CAPSULES AND MUCOID ENVELOPES OF BACTERIA

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(With Plates 10 and 11)

In bacteria, the cultures of which exhibit a mucoid appearance or an opaqueness of growth or both, capsules and slimy envelopes seem to occur. These two components are distinct from the morphological and biochemical point of view. According to Etinger-Tulczynska (1933) the capsule is a part of the bacterium, the slime a secretion. As envelopes of bacteria are usually stained negatively, and as there seems to be some confusion in regard to the differentiation of these two different components, I developed new methods by which they can be fixed and stained positively and differentially. In this paper the methods devised and the results obtained in the examination of altogether fifty mucoid or opaque as well as various non-mucoid and transparent cultures, belonging to different groups of organisms, are reported.

METHODS

A 1. *For capsules*

The cultures are grown on the surface of serum-agar* plates (15–20 % serum content). An addition of 0·5 % of glucose and cultivation in a CO_2-containing atmosphere are often advantageous. So as to obtain a proper spacing of the organisms and a film consisting of one layer only of bacteria, preparations are made from very young growths. Small blocks are cut out of the agar and placed on clean, flamed cover-slips with the growth towards the glass surface. The cover-slips, with their adherent agar pieces upwards, are placed into watch-glasses, covered with Bouin's fixing solution† and kept for 2–3 hr. in a moist chamber at room temperature. Then the agar blocks are pealed off with the tip of a scalpel. The cover-glasses, with their adherent films, are washed and stained in Giemsa solution (Gurr's R 55), diluted with a mixture of tap water and distilled water. The most appropriate dilutions of the Giemsa solution and the time of staining seem to depend on local conditions (properties of the tap water, brand of Giemsa), as they differed widely when carried out at Zürich and in London, respectively. The preparations are mounted in the thin stain or in neutral glycerine-gelatine, in which they sometimes keep for weeks. The bacteria appear dark violet inside a pinkish capsule.

* In this work a trypsin-digest-meat-infusion peptone-agar medium was used.

† 15 c.c. saturated aqueous solution of picric acid, 15 c.c. of formalin and 1 c.c. of glacial acetic acid.

A 2. *For capsules*

In some cases, as, for example, with organisms of the Friedländer group and *B. anthracis*, a very effective staining was obtained when Bouin-fixed preparations (see A 1) were treated with 5 % tannic acid for ½ hr., then washed thoroughly and stained in watery crystal violet (1:10,000) for 15–30 min. or longer. These preparations were mounted as in A 1. The bacteria remain unstained if the process of staining is not continued too long, and the capsules appear in a deep violet tint. Robinow (1946) first used this method for the staining of cell walls.

B. *For slime*

The bacteria are grown on adequate solid media with or without the addition of serum. The time of incubation is unimportant, but obviously must not be so long that the cultures dry out. The material is taken from the culture with a saline-filled loop and spread cautiously and thinly on clean, flamed cover-slips which are allowed to dry in air. The preparations are fixed in Chabaud's (1942) solution‡ for about 10 min., washed and mordanted in 5 % tannic acid solution for 39 min., or, if a stronger effect seems desirable, Löffler's mordant may be used. After thorough washing they are stained in crystal violet 1:10,000 for periods of one to several hours. Eventually the preparations are mounted as in A 1, but for some of the specimens mounting in glycerine-gelatine is not suitable. The bacteria appear a deep violet in their outlines or as a whole, the slime appears delicately pink where it is thin, and deeply red in thick layers.

‡ 60 c.c. of 80 % alcohol, 5 c.c. of formalin, 2 c.c. of glacial acetic acid and 15 g. of crystalline phenol.

ORGANISMS EXAMINED

Pneumococci, streptococci, organisms of the Friedländer, *aerogenes* and *coli* groups, staphylococci, *B. anthracis*, organisms of the *B. subtilis* group, organisms of the Salmonella group, *Haemophilus influenzae* and *B. pertussis*.

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RESULTS

By the application of the methods described it has been shown that capsules of bacteria are of definite shape, of more or less definite density throughout and of definite outline, while slime envelopes are amorphous and can therefore be drawn out into manifold structures, are most concentrated in the vicinity of the bacterial cells, and decrease in density with increasing distance from the cell. Capsules and slime are distinct morphologically; their biochemical difference is obvious, as the methods for the demonstration of capsules by fixation and staining are not applicable in the case of the presence of slime only and vice versa. The various appearances and properties of capsules and slimy envelopes in the organisms examined are briefly outlined below.

Capsules

The size of the capsule, its constitution and the ease with which it can be fixed and stained are widely influenced by conditions of cultivation. Addition of glucose to the medium and presence of CO_2 in the atmosphere greatly stimulate the formation of capsules in some organisms, as, for example, in the Friedländer, *coli* and *aerogenes* groups. As is well known, strains of *B. anthracis*, which do not form capsules under ordinary conditions outside the animal body, will form capsules as soon as they are cultivated on glucose serum agar in an atmosphere of 10–20 % of CO_2. On the other hand, the particular mucoid variant of *B. anthracis* 'HM' (Hanby, Rydon & Bruce-White, 1946) formed capsules readily on 'CCY medium' (casein, casein-hydrolysate, yeast agar).

The serum content has a definite influence on the demonstrability of the capsules. On occasions capsules can be demonstrated from bacterial growth on media devoid of serum, but usually they seem to have collapsed, and the organisms are surrounded by irregular pink material. It is suggested that in cultures grown on media rich in serum, constituents of the serum have unspecifically diffused into the delicate gel of the capsule, thus increasing its solidity. Now they can be fixed and stained easily, and pictures are produced as illustrated in Pls. 10, 11, figs. 1, 2, 3, 5, 6, 7, 8 and 11. Bouin's solution, a watery fixative, seems to be very suitable (if allowed to diffuse through the agar) for the fixation of capsules, but if an alcoholic fixative like Chabaud's is used the collapsed capsules can be seen as an amorphous mass, surrounding the darkly outlined bacillus (see Pl. 10, fig. 4).

The organisms of the Friedländer group are usually characterized by particularly large and dense capsules, as shown in Pl. 10, figs. 1–3. The capsules often show a distinct outline (figs. 1 and 3). Often several rods are enclosed in one extended capsule. Fig. 2 shows that the capsules, if mordanted, acquire a particularly strong affinity for the stain, which they take very deeply. As can be seen, the bacillary edge stains strongly also. It probably consists of the cell wall and adjoining cytoplasmic layer. The penetration of the stain into the cell is apparently inhibited and is only accomplished when the staining process is continued for a very long time.

By the kindness of Dr F. Kauffmann (Copenhagen) the writer was given an opportunity to examine a collection of strains of the Friedländer, *aerogenes* and *coli* groups, previously investigated by him. It was shown that in all strains which possessed an A-antigen (Kauffmann, 1947), a capsule could be demonstrated by the methods described, with one exception only, viz. *coli* strain Bi 316/42 (test strain of O-group 9, Kauffmann), which possesses a capsule and at the same time a thermolabile L-antigen but no A-antigen. The other strains with L-antigens, so far examined, possessed no demonstrable envelopes, neither capsules nor slime. Pl. 10, figs. 1–3, illustrate capsules of strains of the Friedländer type, fig. 5 shows a typical picture of a strain of *B. lactis aerogenes*, while fig. 6 demonstrates the delicate, but nevertheless distinct capsule of a *coli* strain, possessing an A-antigen. These capsular *coli* strains may throw off uncapsulated variants. In one of the strains, obtained from the N.C.T.C., listed as Oezaena E 5051, an A-antigen (Kauffmann) has so far not been demonstrated, and in agreement with this I was not able to find a capsule, but I showed that its mucoid growth was due to amorphous slime, as illustrated in Pl. 11, fig. 12.

Among the cultures of other groups here examined the mucoid variant of *B. anthracis* 'HM' forms a particularly conspicuous capsule on CCY medium, as mentioned before (see Pl. 11, fig. 7); some other *anthrax* strains formed capsules of a rather more irregular shape, when grown on serum agar in a CO_2 atmosphere (Klieneberger-Nobel, 1948).

A study of organisms of the *Pneumococcus-Streptococcus* group revealed that capsules occur in some, slime in other organisms, while a third type produces both components together.

The typical, well-capsulated bacterium of the group, the *Pneumococcus*, type III, showed a very conspicuous, large and readily staining capsule (Pl. 11, figs. 8, 9). Like the Friedländer bacillus this *Pneumococcus* possesses slime in addition to capsules. The capsules with their surrounding slime cannot be demonstrated in the same preparation by any of the methods described; but if an immune serum is first allowed to react with the organisms and the capsule-staining methods are then applied, both components may become visible, as can be seen in Fig. 9. The preparation here illustrated was stained by method A 2; in consequence the cocci appear unstained; they are surrounded by a very darkly

stained capsule, which again is surrounded by delicately stained slime.

For purposes of diagnosis the so-called 'capsule swelling reaction' introduced by Neufeld (1902) is used extensively. It seems that when the immune serum is mixed with capsulated organisms, not only unspecific but also specific serum components diffuse into the capsule and transform it into a body of such solidity that it can now be seen in the untreated bacteria, particularly if a drop of stain such as methylene blue or fuchsin is added. The present author believes that the name 'swelling reaction' is misleading. Neufeld at first seems to have been under the impression that the cocci themselves enlarge under the influence of homologous serum. Later it was assumed that the capsules swell. Recently Mudd, Heinmets & Anderson (1943) tried to demonstrate the 'capsule swelling reaction' by means of the electron microscope. Yet their illustrations are unsatisfactory, as they show no differentiation whatsoever; the coccus is not distinct from the capsule, and the capsulated organisms are not distinct from the embedding slime; nothing else but one dark mass is seen in the photographs. As the immune serum, according to the present findings, reacts not only with the capsules but also with the surrounding slime, it is suggested that what Mudd and collaborators interpret as the swollen capsule is a mass consisting of the coccus, the capsule and the surrounding slime. In numerous tests in which immune serum was added to the homologous bacteria, actual swelling was never demonstrated. Even on agar devoid of serum it could be shown—as mentioned before—that the capsules of Friedländer bacilli developed to their full size. This suggests that capsules, however delicate they may be, usually develop to their full extent and do not swell, though they solidify considerably by addition of immune serum.

It seems of interest to mention *Streptococcus parafaecalis*, several strains of which I received by the kindness of Dr Skadhauge (Copenhagen). According to him (1948) they possess an A-antigen. They were found to be capsule-positive by me, though their capsules were very delicate. Pl. 11, fig. 11, illustrates the appearance of one of these strains when fixed and stained according to method A 1. The outline of the capsule can be seen.

Slime

Among the *Streptococcus* cultures examined, one, kindly supplied and previously examined by Prof. Grumbach, Zürich, was characterized by particularly mucoid growth (see Grumbach, 1945). This organism, *Str. equi*, gave a strong slime reaction, but capsules were not present (see also Klieneberger-Nobel, 1948). In Pl. 11, fig. 10, the amorphous slime surrounding the streptococci can be seen; the inner part of the cocci is unstained, the cell walls with probably adjoining cytoplasmic layer are deeply stained. In other slime-producing organisms, as, for example, *Leuconostoc mesenteroides* and *Salmonella paratyphi* B (grown at room temperature!), similar pictures were obtained (see again Pl. 11, fig. 12). In all these organisms the bacterial edge was conspicuously stained while the interior of the bacterium was unstained. An exception to this rule is illustrated in Pl. 11, fig. 13. It shows the edge of a microculture of *Myxococcus fulvus*, a culture of which was kindly supplied by Dr Singh (Rothamsted). Vegetative rods and microcysts can be distinguished. It is generally agreed that Myxococci produce slime; the microcysts, building up the fruiting bodies, cohere by means of this slime. The amorphous mucoid substance surrounding each bacillus and even more conspicuously each microcyst is distinctly seen in the illustration. It is of particular interest that the microcysts which, as is generally known, possess a kind of wall, show a deeply stained outline, while the bacilli, which are supposed to possess no cell wall and are naked, show no outline, but are encased by slime. This distinguishes them from the eubacteria so far examined.

The various capsules stained by the methods described are of similar appearance in regard to even density and outline. One exception, however, was found, viz. the capsule of *Haemophilus influenzae*. First, its capsule could not be demonstrated in the ordinary way. It was necessary to subject the grown culture first to its immune serum and then to carry out the capsule-staining methods. In this way an envelope was demonstrated in both types, *a* and *b*, kindly supplied by Dr Zinnemann (Leeds), which distinguished itself from slime by an even density throughout and by surrounding the bacilli evenly. Yet its capsule showed no definite outline which distinguished it from those of the other capsulated bacteria investigated (see Pl. 11, fig. 14). If the variance of the capsule of *H. influenzae* is due to its particular delicateness, or if it is really of a different nature could not be decided here. In fresh cultures of *B. pertussis* on Bordet-Gengou medium a delicate slime could be demonstrated, but a capsule was not found.

In various non-mucoid cultures of streptococci, staphylococci and members of the *coli-Salmonella* groups capsules or amorphous slime were not demonstrated.

SUMMARY

Methods have been described by which capsules and mucoid envelopes of bacteria can be fixed and stained positively and differentially, proving they are of different natures. The capsule is of definite shape and outline, the slime is amorphous. Some bacteria may have capsules, some may produce slime, and in

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others the capsulated organisms may be embedded in amorphous slime, as, for example, in the Friedländer bacillus and in the *Pneumococcus*, type III. These properties are probably more or less characteristic for certain organisms or groups of organisms. Of course, loss of capsules, and loss of slime production do occur.

All bacteria which have capsules seem to possess special antigens (cp. Kauffmann's A-antigen). This was particularly shown for members of the *coli-aerogenes*-Friedländer groups and *Streptococcus para-faecalis* (Skadhauge). Organisms with L-antigens possess generally no capsular or mucoid envelopes of the type described, with one exception, viz. strain Bi 316/42.

By means of the capsule- and slime-staining methods here described, a densely staining edge was demonstrated in the bacteria examined, which—it is suggested—may comprise the cell wall and its adjoining cytoplasmic layer. Organisms supposed to have no cell walls (Myxococci) do not show this appearance.

The so-called 'swelling reaction' is a reaction between antiserum and homologous bacteria, by which the capsule becomes easily demonstrable. It seems doubtful that actual swelling takes place.

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EXPLANATION OF PLATES 10 AND 11

(Final magnification of photographs × 2500, with the exception of fig. 9, the magnification of which is × 1700.)

Plate 10

Fig. 1. Oezaena bacillus 5052, N.C.T.C., method A 1. This photograph was focused on the edge of the capsule, which therefore appears sharp, in consequence the bacillus is slightly out of focus and appears wider than it really is.

Fig. 2. Friedländer bacillus, freshly isolated from sputum, supplied by Prof. Grumbach (Zürich), method A 2. Here the bacillus is in focus and shows its darkly staining edge; in consequence the edge of the capsule is out of focus.

Fig. 3. The same Friedländer bacillus as in fig. 2, but, after having been kept on artificial media for some months, the capsule is more slender. Method A 1.

Fig. 4. The same Friedländer bacillus, method B. The capsule is destroyed and cannot be distinguished from the surrounding slime; the edge of the bacillus is distinctly and deeply stained.

Fig. 5. *B. lactis aerogenes* 243, N.C.T.C., method A 1. The slender capsule is delicately stained. Note its outline in some of the organisms.

Fig. 6. *B. coli* Bi 449, supplied by Dr Kauffmann (Copenhagen), method A 1. Note the very delicate capsule with distinct outline.

Plate 11

Fig. 7. Mucoid variant of *B. anthracis* 'HM', supplied by Dr Herbert (London). Method A 1.

Fig. 8. *Pneumococcus*, type III, supplied by Prof. Grumbach (Zürich); method A 2. Chains of organisms are enclosed in one capsule, the shape of which is influenced by the arrangement of the cocci.

Fig. 9. The same *Pneumococcus*, type III, method A 2, following application of the homologous serum. Note the unstained cocci, the darkly stained capsules and the delicate surrounding slime.

Fig. 10. *Streptococcus equi*, supplied by Prof. Grumbach (Zürich), method B. Note the irregularly arranged slime and the darkly stained edge of the unstained cocci.

Fig. 11. *Streptococcus para-faecalis*, supplied by Dr Skadhauge (Copenhagen), method A 1. Note the delicate capsules with their definite outlines.

Fig. 12. So-called Oezaena bacillus 5051, N.C.T.C., method B. The bacilli appear embedded in slime. Note their dark outlines where in focus.

Fig. 13. *Myxococcus fulvus*, supplied by Dr Singh (Rothamsted), method B. Note the long bacillary forms, which show no outline but are surrounded by slime, and the microcysts showing a darkly stained edge as well as a slime envelope.

Fig. 14. *Haemophilus influenzae*, supplied by Dr Zinnemann (Leeds), method A 1, following application of immune serum. Note the delicate envelope showing no outline.

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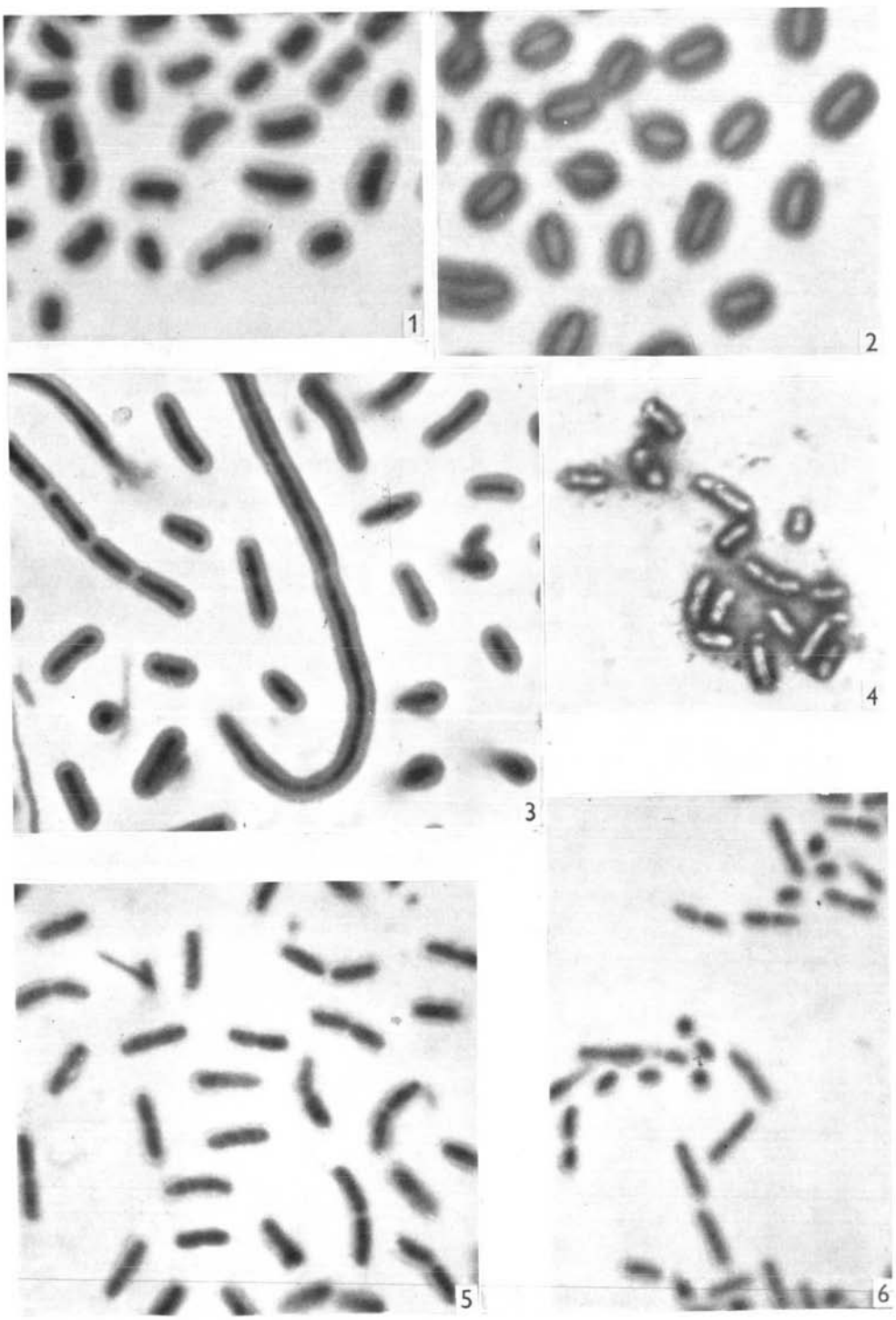

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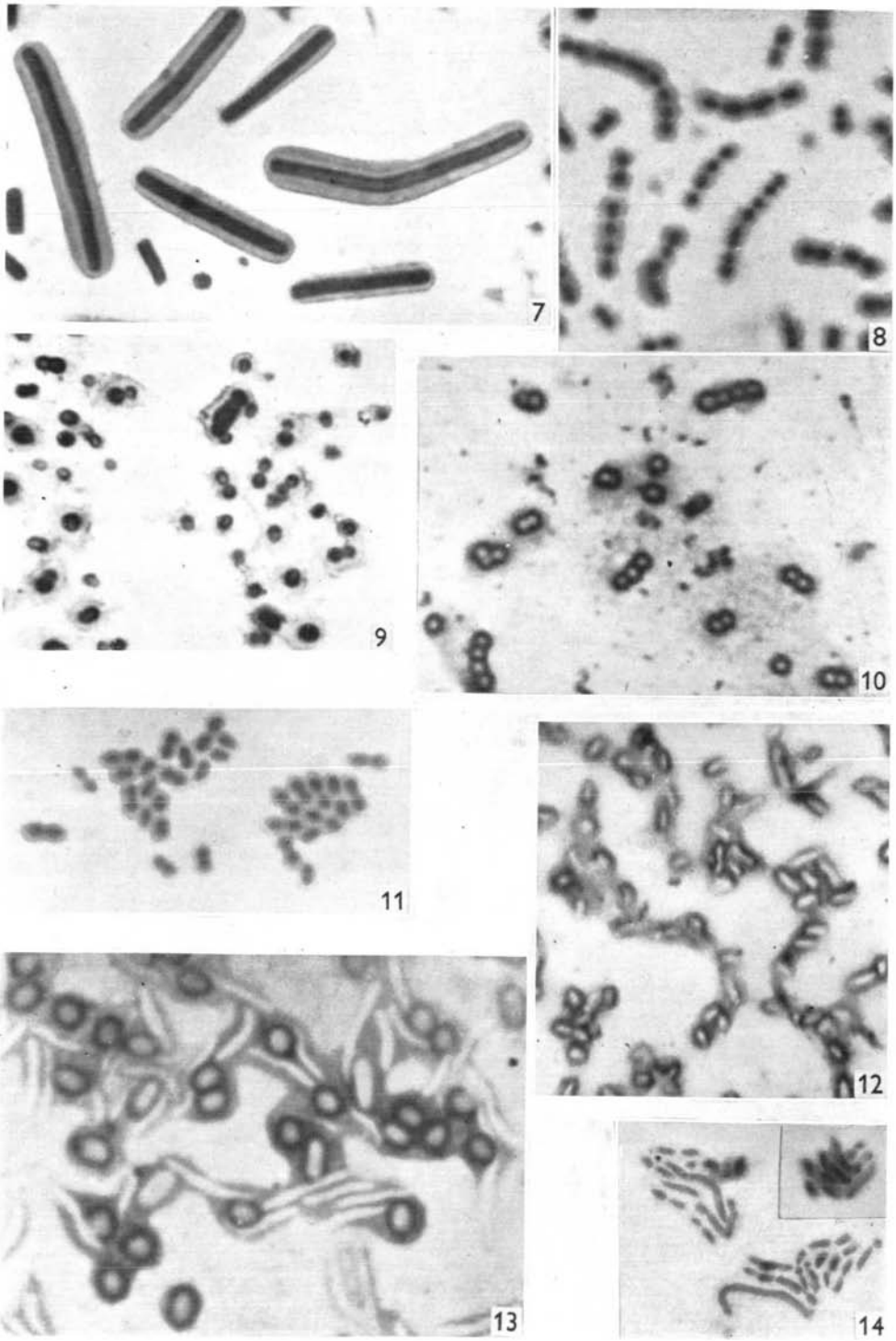

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