STREPTOCOCCAL M ANTIGEN LOCATION AND SYNTHESIS, STUDIED BY IMMUNOFLUORESCENCE

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PLATES 70 TO 72

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Streptococcal M antigen is a low molecular weight, alcohol-soluble protein which is responsible for type-specificity of Group A streptococci and, in part, for virulence by virtue of an antiphagocytic action when present on intact cells. Its properties have been recently reviewed by Lancefield (1). Although indirect evidence (2–5) indicates that it is a surface antigen, its location has been visualized only recently (6) by type-specific immunofluorescence. It has been reported to be present in the culture medium of old cells (7) and of protoplasts (5), and in the hyaluronic acid capsule (8). After its ready removal by trypsin from the living streptococcal cell, it has been said to be "resynthesized" by "non-proliferating" cells (9–11).

Many of these observations have been made, however, by indirect methods entailing mass procedures, drastic manipulation of the bacteria and their antigens, and the reading of secondary manifestations of antigen-antibody combination. By using the simple and sensitive technique of specific fluorescent antibody application, we have been able to observe directly, at a cellular level, some properties of M protein related to the above reported findings.

Materials and Methods

Streptococci.—T1/155/4 (Type 1), D58x/11/2 (Type 3), S43/155/2 (Type 6), S23/94/3 (Type 14) were obtained through the courtesy of Dr. Rebecca C. Lancefield of The Rockefeller Institute. Strains 5228 (Type 19) and 5326 (Type 30) were obtained from Dr. Elaine Updyke, Laboratory Branch, Communicable Disease Center, Chamblee, Georgia. Strain NTCC 8301 (Type 23) was originally from Dr. D. G. Fleck, Streptococcal Reference Laboratory, Colindale, London. Strain K56 (Type 12) was obtained from Dr. Ebbe Kjems, Copenhagen, Denmark. Strain 59154 (Type 18) is from the culture collection of the Laboratory of Infectious Diseases, National Institutes of Health, Bethesda. All cultures were stored on rabbit blood agar at 5°C. Single colonies were subcultured to Todd-Hewitt broth as needed.

Production of Antisera.—Single colonies of streptococci were inoculated into 50 cc of Todd-Hewitt broth and incubated for 24 hours at 37°C. The bacteria were washed twice with physi-

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ological saline and heat-killed by incubating for 30 minutes at 56°C. They were washed again and resuspended to 1/5 the original volume of saline and stored in the cold until used. White rabbits (2 kg, NIH strain) were injected intravenously with 0.5 cc of heat-killed vaccines of the appropriate organisms on alternate days during the week. The rabbits were test-bled after 4 weeks of injections and when strong precipitin reactions were noted with homologous acid extracts, 50 to 60 cc of serum were collected from each animal.

Type 1 streptococci autoclaved at 127°C for 120 minutes (12) were washed three times with physiological saline, resuspended in 1/5 the original volume of saline, and used as vaccine for preparation of group-specific antisera. In addition, Type 1 streptococci were digested with 1 per cent trypsin (tryptar®); trypsin crystallized, Armour Pharmaceutical Company, Kankakee, Illinois) for 2 hours at 37°C, and washed and resuspended as above, and also used to produce group antibody. Injection and testing schedules were as described for heat-killed vaccines. Both vaccines for production of group antibody were tested for the presence of M protein by both precipitation of acid extracts of the cells and type-specific immunofluorescence; and were found to be devoid of this antigen by these methods.

Preparation of Fluorescein-Conjugated Antibody Globulins (FAG)—The globulin fractions of rabbit antisera were precipitated with 50 per cent ammonium sulfate (5°C for 2 hours), dissolved in 0.15 M saline and dialyzed free of ammonium sulphate. These fractions were conjugated with fluorescein isothiocyanate according to the method devised by Marshall *et al.* (13). The unbound fluorescein and buffer were removed by passing the solution through a Sephadex G-25 column (1 × 40 cm) equilibrated with 0.15 M saline buffered with 0.05 M phosphate pH 7.5 (14).

Absorption of Conjugated Antibody Globulin.—Groups A, C, and G streptococci grown in 3-liter lots of beef infusion broth, were centrifuged, washed twice with saline, and heat-killed (56°C for 60 minutes). The killed cells were suspended in saline to 1/10 the original volume and stored in the cold until used. 5 to 10 cc of conjugated globulin was added to the sediment from 10 cc of the concentrated bacterial suspension, thoroughly mixed and incubated for 2 hours at 37°C. The cells were removed by centrifugation and the procedure repeated as necessary.

Labeled globulin fractions of sera from rabbits immunized with trypsinized or autoclaved Group A cells were absorbed with cells of Groups C and G. The globulins were regarded as group-specific if they gave brilliant fluorescence with Group A cells and did not react with cells of Groups B, C, D, G, K, and L.

Labeled globulins of sera from rabbits immunized with heat-killed organisms for production of type-specific antibody were absorbed with cells of at least three heterologous types of Group A. A globulin was considered adequately absorbed if it produced cell wall fluorescence of homologous cells and did not produce such fluorescence with streptococci of Groups C and G, and of at least 15 heterologous types of Group A.

Preparation of Bacteria for Fluorescent Microscopy.—Single colonies from rabbit blood agar were incubated 18 hours in Todd-Hewitt broth. 1.0 cc of the broth culture was diluted 1:10 with fresh broth and incubation continued for 2 to 3 hours. The cells were recovered by centrifugation and washed twice with distilled water. The washed bacteria were resuspended to the original volume with water and 1) drop placed on a clean slide and allowed to dry. The slides were heat-fixed and ringed with collodion (the collodion was found to be an effective method for keeping the antisera localized over the bacteria). 1) or 2) drops of conjugated antisera were placed over the fixed bacteria and slides incubated for 30 minutes in a moisture chamber. The slides were rinsed twice with saline and finally with water. A drop of buffered glycerol (9 parts glycerol and 1 part phosphate buffer pH 8.5) was placed over the bacteria and covered with a No. 1 coverslip. The slides were examined under oil immersion with a Leitz Ortholux microscope. The light source was an Osram HB0200 mercury vapor lamp, and darkfield illumination was employed with a Schott exciter filter BG-12 and barrier filter OG-1. Photographs were

made on Kodak panatomic X film, exposed for 2 minutes, and developed 2½ to 3 minutes at 68F in acufine. (Baumann Photo-Chemical Corporation, Chicago)

RESULTS

Production of M Protein by Trypsinized Cells.—Group A, Types 1, 3, and 18, streptococci were incubated 18 hours in Todd-Hewitt broth containing 0.1 per cent trypsin (3 times crystallized trypsin, Sigma Chemical Co., St. Louis). The cells were subcultured into fresh trypsin broth and incubated for another 2 to 3 hours. After centrifugation, they were washed free of broth and suspended in Hanks' solution containing 500 μg/ml of trypsin at pH 7.5 for 1 hour at 37°C. The tubes were then placed in ice water, centrifuged, and washed with cold Hanks' solution three times at 5°C. Iced normal rabbit serum was added and the cells brought into suspension. One sample was taken from the ice bath and immediately placed in a water bath at 56°C for 30 minutes. The remainder of the suspension was placed in the 37°C water bath and samples removed at intervals and immediately placed in the water bath at 56°C for 30 minutes. The heat-killed cells from each sample were washed twice in saline and finally resuspended in water. Slides were prepared from the water suspension and, after drying, stained with fluorescein-tagged group- or type-specific antibody globulins.

The photographs in Figs. 1 *a* to 1 *f* show the sequence of events in washed, trypsin-treated Type 3 cells during subsequent incubation at 37°C in the absence of trypsin. (Identical results were obtained with Types 1 and 18.) The preparation at zero time shows that the trypsin has denuded the cells of reactive M protein. At subsequent intervals of time M protein appears and covers an increasing area of the chains. The photographs taken at 5 and 10 minutes show that M protein first appears at the sites of new cell wall and septum formation and at the sites of completion of the previous division (6). Throughout the period of observation, non-fluorescent gaps are apparent, indicating that trypsin-removed M protein is not resynthesized on old cell wall but is synthesized only at sites of new cell wall growth in the absence of trypsin. The same effects were seen when the trypsinized and washed cells were resuspended in a variety of media instead of in normal rabbit serum. Throughout, cell wall staining with group-specific labeled antibody was always complete without non-fluorescent gaps.

The apparent inability of the streptococci to resynthesize M protein on cell walls previously exposed to trypsin, without cellular growth, differs from the reports of Fox and Krampitz (9) and later Fox (10, 11). Their work suggested that "... apparently non-proliferating cells" can, with proper nutrients, resynthesize M protein. In order to ascertain whether resynthesis of M protein without cellular growth took place in their media, we repeated their experiments and monitored the production of M protein with fluorescent type-specific antibody.

The preparation of trypsinized streptococci, the conditions of incubation, and the media used were identical with those described by Fox and Krampitz (9). Optical density was measured at 660 mμ with a Coleman junior spectrophotometer.

Strains of Types 1, 3, 12, 18, and 23 were tested for their ability to "regenerate" M protein under these conditions. After the 3 hour period of incubation, only the Type 3 strain had formed M protein, as determined by the precipitin reaction of type-specific antiserum with acid extracts of the incubated cells. There was no detectable change in optical density during the incubation of these cells in the media. The photograph in Fig. 2 demonstrates the cellular distribution of M protein found with this strain. This distribution of M protein visualized with fluorescent antibody (identical with, but more limited than, that seen in complete media) suggests to us that M protein production in the system described by Fox and Krampitz (9) represents limited growth of cells in a nutritionally deficient media. The other strains failed to resynthesize M protein as measured by the precipitin reaction but on examination with fluorescent antibody, very faint fluorescence, with a distribution identical with that seen with Type 3 was found.

Effect of Hyaluronic Acid Capsules on the Combination of Cell Wall Antigens and Their Antibodies.—Known mucoid strains (T12, T14, T18, and T23 of Group A streptococci were grown in 10 per cent normal rabbit serum broth overnight. The cultures were diluted 1:10 in fresh serum broth and allowed to incubate 2 to 2½ hours at 37°C. 0.1 cc of the log phase culture and 0.1 cc group of homologous type FAG were mixed in a 20 cc test tube and placed in ice water for 30 minutes. 20 cc of cold saline was added to the tube and the contents gently mixed by inversion of the tube. The cells were recovered by decanting the supernate after centrifugation. India ink preparations were made of the cells and photographed under phase and darkfield microscopy.

The photographs in Figs. 3 *a* and 3 *b* show that large capsules surround the cells. It is evident from Figs. 3 *c* and 3 *d* that fluorescein-labeled type-specific and group-specific antibodies are able to penetrate the capsule and combine with the cell wall antigens. The absence of any noticeable fluorescent staining in the capsular material (Figs. 1 *c* and 1 *d*) indicates that very little, if any, M or C antigen diffuses into the capsule from the cell wall in cultures of this age.

Inhibition of C Anti-C Combination by Prior Combination of M Anti-M.—Slides of Types 1, 3, 6, 12, 18, 19, and 30 were prepared as described (Materials and Methods) and treated in the following manner:

1. Two slides of each type were incubated 10 minutes at room temperature in moisture chambers with 3 drops each of, respectively: (*a*) unlabeled normal rabbit globulin; (*b*) unlabeled homologous type-specific rabbit globulin; (*c*) unlabeled heterologous type-specific rabbit globulin; and (*d*) unlabeled anti-Group A (anti-C, or anticarbohydrate) rabbit globulin.

2. To one slide of each pair was added 2 to 3 drops of homologous type-specific FAG; and to the other, 2 to 3 drops of Group A FAG.

3. After an additional 30 minutes' incubation at room temperature in the moisture chamber, the slides were washed and prepared for darkfield ultraviolet examination as previously described.

As expected, unlabeled normal serum did not inhibit either type-specific or group-specific immunofluorescence. Homologous type-specific globulin, however, inhibited both homologous type-specific and group-specific immunofluorescence, whereas globulin prepared against heterologous types did not inhibit either. Anti-group globulin inhibited only group-specific immunofluorescence. Typical results with cells of one type are shown in Table I: the same results were obtained with each of the 7 Group A serotypes tested. Heterologous type-specific globulin against each of the other 6 serotypes was tested against cells of each serotype with equally negative results, thus demonstrating the type-specificity of the inhibition seen.

TABLE I

Inhibition of Specific Immunofluorescence by Prior Reaction with Non-Fluorescent Rabbit Globulins

Streptococcal cells	Incubated with unlabeled globulin:	Inhibition of fluorescence of:	
		Type-specific FAG (T1)	Group-specific FAG (Group A)
Type 1	Normal	0	0
Type 1	Homologous type-specific (T1)	+	+
Type 1	Heterologous type-specific (T 3, 6, 12, 18, 19, 30)	0	0
Type 1	Group-specific (Group A)	0	+

0 = no inhibition; immunofluorescence of cells with indicated FAG seen.
+ = inhibition: *no* fluorescence of cells seen.

When trypsin or pepsin-treated cells were used, the inhibition of the C anti-C (Group) immunofluorescence by prior incubation with unlabeled homologous type-specific globulin, could not be shown.

DISCUSSION

Our findings establish, by direct observation, a number of facts concerning M protein of Group A streptococci. It is indeed a surface antigen of the coccus, as shown by the concentration of its homologous specific fluorescein-labeled antibody (6; this report) in or on the cell wall (seen microscopically, in fluorescent optical section); and by the failure to obtain such localizing fluorescence after treatment of living cells with trypsin. When such treated cells are washed

free of trypsin and reincubated, M protein reappears in the cell population,—but not at sites on individual cell walls which had been previously exposed to trypsin. Instead, M appears only in areas previously shown (6) to be sites of new cell wall formation. It thus appears that some growth is essential to the reappearance of M, and that its "resynthesis" by "non-proliferating" cells as reported by Fox and Krampitz (9–11) is a misnomer. Their results may be explained by limited growth of, and therefore cell wall formation in, cells of a good M-producing strain. This growth was limited by the partially defined and deficient medium used, and was insufficient to result in a detectable change in optical density of the cell population, although producing enough M per cell to be detectable in the aggregate by gross immunologic methods.

By similar direct observation of specific immunofluorescence, we have been unable to verify the contention of Morris and Seastone (8) that M protein is distributed throughout the cell, including the capsule. These authors demonstrated release of M into the medium after enzymatic decapsulation of log phase (but not older) cultures. Olarte (7) has reported M in supernates of old cultures, which have presumably become spontaneously decapsulated. Our failure to visualize M within the capsule may mean: (*a*) the amount of M present in each capsule is too small; (*b*) M was washed out of the capsules during preparation of the cells for smears; (*c*) M is not in the capsule, but the capsule, while present in young cultures, aids physically in retaining M on the cell surface proper. In this latter condition, any decapsulation, either by added enzyme or spontaneously with ageing, would allow release of M into the medium and would explain the findings of both Morris and Seastone and of Olarte.

Evidence presented by Freimer *et al.* (5) suggests that at least some part of the streptococcal protoplast, presumably membrane, is continuously producing M protein which, in the absence of a cell wall, passes freely into the medium. The question naturally arises as to the site of arrest of M in the wall of normal cocci and the nature of its binding, if any, therein. It is possible that M is always in a state of passage through the cell wall, that passage is physically blocked by the capsule of young cells, and that its appearance in the medium of ageing cultures is the result, as noted above, of the usual decapsulation which occurs in most strains with age. Strains vary in their capacity to produce M, and perhaps also in its release, even though the cells are decapsulated. A further possibility, and a purely speculative one, is that the hyaluronidase preparation used by Morris and Seastone contained additional impurities, enzymatic or other, which accelerated the release of M from the cell surface, apart from the decapsulation effect.

The ability of anti-M (type-specific), but not anti-C (group-specific), antibody to agglutinate whole streptococcal cells (15) has been additional indirect evidence that M is the more superficial of the two antigens. Also, recent studies (16) on the mechanism of the long chain reaction in type-specific antibody are consistent with a superficial location of M. Finally, the blocking by unlabeled

antibodies of type- or group-specific immunofluorescence, as reported in the present study, supplies further evidence. The combination of unlabeled type-specific antibody with M blocked the subsequent combination of labeled anti-group antibody with its polysaccharide antigen, and is most readily explained by a superficial location of M. If the type-specific protein contained combining sites in common with the group polysaccharide, inhibition of group anti-group combination on heterologous as well as on homologous type cells by type-specific antibody would be expected,— a result which did not occur. The lack of inhibition of group-specific fluorescence by type-specific antibody noted when homologous cells were treated with trypsin or pepsin, indicates that the inhibition requires the presence of specific protein accessible and sensitive to these proteolytic enzymes.

The inability of unlabeled group antibody to prevent the subsequent combination of M anti-M suggests that the reverse phenomenon (*e.g.* inhibition of group reaction by type-specific antibody) is not merely a steric one due to spatial proximity of M and C determinant sites in or on the cell wall. If this were true, one might expect "steric hindrance" in either direction, although in the absence of data on the number, size, and distribution of the antigenic determinants, such an expectation cannot be stated with any certainty. The most likely alternative consistent with all observed phenomena is that M determinant sites are superficial to C determinant sites.

SUMMARY

Streptococcal M protein has been studied directly in the intact streptococcal cell by specific immunofluorescence. By this method, it can be seen to be concentrated in or on the cell wall, but cannot be detected in the capsule. The lack of type-specific (but not group-specific) immunofluorescence after trypsinization; and the inhibition of group-specific immunofluorescence by unlabeled type-specific antibody, are observations most compatible with a location of the M antigen determinants on the cell surface superficial to the group antigen.

M antigen is not "resynthesized" after trypsinization of living cells, but appears anew only at sites of new cell wall growth. A limited amount of such growth, leading sometimes to detectable amounts of M in the gross, can take place in deficient media without detectable increases in optical density of the cell population.

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EXPLANATION OF PLATES

PLATE 70

FIGS. 1 *a* to 1 *f*. Trypsinized cells (59165 Type 3) were washed three times with cold Hanks' solution and resuspended in cold normal rabbit serum. One sample was taken from the iced serum and immediately placed in a water bath at 60°C for 60 minutes. The remainder of the suspension was placed in the 37°C water bath and samples removed at intervals, heat-killed, and stained with fluorescein-labeled type-specific antiserum. Approximately × 4000.

Fig. 1 *a*. Trypsinized cells, control.

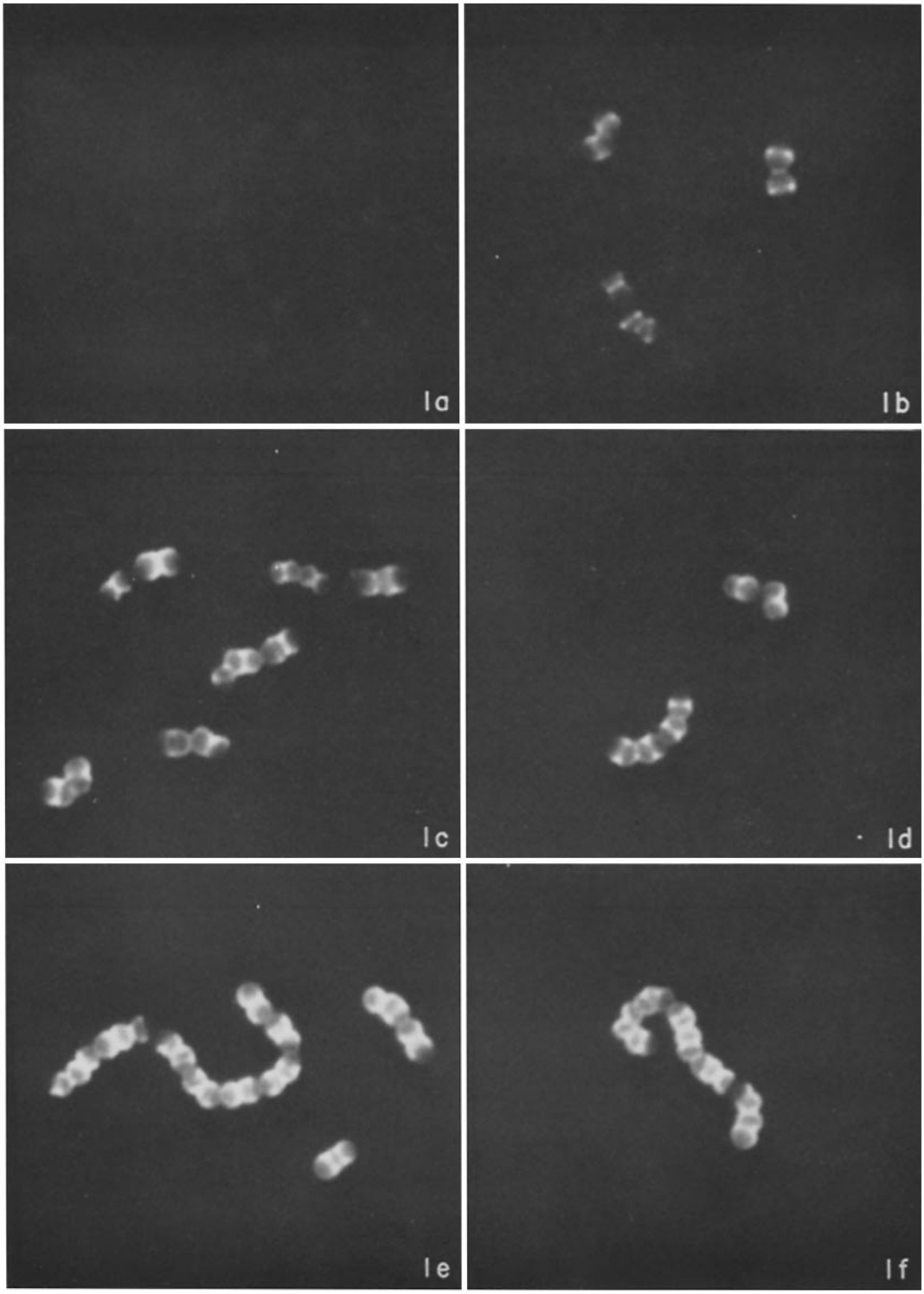
Fig. 1 *b*. Trypsinized cells, 5 minutes' incubation at 37°C.

Fig. 1 *c*. Trypsinized cells, 10 minutes' incubation at 37°C.

Fig. 1 *d*. Trypsinized cells, 15 minutes' incubation at 37°C.

Fig. 1 *e*. Trypsinized cells, 20 minutes' incubation at 37°C.

Fig. 1 *f*. Trypsinized cells, 30 minutes' incubation at 37°C.



(Hahn and Cole: Streptococcal M antigen location and synthesis)

PLATE 71

FIG 2. Strain 59165, Type 3, trypsinized and incubated for 3 hours at 37°C in media described by Fox and Krampitz. Preparation stained with fluorescein-labeled type-specific antiserum. Approximately × 2000.

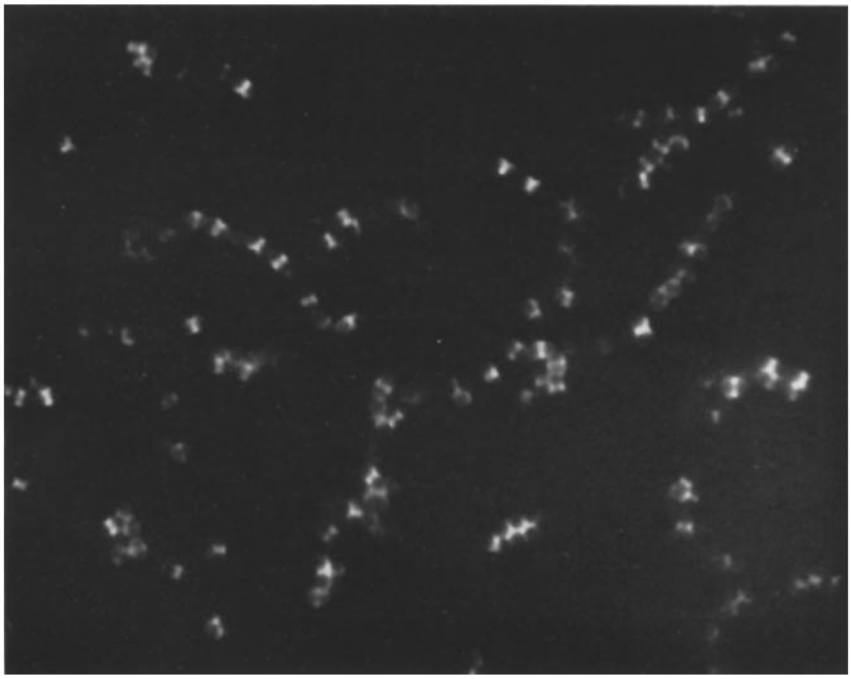


FIG. 2

(Hahn and Cole: Streptococcal M antigen location and synthesis)

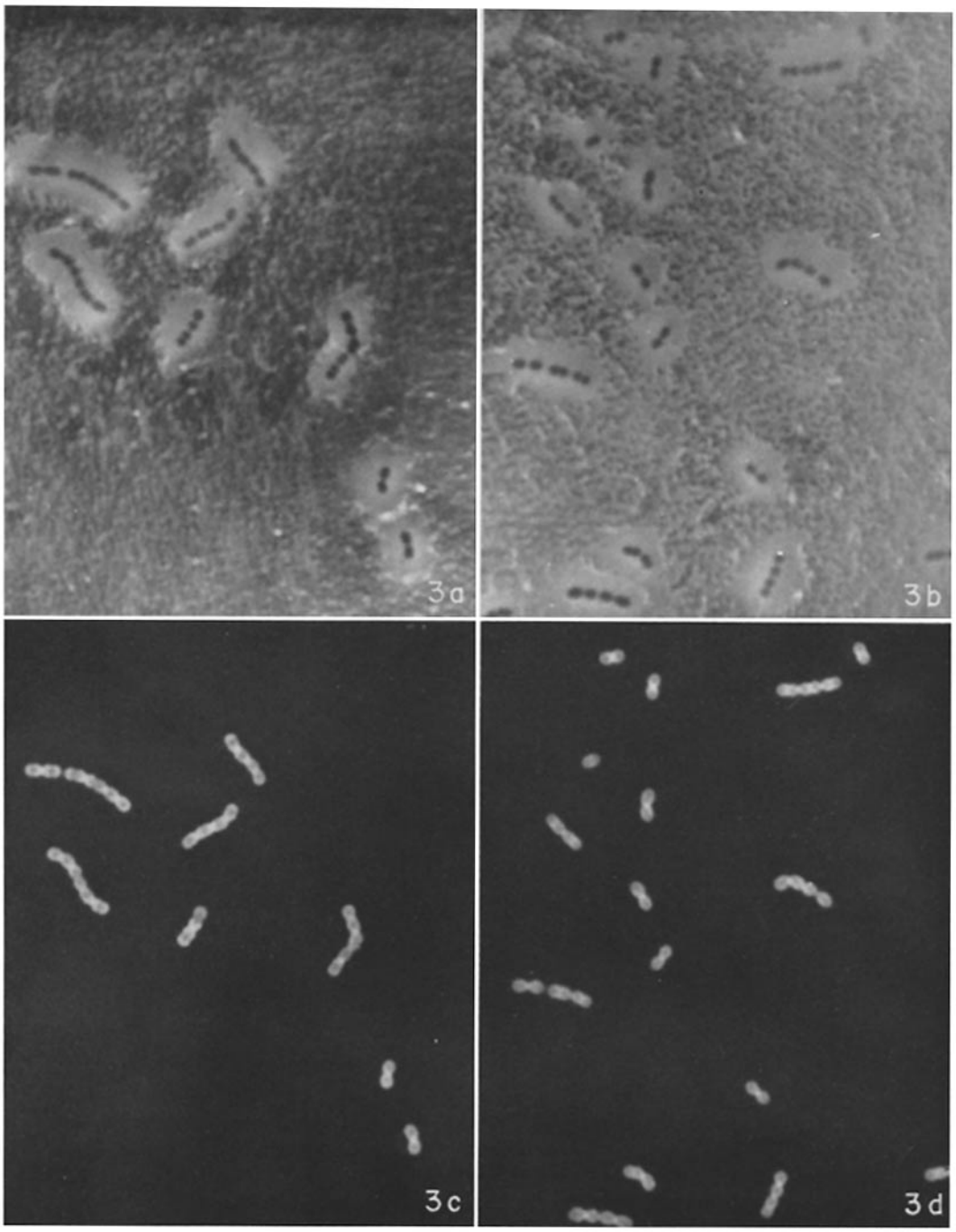
PLATE 72

FIG. 3 *a*. India ink preparation of strain 59154 Type 18 stained with fluorescein-labeled type-specific antiserum. Phase contrast microscopy, white light illumination. Approximately × 2000.

FIG. 3 *b*. India ink preparation of strain 59154 Type 18 stained with fluorescein-labeled group-specific antiserum. Phase contrast microscopy, white light illumination. Approximately × 2000.

FIG. 3 *c*. Same field as in Fig. 3 *a*, photographed under darkfield ultraviolet light illumination. Approximately × 2000.

FIG. 3 *d*. Same field as in Fig. 3 *b*, photographed under darkfield ultraviolet light illumination. Approximately × 2000.



(Hahn and Cole: Streptococcal M antigen location and synthesis)