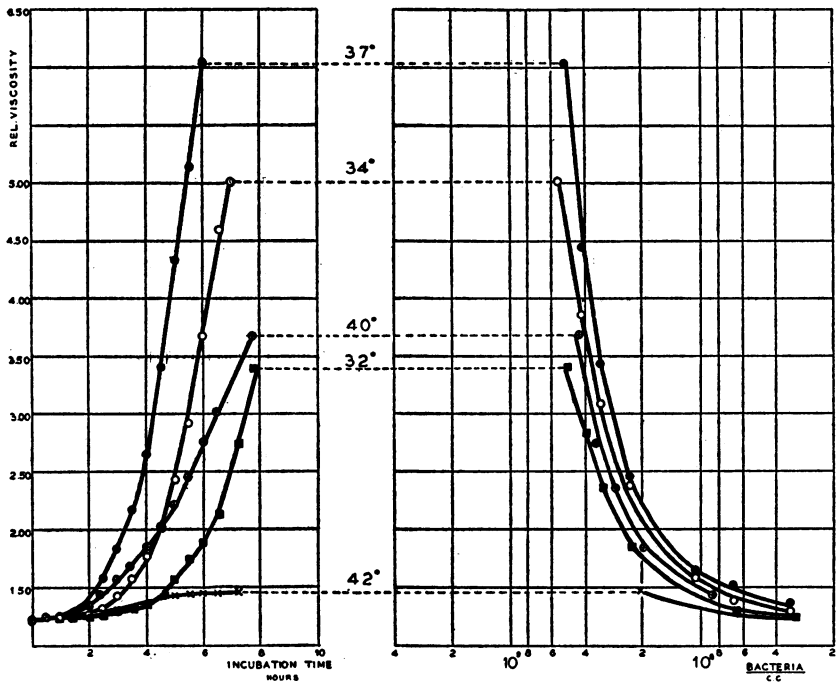

FIG. 9-a FIG. 9-b

FIG. 9-a. Increase in relative viscosity of cultures of Friedländer's bacterium in 4 per cent neopeptone-1 per cent glucose incubated at varying temperatures (32–42°C.) (initial pH 7.5).

9-b. Viscosities of dilutions of the same cultures taken at the conclusion of the experiment (diluent: original nutrient medium).

pH 7.5. It appears, however, that encapsulation is practically independent of the pH of the medium. The same capsule size was obtained in 4 per cent neopeptone-1 per cent glucose, neutralized to pH 6.5, 7.0, 7.5 and 8.0. When not otherwise stated, in all experiments the medium was neutralized to pH 7.5.

Friedländer's bacterium will develop over a wide range of

temperature up to 42°C. Its optimum temperature is 37°C. A sharp drop is observed above 37°C. Below 37°C., growth is retarded but the decline is a gradual one. The same may be said for the increase in relative viscosity. Growing in 4 per cent neopeptone-1 per cent glucose at pH 7.5, the greatest increase in viscosity is observed when incubated at 37°C. At 40°C., this increase is markedly retarded, and at 42°C. almost no increase in viscosity is observed (fig. 9). Capsules of bacteria grown at 40°C., and especially at 42°C., are much smaller than at 37°C. It is probable that at higher incubation temperatures, sugar is fermented to a greater extent (more acid is formed) and less synthesis to polysaccharide takes place. At temperatures lower than 37°C., capsule size diminishes gradually with decrease in temperature, at 32°C. the size being considerably smaller than at 37°C. (When not otherwise stated all experiments have been performed at 37°C.)

THE INFLUENCE OF BUFFER IN THE MEDIUM

A. $NaHCO_3$

The addition of $NaHCO_3$ up to 1 per cent to the nutrient broth increases growth markedly, probably by neutralizing part of the acids formed during fermentation. Viscosity in the presence of $NaHCO_3$ increases tremendously, 5 hours after inoculation the relative viscosity having increased to a value of almost 12 (fig. 10).

Since capsule size is not at all, or only slightly, affected by the addition of $NaHCO_3$ such an addition may be advocated, for instance, in the isolation of capsular polysaccharides from cultures of Friedländer's bacterium.

B. Na_2HPO_4

The addition of Na_2HPO_4 to the nutrient broth also causes a marked increase in growth and viscosity compared with the phosphate-free culture. However, this increase is less than that caused by $NaHCO_3$. A slight precipitate is formed, probably of Ca-phosphate. When this precipitate is not removed, capsule size is the same as in the medium without phosphate, although

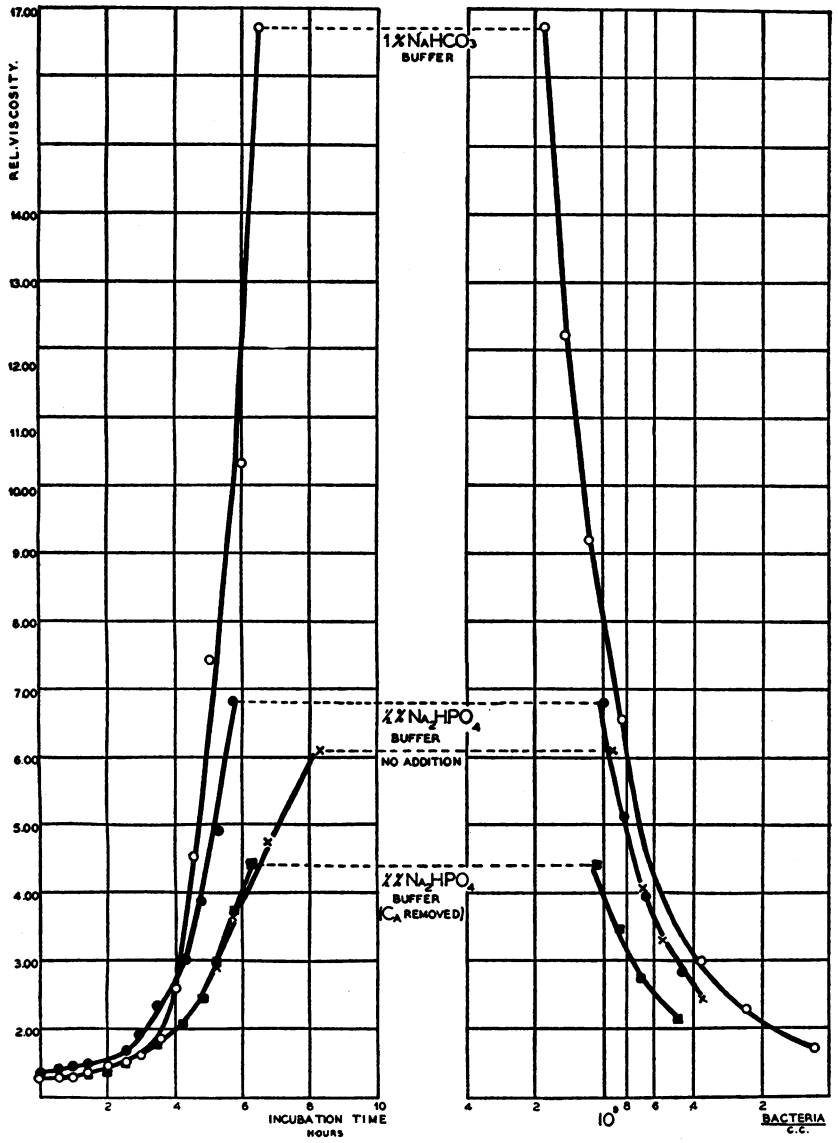

FIG. 10-a FIG. 10-b

FIG. 10-a. Increase in relative viscosity of cultures of Friedländer's bacterium in 4 per cent neopeptone-1 per cent glucose medium with and without addition of carbonate or phosphate buffer during 8 hours incubation at 37°C. (initial pH 7.5).

10-b. Viscosities of dilutions of the same cultures taken at the conclusion of the experiment (diluent: original nutrient medium).

the viscosity is much higher, due to the better growth. When, however, this precipitate is removed from the culture medium, a marked inhibition of capsule formation is observed. This inhibition is due to the removal of practically the entire calcium content of the culture medium. This was proved to be true by a preliminary treatment of the culture medium with Na oxalate in such amount that all of the calcium originally present was precipitated. Capsule formation in such a medium deprived of calcium is much inferior to that in the original medium, but by adding calcium in the form of calcium chloride in the amount that originally was present, capsule formation becomes normal. This indicates that calcium may play an important role in the formation of capsular substance.

IS HEAVIER ENCAPSULATION DUE TO SWELLING OF THE CAPSULES OR TO AN INCREASED PRODUCTION OF CAPSULAR SUBSTANCE?

The question arises whether varying capsule size is really due to an increased production of capsular material or whether it can be ascribed to a greater or lesser swelling of the capsules. This possibility must be taken into consideration, since it is possible to demonstrate a marked swelling of capsules in general by addition of suitable amounts of antiserum (Neufeld reaction). This reaction, however, is specific inasmuch as it can be brought about only by type-specific antisera. Its mechanism is entirely unknown. Consequently the amount of capsular material produced in several different culture media was determined by the combining equivalent method of Felton and Stahl (1935).

Cultures of Friedländer's bacterium were prepared in 4 per cent neopeptone with and without addition of 1 per cent glucose, and also in 4 per cent yeast extract-1 per cent glucose. These cultures were incubated for 7 hours, after which counts were made to determine the bacterial population. Each culture was then diluted with saline until the bacterial concentration was 10^8 bacteria per ml. The cultures were subjected to a 10-minute treatment with ultrasonic vibration, which brings the capsules completely into solution.

Dilution series of these polysaccharide solutions were prepared and 0.3 ml. of each dilution were mixed with 0.3 ml. of antiserum. It was then determined which dilution contained just enough polysaccharide to combine with all the antibodies present in a standard amount of anti-serum. A culture of 4 per cent neopeptone without glucose (10^8 bacteria per milliliter) had to be diluted 1:2; the culture in 4 per cent yeast extract—1 per cent glucose had to be diluted 1:4; a three-hour culture in 4 per cent neopeptone—1 per cent glucose, (10^8 bacteria per milliliter) also had to be diluted 1:4; however, a seven-hour culture in 4 per cent neopeptone—1 per cent glucose had to be diluted 1:16 in order to precipitate all of the polysaccharide present.

This makes it clear that the amount of polysaccharide produced per bacterium in 4 per cent neopeptone—1 per cent glucose, was about 4 times that in 4 per cent yeast extract, and about 8 times that in 4 per cent neopeptone without glucose.

Since the results of these polysaccharide determinations on cultures in various media are in agreement with the viscosity measurements, and with the sizes of capsules as demonstrated by India ink smears, it seems, that heavier encapsulation means greater production of polysaccharide and not merely swelling of the capsules.

Similar results were obtained by estimation of the dry weight of the precipitated bacteria from 20 ml. of cultures of Friedländer's bacterium in the media mentioned above, after precipitation with 20 ml. of alcohol. The highest dry weight for a standard amount of bacteria was obtained from a seven-hour culture in 4 per cent neopeptone—1 per cent glucose.

DISCUSSION

Many investigators are of the opinion that heavy capsule formation takes place only in the animal body as a protection against phagocytosis, that continued subculturing on artificial media gives rise to a gradual diminution in capsule formation, and that repeated animal passages may completely or partially restore the loss.

As a matter of fact, there is little reason to assume that body fluids would possess such a magical action by which pathogens could encapsulate themselves. This study indicates that the reason why many encapsulated organisms lose their capsules by continued subculturing on artificial media may be that the media upon which they are cultivated contain such abundance of nutrient substances that there is no tendency towards encapsulation.

In many cases, it has been shown that encapsulation occurs under conditions unfavorable for growth and not when active proliferation is occurring. Instead of transplanting freshly isolated mucoid strains frequently, it seems more reasonable to store them as long as possible and to transplant them no oftener than is absolutely necessary.

The abundance with which Friedländer's bacterium forms capsular polysaccharides under certain circumstances offers no reason to believe that the capsule is a cell constituent; it is more reasonable to consider it as a metabolite. Under certain conditions the normal bacterial metabolism is changed and instead of the normal assimilation to new cell-material (growth), abnormal assimilation takes place in which polysaccharides are synthesized from the intermediates of the sugar break-down.

To what extent the formation of capsules only after the logarithmic phase of growth is a general phenomenon is not known, but it may be stated that *Betacoccus dextranicus* (*Leuconostoc mesentericus*) behaves similarly to Friedländer's bacterium in this respect.

SUMMARY

1. Friedländer's bacterium was grown in Ostwald viscosity tubes, and, from the determinations of relative viscosity and simultaneous bacterial counts, deductions could be made as to the degree of encapsulation.

2. The largest capsules were obtained by cultivation for about 8 hours at 37°C. in a medium containing 1 to 4 per cent peptone and 1 per cent glucose, initial pH 7.5.

3. Capsule formation took place mainly after the logarithmic

phase of growth when the multiplication of the bacteria became restricted.

4. In the absence of sugars, the capsules were very small. The presence either of glucose, maltose, sucrose, mannitol, or to a lesser extent, pyruvate or glycerin, was necessary to ensure the formation of large capsules.

5. The protein source was also important for encapsulation. Neopeptone, Witte peptone or bactopeptone served equally well as sources of protein. However, several media such as yeast extract, Loeffler's blood serum, and brain-heart infusion contained factors, as yet unknown, which inhibited encapsulation markedly, although they did not affect the growth of the bacteria.

6. In a synthetic medium, capsule formation took place only under special conditions, i.e., when the phosphate concentration was below the limits of optimal bacterial growth.

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PLATES 1-4

INDIA INK SMEARS OF ENCAPSULATED FRIEDLANDER'S BACTERIUM GROWN UNDER DIFFERENT CONDITIONS

PLATE 1. Encapsulated bacteria from a 4 per cent neopeptone culture without addition of carbohydrates.

PLATE 2. Encapsulated bacteria from a 4 per cent neopeptone-1 per cent glucose culture (3 hours after incubation).

PLATE 3. The same culture 7 hours after incubation.

PLATE 4. Encapsulated bacteria from a 4 per cent yeast extract-1 per cent glucose culture.

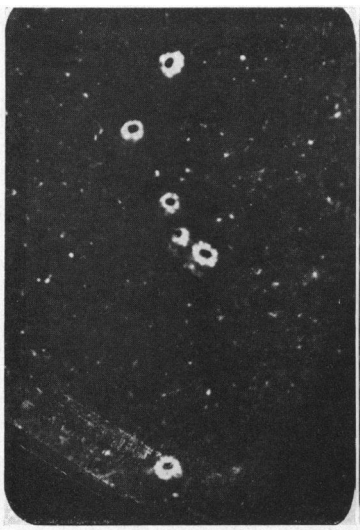

PLATE 1

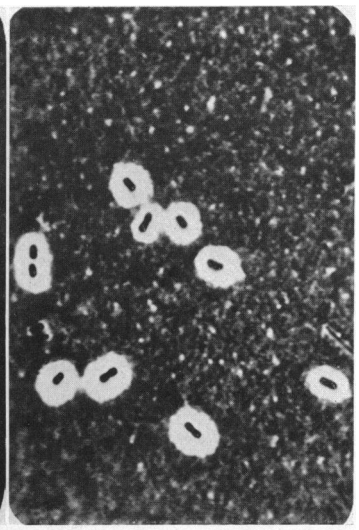

PLATE 2

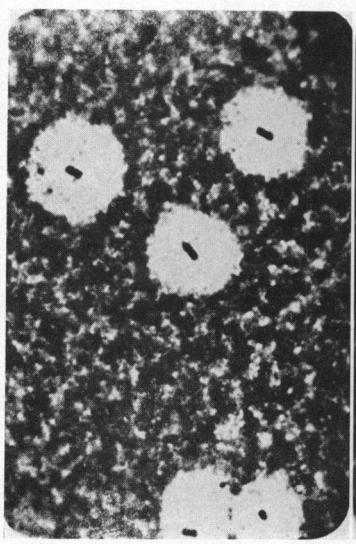

PLATE 3

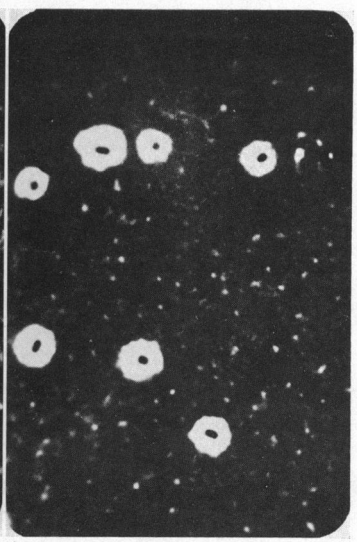

PLATE 4

(J. C. Hoogerheide)