THE PNEUMOCOCCAL CAPSULAR SWELLING REACTION, STUDIED WITH THE AID OF THE ELECTRON MICROSCOPE

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Plates 12 to 14

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The pneumococcal specific capsular swelling reaction was discovered by Neufeld (1) in 1902 and applied to practical diagnosis by Neufeld and Etinger-Tulczynska (2), Armstrong (3), Logan and Smeall (4), Sabin (5), and subsequently many others. The phenomenon was investigated in detail by Etinger-Tulczynska (6). Capsular swelling has been described as reversible by heat (Neufeld, see reference 6), by addition of excess of specific soluble substance (7), and by digestion of the serum protein by papain (8).

In the present study the phenomenon is investigated with the aid of the electron microscope; brief reports of this work have appeared (9, 10).

Technique

The pneumococci used were capsulated Type 1 and Type 3 strains; they were grown for 18 to 25 hours on fresh 5 per cent horse blood extract agar plates incubated in large air-tight jars to preserve moisture. We are indebted for the purified rabbit Type 1 antipneumococcal globulin used to Dr. Michael Heidelberger, and to Dr. H. E. Morton and Mr. Frank B. Engley, Jr. for help with the cultures.

The electron microscopes used were RCA 60 kv. Type B commercial instruments, first at the RCA Research Laboratories in Camden, later at the Johnson Foundation for Medical Physics, University of Pennsylvania. Of several procedures for preparing the bacteria for study the following has been found to be most useful:

A clean platinum inoculating loop was dipped in distilled water. The tip of an inoculating wire was touched to the surface of a pneumococcal colony on the plate; the tip of the wire was then thrust several times through the film of distilled water in the inoculating loop; the film, thus inoculated, was touched to the collodion mount on the usual 200 mesh wire supporting screen; this preparation was allowed to dry and was washed in distilled water. If treatment with serum was desired, a film of the appropriate dilution was taken up in an inoculating loop and transferred to the dried specimen; after the desired time interval, the specimen was dipped repeatedly into isotonic saline solution and then into distilled water in a test tube; the reaction with serum was thus stopped at the desired time and the excess of serum was removed from

the mount. The specimen when dried was ready for micrographing. Pictures were usually taken at an electron magnification on the plate of 7,000, and later enlarged optically as required.

EXPERIMENTAL

Fig. 1 shows pairs and individual cells of Pneumococcus Type 3. The bacterial protoplasm in this preparation fills the cell walls completely so that the cell wall is not visible as a separate structure; the capsule surrounds the cell wall as, in the words of Pasteur's (11) original description, a sort of aureole. In Fig. 2 the diplococci have been exposed for 30 seconds to the action of Type 1 antipneumococcal rabbit serum in 1:10 dilution. Although this considerable concentration of protein has left an obvious deposit on the collodion mount, the capsules of the pneumococci are little affected by the heterologous serum.

In Figs. 3 to 7 the Type 3 pneumococci have been exposed for 2 minutes in each case to Type 3 rabbit serum in dilutions, respectively, of 1:200, 1:100, 1:10, 1:3, and 1:3. Successive stages in the permeation of the capsule by serum, with increase in size and density, are obvious. After treatment with homologous serum in dilution of 1:3 (Figs. 6 and 7) the density of the swollen capsule is such as completely to obscure the pneumococcal cell or cells contained within.

Figs. 8 to 11 are micrographs of Pneumococcus Type 1 cells. In Fig. 8 the cells have not been exposed to antibody. In Figs. 9 and 10 the pneumococci have been exposed for 2 minutes, respectively, to 1:5 and 1:2 dilutions and in Fig. 11 to an undiluted solution containing 43 mg. of purified rabbit Type 1 antibody globulin per 100 ml. of 0.9 per cent NaCl solution. The diplococci in this series are plainly visible even though inside capsules permeated with the antibody globulin.

Figs. 12 to 14 are micrographs of Type 1 pneumococcal cells from the same culture after exposure in each case for 3 minutes to a saline solution containing 11 mg. per 100 cc. of the purified Type 1 rabbit antibody globulin. In Fig. 13 the antibody globulin solution was dissolved in 0.9 per cent NaCl solution; in Figs. 12 and 14 the saline globulin solution contained also fresh normal rabbit serum in final dilution of 1:4. The capsules appear to be swollen almost equally in the three micrographs; the capsules are, however, strikingly greater in density in Figs. 12 and 14 in which fresh normal rabbit serum had been present in addition to the specific antibody.

Figs. 15 to 17 are of another culture of Pneumococcus Type 1 treated for 2 minutes with a saline solution of the rabbit antibody globulin containing 11 mg. per 100 ml. of solution. Figs. 15 and 17 were without added serum; the preparation in Fig. 16 had in addition fresh normal rabbit serum in final concentration of 1:4. The greater density of the swollen capsules in the preparation treated with antibody plus normal rabbit serum is strikingly apparent.

Figs. 18 and 19 are pneumococci of Type 1 after exposure for 2 minutes to

horse Type 1 antipneumococcal serum. For Fig. 18 the horse serum was diluted 1:3, for Fig. 19 it was undiluted. Some permeation of the capsule by serum, with swelling and increased density, has occurred; the increase in capsular thickness and density is, however, considerably less than with rabbit antiserum of similar concentration.

DISCUSSION

The pneumococcal capsular polysaccharide, when prepared with meticulous precautions against alteration (12), has been found to be in the form of thread-shaped carbohydrate polymers. The micrographs in this study and another published elsewhere (13) indicate that the pneumococcal capsule is a gel of low density outside of and closely enveloping the cell wall; the cell wall in turn envelops the bacterial protoplasm with its outer limiting membrane. Whether or not the cell wall and the inner protoplasm with its limiting membrane are actually distinguishable in electron micrographs as separate structures depends in part upon how closely the protoplasm is applied to the cell wall. Slight shrinkage of the protoplasm due to drying or plasmolysis, or escape of the inner protoplasm following injury of the cell, clearly shows the distinctness of the cell wall, which is in the solid state, from the inner protoplasm, which is either in a fluid state or is a hydrogel which may readily become a hydrosol.

The increase in thickness of the capsular gel on treatment with homologous immune serum may be relatively great; for instance in Figs. 12 and 14 the swollen capsule was of the order of 1 cm. in thickness at 14,000 magnification, or an absolute thickness of about 700 mμ. Neurath (14) has estimated the (unhydrated) rabbit antibody globulin molecule to be an ellipsoid with major and minor axes, respectively, 27 mμ and 4 mμ. The increase in capsular thickness thus may be twenty-five or more times the length of the major axis of the antibody globulin molecule.

This result is in contrast to the action of serum upon a non-capsulated bacterial cell; *e.g.*, the action of somatic and flagellar antibodies on *Eberthella typhosa*. We have succeeded thus far (15) only in depositing specific antibody on *E. typhosa* to a thickness which is within the dimensions of a possible monomolecular film; the possibility that even such a film may be a composite of tangentially disposed antibody molecules and secondarily combined (16) or adsorbed serum components has not been excluded, however.

The presumptive permeation of the pneumococcal capsular gel by antibody would of course afford a much greater effective surface, per bacterium, for the interaction between antigen and antibody than is afforded the antigens in the cell wall of a non-encapsulated bacterium. This, together with the high antibody combining capacity of pneumococcal polysaccharide (17), would seem to explain the relatively low agglutinating titers of antipneumococcal sera even when high in antibody content.

It is interesting in this connection that Hershey (18) found that the amount

of antibody actually absorbed by an encapsulated pneumococcal cell was considerably greater than the amount necessary to form a single surface layer of closely packed molecules, and his tentative explanation was "that the pneumococcal surface is covered with projecting molecules or fibers of soluble specific substance, to which antibody attaches itself."

The pneumococcal capsules acted upon by specific antibody in the presence of diluted fresh normal rabbit serum have been strikingly more increased in density than have those capsules acted upon by the specific rabbit antibody alone (*cf.* Figs. 12, 14, and 16 in comparison with Figs. 13, 15, and 17). This indicates, we believe, that non-specific components of the normal serum are secondarily combined (16) or adsorbed[1] on the primary capsular carbohydrate-antibody complex.

In apparent contradiction to this conclusion it may be recalled that Heidelberger and Kendall (19), working with specific Type 1 and Type 3 polysaccharides and equine antibody, found that the N:SS ratios in specific precipitates were the same whether whole immune serum or relatively purified antibody was used. However, pneumococcal carbohydrate-equine antibody complexes are exceptional in that they do not adsorb those components of normal serum which are collectively known as complement (20). Pneumococcal carbohydrate-rabbit antibody complexes do fix complement (21, 22). Indeed, Heidelberger and Kabat (17), working with Type 1 pneumococci and raw immune rabbit serum, write of "absorption of a small amount of complement" by the sensitized bacteria. Ecker, Pillemer, and Kuehn (23) have shown that staphylococci specifically sensitized with rabbit antibody adsorb non-specific components from fresh rabbit serum. Robertson and his coworkers (24) working with the pneumococcidal effects of serum-leucocyte mixtures have "shown that, in general, immune serum is effective in higher dilutions when used with normal serum and leucocytes obtained from the same animal." Horsfall and Goodner (25) have shown that the specific precipitate formed from Pneumococcus Type 1 carbohydrate and immune rabbit serum contains an adsorbed non-amino phosphatide, believed to be lecithin, and other lipids; the adsorbed lipid forms from 4 to 51 per cent by weight of the specific precipitate (26).

Figs. 1 to 19 show that the interaction of specific antiserum with pneumo-

[1] Heidelberger, Weil, and Treffers (16) have recorded evidence that the combining component of complement (C'1 or mid-piece) is combined in the antigen-antibody complex "either through the attraction of approaching ionized groupings of opposite sign, through hydrogen bonding, through spatial accommodation of large groupings on C'1 and A, or through the presence, on C'1, as on antigen and antibody, of more than one grouping capable of reacting with A molecules brought into apposition." Phospholipin and other components of normal serum have also to be considered as possibly contributing to the observed increase in density of pneumococcal capsules acted upon by specific antibody in the presence of diluted fresh serum.

coccal capsular substance is responsible for changes in the pneumococci covering a considerable range, dependent upon the relative concentrations of the reactants and upon the combination or adsorption of non-specific serum components by the specific antigen-antibody complex. The biological consequences of this interaction also cover a wide range and may include, according to circumstances, specific precipitation, agglutination, enhanced phagocytosis by polymorphonuclear leucocytes and macrophages, allergic reactions, the pneumococcidal action of the whole blood *in vitro*, and improved clearing capacity *in vivo*. It would be a strange coincidence, indeed, if the relative concentrations of antibody required for the detection of these various effects were the same. Indeed the conclusion may be drawn from the thorough, comparative study of H. D. Wright (27) on the interaction of Pneumococcus Type 1 and its homologous antiserum, that the threshold sensitivities of the various manifestations of the antigen-antibody reaction are very different. Thus Wright records regularly finding enhanced type-specific pneumococcidal action of the blood *in vitro* and improved clearing capacity *in vivo* before the "appearance of agglutinins, precipitins, opsonins and complement fixing antibodies" and that the improved clearing capacity far outlasts "the ordinary antibodies." The conclusion should certainly not be drawn, as has so often been the case, that these several reactions need be due to different antibodies. The conclusion that can be drawn is that the various reactions by which the interaction of specific carbohydrate with its antibody is detected may have very different sensitivities, and hence may become manifest at different stages in immunization: this conclusion may perhaps seem more acceptable after consideration of the electron micrographs presented.

SUMMARY

Electron micrographs indicate, in harmony with previous findings, that the pneumococcal capsule is a gel of low density outside of and closely applied to the bacterial cell wall. Interaction with homologous immune rabbit serum greatly increases the thickness and density of this capsular gel; the increase in thickness of the specifically swollen pneumococcal capsule may exceed by 25-fold the thickness of the surface deposit caused by rabbit immune serum on the cell walls and flagella of homologous non-capsulated bacteria.

Conclusions drawn from these and earlier data are that homologous immune serum permeates the pneumococcal capsular gel; the specific antibody combines with the capsular polysaccharide; non-specific serum components are secondarily adsorbed to or combined with the specific antigen-antibody complex. The relatively low antibacterial titers characteristic of pneumococcal antisera can be explained in part by the permeation of the capsule by antiserum, in part by the high combining capacity of pneumococcal carbohydrate for antibody (17).

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EXPLANATION OF PLATES

Pictures Made with the Electron Microscope

PLATE 12

FIG. 1. Capsulated cells of Pneumococcus Type 3. Not exposed to serum. × 14,000.

FIG. 2. Pneumococcus Type 3 after 30 seconds' exposure to 1:10 dilution of heterologous (Type 1) rabbit antiserum. × 11,000.

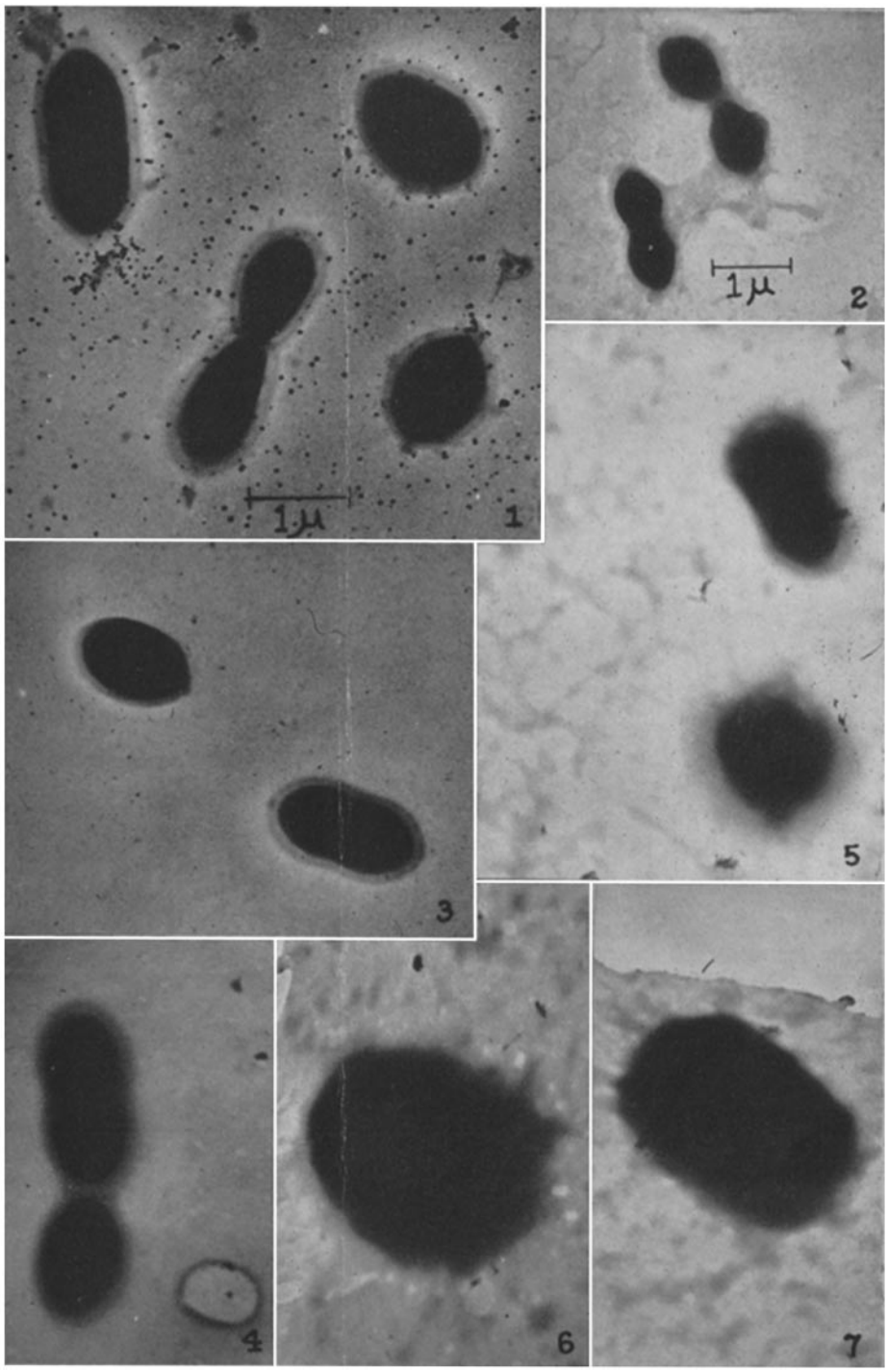
FIG. 3. Pneumococcus Type 3 after 2 minutes' exposure to 1:200 dilution of homologous (Type 3) rabbit antiserum. × 14,000.

FIG. 4. Pneumococcus Type 3 after 2 minutes' exposure to 1:100 Type 3 rabbit antiserum. × 14,000.

FIG. 5. Pneumococcus Type 3 after 2 minutes' exposure to 1:10 Type 3 rabbit antiserum. × 14,000.

FIG. 6. Pneumococcus Type 3 after 2 minutes' exposure to 1:3 Type 3 rabbit antiserum. × 14,000.

FIG. 7. Same as Fig. 6.



(Mudd, Heinmets, and Anderson: Pneumococcal capsular swelling reaction)

PLATE 13

FIG. 8. Pneumococcus Type 1. Not exposed to serum. × 10,500.

FIG. 9. Pneumococcus Type 1 after 2 minutes' exposure to purified rabbit antibody globulin in 0.9 per cent NaCl solution containing 8.6 mg. antibody per 100 ml. × 10,500.

FIG. 10. Pneumococcus Type 1 after 2 minutes' exposure to purified globulin solution containing 21.5 mg. antibody per 100 ml. × 10,500.

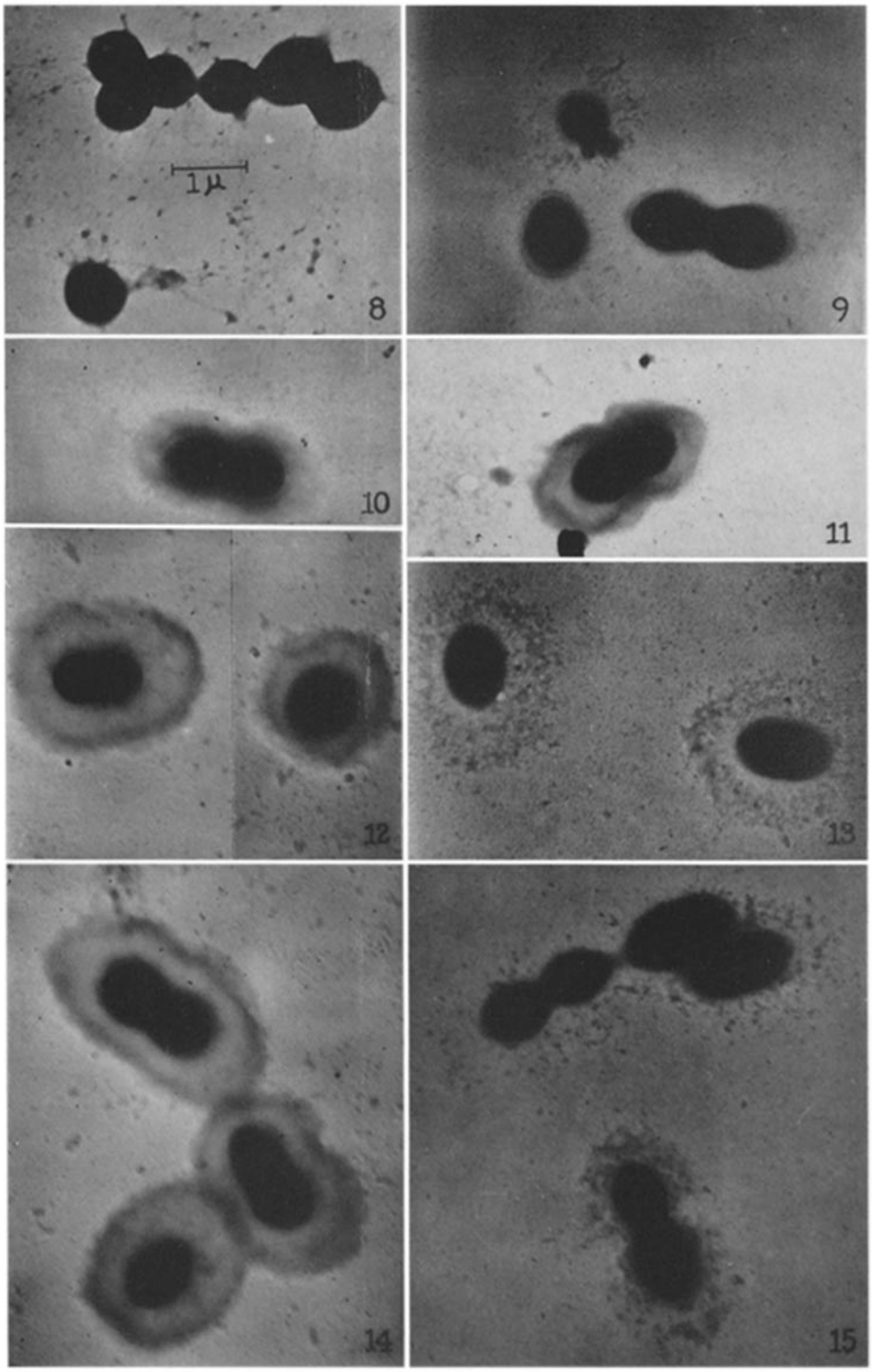
FIG. 11. Pneumococcus Type 1 after 2 minutes' exposure to purified globulin solution containing 43 mg. antibody per 100 ml. × 10,500.

FIG. 12. Pneumococcus Type 1 after 3 minutes' exposure to 0.9 per cent NaCl solution containing 11 mg. purified globulin per 100 ml. and normal rabbit serum in final dilution of 1:4. × 10,500.

FIG. 13. Pneumococcus Type 1 after 3 minutes' exposure to 0.9 per cent NaCl solution containing 11 mg. purified globulin per 100 ml. × 10,500.

FIG. 14. Same as Fig. 12.

FIG. 15. Pneumococcus Type 1 after 2 minutes' exposure to 0.9 per cent NaCl solution containing 11 mg. purified rabbit antibody per 100 ml. solution. × 10,500.



(Mudd, Heinmets, and Anderson: Pneumococcal capsular swelling reaction)

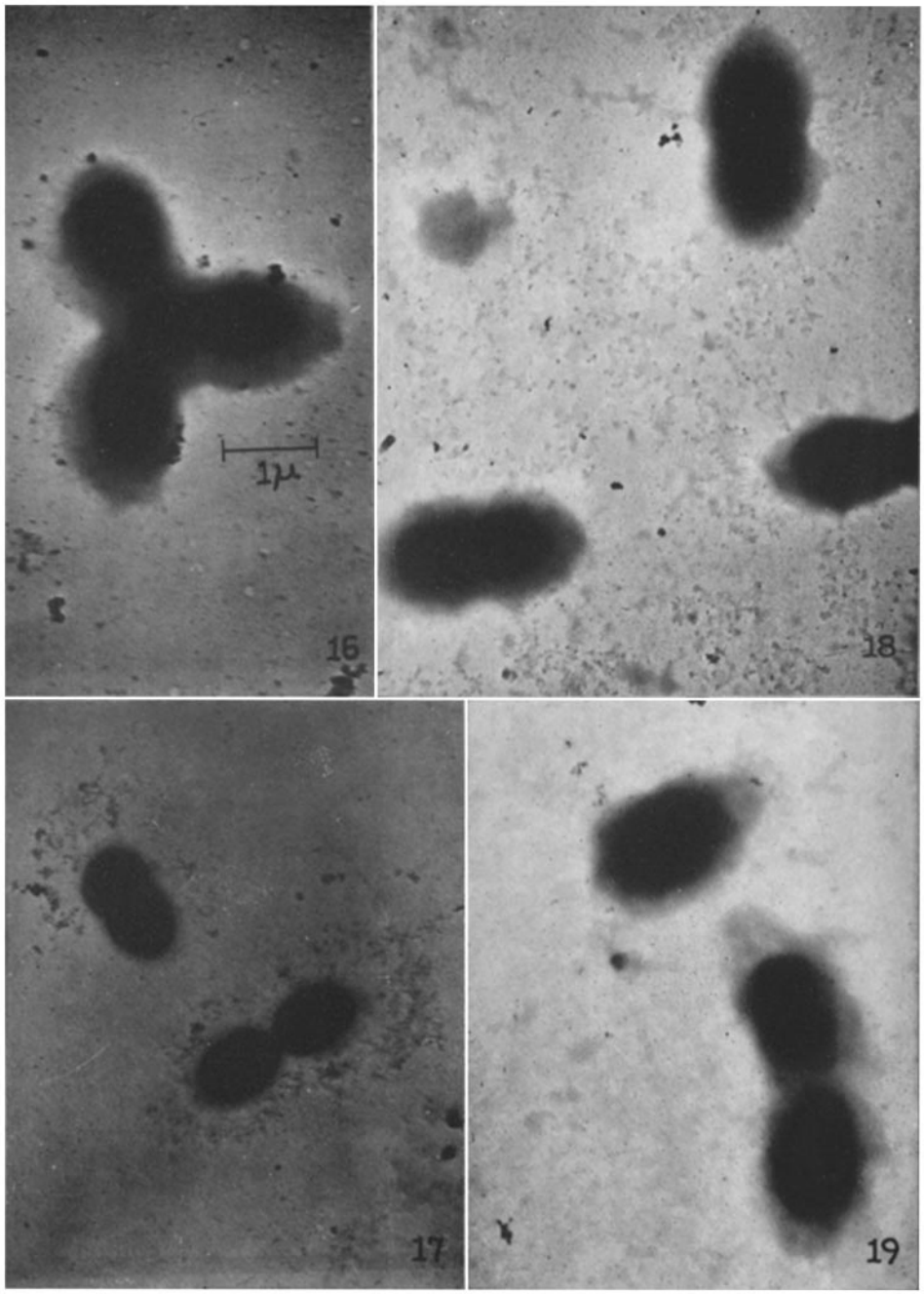
PLATE 14

FIG. 16. Pneumococcus Type 1 after 2 minutes' exposure to 0.9 per cent NaCl solution containing 11 mg. purified antibody per 100 ml. solution and fresh normal rabbit serum in final dilution of 1:4. × 12,800.

FIG. 17. Same as Fig. 15.

FIG. 18. Pneumococcus Type 1 after 2 minutes' exposure to Type 1 antipneumococcal horse serum diluted 1:3. × 12,800.

FIG. 19. Pneumococcus Type 1 after 2 minutes' exposure to Type 1 antipneumococcal horse serum, undiluted. × 12,800.



(Mudd, Heinmets, and Anderson: Pneumococcal capsular swelling reaction)