Structure and Composition of the *Bacillus anthracis* Capsule

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ABSTRACT

AVAKYAN, A. A. (Academy of Medical Sciences, Moscow, USSR), L. N. KATZ, K. N. LEVINA, AND I. B. PAVLOVA. Structure and composition of the *Bacillus anthracis* capsule. *J. Bacteriol.* **90**:1082-1095, 1965.—Observations by various methods of light microscopy (phase contrast, dark-field, and fluorescence) revealed the complex structure of the *Bacillus anthracis* capsule, which changes regularly during the growth cycle of the culture. Special cytological methods of staining the capsule made it possible to study its fine structure, which is not revealed by negative staining with India ink. For example, the capsule shows a membranelike outline, fine transverse lines, and interruptions and transverse septa traversing the entire capsule. By using cytochemical methods, it was found that the capsule has a stratified structure and that the various layers of the capsule differ as to the value of the isoelectric point, metachromatic ability, sensitivity to various enzymes, and, consequently, chemical composition. It was thus shown that the membranelike outline of the capsule consists of peptides and neutral mucopolysaccharides. The middle part of the capsule consists of a complex of substances of both polysaccharide and protein nature, and the inner part consists of acid mucopolysaccharides. Observation of the capsular forms of *B. anthracis* by means of an electron microscope revealed differences in the osmiophilia and submicroscopic structure of the membranelike outline and the middle and inner parts of the capsule. Immunochemical studies conducted by the fluorescent-antibody method revealed localization of antigens in different parts of the capsule, and made it possible to differentiate the capsular antigens according to their serum-staining ability and according to their relations to enzymes, i.e., their chemical composition. This paper concerns the possibility of studying the fine structure of bacterial capsules in fixed preparations, and the differences and similarities of the antigens of the capsule and cell wall of *B. anthracis* and of the related species, *B. megaterium*.

It is well known that *Bacillus anthracis* forms a capsule in special nutrient media and in infected animals. Many investigators associate the virulence of the microorganism with the capsule and its ability to produce the anthrax toxin (Thorne, 1956; Keppie and Smith, 1963).

However, the literature contains limited information on the structure and composition of the *B. anthracis* capsule. In cytological studies of the capsules, most investigators have resorted to negative staining with India ink without preliminary fixation (e.g., Duguid, 1951). This method does not permit observation of structural details in the capsule, because it reveals the capsule negatively, as a white trace against a dark background. Klieneberger-Nobel (1948) suggested cytological observations of the capsules in preparations fixed by Buin's solution and then stained with Giemsa solution or with

crystal violet after treatment with tannin. But the treatment with tannin resulted in coagulation of the proteins and therefore made it impossible to observe the fine structure of the capsule. Observations of the capsular forms of *B. anthracis* in ultrathin sections also failed to contribute anything new to the studies of the capsular structure, because the main part of the capsule was destroyed while the preparations were being made (Roth, Levis, and Williams, 1960; Roth and Williams, 1964).

Information on the composition of capsules was obtained mainly by chemical and immunochemical methods. Tomcsik (1954), Nordberg and Torsell (1955), and others have shown that the *B. anthracis* capsule consists of glutamyl polypeptide and that it possibly also contains lysozyme-sensitive polysaccharides.

The present investigation was aimed at ob-

serving the *B. anthracis* capsule by light and electron microscopy, as well as cytochemical and immunochemical methods. An attempt was made to compare the data on the fine structure of the capsule with its chemical and antigenic composition.

MATERIALS AND METHODS

To produce capsular forms, the 71/12 vaccinal strain of *B. anthracis*, obtained from the second Tsenkovsky vaccine, was grown in Hanks medium with 40% ox serum (Arkhipova, 1962). To produce noncapsular forms, the culture was inoculated on beef extract-agar.

The cytological study was conducted on unfixed slide preparations with the aid of a phase-contrast microscope or dark-field illumination. In some cases, negative staining with India ink was resorted to. Staining with crystal violet after treatment with tannin (Gutstein, 1924) or with Löffler's alkaline methylene blue, after fixation with ethyl alcohol, methanol, or formalin, was also utilized.

The cytochemical study made use of the isoelectric point (IEP) method (Pischinger, 1926). To determine the relative IEP value of the capsule, the capsular cells were stained with diluted solutions of Toluidine Blue and Acid Fuchsin at pH 10.0 to 1.4 at 0.2 intervals. To reveal the polysaccharides, the periodic acid-Schiff technique of Hotchkiss was used (Pearse, 1960); to reveal the metachromatic substances (mainly polysaccharides), Schmorl's thionine method was employed (Pearse, 1960). The Alcian Blue method (after Steedman) and metachromatic staining with thionine and Toluidine Blue at pH 4.0 and 2.0 were used (Pearse, 1960) to reveal acid mucopolysaccharides; Fast Green at pH 2.2 after Alfert and Geschwind (1953) or bromophenol blue at pH 2.3 (Bloch and Hew, 1960) was used to reveal proteins; the propylene glycol-Sudan Black method of Chiffelle and Putt was used to reveal lipids (Pearse, 1960).

For immunochemical studies, the fluorescent-antibody method (Coons and Kaplan, 1950; Riggs et al., 1955) was used. The smears were fixed on glass slides with ethyl alcohol, and were treated with fluorescent sera labeled with fluorescein-isothiocyanate. The sera were obtained by immunizing rabbits with the capsular cells, and were prepared from commercial precipitable sera (Levina and Arkhipova, 1964).

The cytological and serological studies were conducted on preparations treated with enzymes, as well as on untreated preparations. The following enzymes were used: pepsin (E. Gurr), trypsin (Merck), chymotrypsin (Chempl), lysozyme (Lawson), and hyaluronidase (Light).

For electron microscopy, whole unsectioned capsular cells were fixed in formalin vapor, and were later overfixed in osmium tetroxide vapor or were negatively stained with 2% phosphotungstic acid (pH 11.5) without preliminary fixation. The

preparations were examined with a JEM-6C electron microscope, at 80 kv and $\times 10,000$ magnification.

The light-microscope studies were carried out with a MBI-6 microscope; the preparations were photographed with an FMH-3 microcamera (oil immersion, $10\times$ ocular, $100\times$ objective). A usual aplanatic condenser, an OI-13 dark-field condenser, and a KF-4 phase-contrast device were used for photography. An ML-2 fluorescence microscope was used, and the photographs were made on PF-3 fluorographic film.

RESULTS

Cytological study of capsules by light microscopy. Within 2 to 3 hr after inoculation of the nutrient medium with *B. anthracis* spores, the formation of capsules begins. Young capsules are easily seen with phase contrast. At first they form in various areas near the transverse septa, and have an interrupted structure. Within 12 to 18 hr, the capsules completely surround the streptobacilli, and have no interruptions. Often, streptobacilli form pairs or chains inside a capsule. The lysis of the capsules begins within 24 to 30 hr. The capsules lyse first in areas corresponding to the dead cells. The capsules of old cultures, therefore, also have an interrupted structure, like the newly forming capsules of young cultures.

An 18-hr *B. anthracis* culture with well-formed capsules was used for the cytological study. The living cells, observed with a phase-contrast microscope, clearly show capsules in the form of a white halo about the streptobacilli. They are bounded on the outside by a barely observed membranelike outline (Fig. 1). In preparations negatively stained with India ink, the capsule is of the same size as in unstained preparations. Preliminary treatment of the capsular cells with ox serum leads to a noticeable nonspecific capsular swelling (Fig. 2). This phenomenon was described by Tomcsik and Guex-Holzer (1953, 1954a) for various capsular bacteria as a nonspecific swelling reaction, and was made a special basis for revealing capsules by treating them with sera containing proteins. All of the above-described methods of studying capsules in living cells have failed to show the internal organization of the capsules.

In observations of living organisms by dark-field illumination, capsular cells show white punctate structures in the membranelike outline (Fig. 3) and in the inner part of the capsule (Fig. 4). In noncapsular forms of *B. anthracis*, only the cell wall is clearly seen in dark-field illumination (Fig. 5).

Fixed capsular cells were also studied in a fluorescence microscope. Careful fixation of non-

FIG. 1. *Bacillus anthracis* capsular cells from an 18-hr culture. Membranelike outline of capsule indicated by arrows. Phase contrast. $\times 2,000$.

FIG. 2. Capsular cells from a culture of the same age after treatment with ox serum. Negative staining with India ink. Note swelling of capsule. Light microscope. $\times 1,200$.

FIG. 3 and 4. Capsular cells in dark-field. Granules showing in the cytoplasm, membranelike outline, and middle part of the capsules. $\times 1,500$.

FIG. 5. Noncapsular cells in dark-field. The cell wall is easily seen. $\times 1,500$.

FIG. 6. Capsular cells after treatment with capsular serum labeled with fluorescein isothiocyanate. Punctate fluorescence of the capsule can be seen. Fluorescence microscope. $\times 2,000$.

FIG. 7. Capsular cells after treatment of the cell-wall serum labeled with fluorescein isothiocyanate. Fluorescence of transverse septa in the capsule and of punctate structures in the membranelike outline of the capsules is noticeable. Fluorescence microscope. $\times 2,000$.

desiccated preparations in formalin vapors with methanol or ethyl alcohol revealed a slight shrinkage of the capsule, compared with living cells. For study under the fluorescence microscope, the preparations were stained with anthrax sera labeled with fluorescein-isothiocyanate. After this treatment, the whole capsule was brilliantly fluorescent (Fig. 6), or a weak punctate fluorescence of the membranelike outline, the transverse septa in the capsule, and the cell wall was observed (Fig. 7).

Various staining techniques were used in studying fixed capsular cells under the light microscope. On staining with Löffler's blue, the middle part of the capsule stained metachromatically a light purple, the membranelike outline of the capsule stained dark blue, and the inner part stained violet (Fig. 8). The membranelike outline of the capsule (Fig. 9, 10, and 29), which sometimes has filaments, is seen when stained with Toluidine Blue or crystal violet after treatment with tannin.

Thus, the use of various methods of light microscopy and specific staining has made it possible to observe the fine structure of the *B. anthracis* capsule, which is not revealed when negatively stained with India ink.

Cytochemical study of the capsule. The use of cytochemical methods has made it possible to determine the chemical composition of the *B. anthracis* capsule, and to establish its stratified structure.

Figure 35 diagrammatically shows the stratified structure of the *B. anthracis* capsule, and Table 1 summarizes the results of the cytochemical studies of the various layers of the capsule, obtained by IEP determination and by cytochemical staining methods specific for polysaccharides, acid mucopolysaccharides, lipids, and proteins, as well as by treatment of fixed capsular cells with enzymes.

Staining with Toluidine Blue at pH 7.0 to 8.0 often reveals a delicate outer fringe which bounds the capsule on the outside and which is easily destroyed when the preparations are being made. This fringe is not metachromatic when stained by Toluidine Blue and thionine (which is characteristic of polysaccharides), does not react with Schiff's reagent (test for polysaccharides), has an IEP at pH above 3.8, does not stain with Alcian Blue (test for acid mucopolysaccharides), gives positive reactions for proteins and lipids, and is destroyed by pepsin (Table 1). All of the foregoing suggests that this part of the capsule is formed by peptides, possibly along with lipoproteins.

Directly under the fringe is a thin dense membrane which is not metachromatic, weakly reacts with Schiff's reagent, has an IEP at pH

below 2.3, does not stain with Alcian Blue, gives a positive reaction for proteins, and gives a negative reaction for lipids. This suggests that the membrane contains polysaccharide and protein components which are bound to each other, apparently after the fashion of neutral mucopolysaccharides or mucopeptides. The mucopolysaccharides of this membrane resemble the mucopeptides of the cell wall, but differ from them by their susceptibility to destruction by pepsin and insusceptibility to lysozyme. The outer fringe and the aforesaid membrane together form the membranelike outline.

The second layer forming the main, middle part of the capsule is located under the membranelike outline. This part of the capsule is γ -metachromatic when stained with thionine (Fig. 12) and β -metachromatic when stained with Toluidine Blue; it does not react with Schiff's reagent, has an IEP in the very acid region (below pH 2.0), stains with Alcian Blue (Fig. 13), does not give positive reactions for proteins or lipids, and is destroyed by lysozyme. It is well known that, as a rule, acid mucopolysaccharides do not stain with Schiff's reagent but are γ -metachromatic at pH below 4.0 to 3.0 (Pearse, 1960). It may therefore be quite logically assumed that this layer of the capsule is formed by acid mucopolysaccharides.

Lastly, the fourth and innermost layer of the capsule, directly adjoining the cell wall, is clearly γ -metachromatic in the acid and neutral regions, has an IEP at pH below 2.3, does not react to Schiff's reagent, weakly stains with Alcian Blue, and is destroyed by lysozyme and hyaluronidase. This capsular layer is thus also formed by acid mucopolysaccharides, which differ, however, from the acid mucopolysaccharides of the preceding capsular layer by their sensitivity to hyaluronidase and the position of the IEP in a somewhat more alkaline region. By its position in the cell, this innermost layer of the capsule resembles the cell wall, but we ascribe it to the capsule because it is absent in noncapsular forms and stains with crystal violet without preliminary treatment with tannin. (The bacterial cell wall stains, as is well known, only after treatment with tannin.)

In addition to the aforementioned layers of the *B. anthracis* capsule, we observed delicate transverse lines which are γ -metachromatic at pH 2.3 to 6.0 and are apparently also of a polysaccharide nature (Fig. 11). Similar lines in the capsules were observed by Ivanovics and Horvath (1953a) in *B. megaterium*, and by Tomcsik and Guex-Holzer (1952) in *Bacillus M* (an organism allied to *B. anthracis*) treated with serum.

Thus, the cytochemical study made it possible

FIG. 8. Capsular cells from an 18-hr culture. Stained with Löffler's blue. Membranelike outline (first layer), middle part (second layer), and innermost (fourth layer) part of capsules are seen. Light microscope. $\times 1,300$.

FIG. 9. Capsular cells from a 30-hr culture, stained by Gutstein's method. Several cells with lysed capsule and cell wall in the middle part of the streptobacillus. Light microscope. $\times 1,300$.

FIG. 10. Capsular cells from an 18-hr culture. Stained with Toluidine Blue at pH 6.0. Membranelike outline and innermost (fourth) layer of capsule are seen. Light microscope. $\times 2,000$.

FIG. 11. Capsular cells from an 18-hr culture. Stained with Toluidine Blue at pH 2.3. Transverse lines can be seen in capsule. $\times 1,700$.

FIG. 12. Capsular cells stained by Schmorl's thionine method. Mainly the inner part (third layer) of the capsule is seen. $\times 1,500$.

FIG. 13. Capsular cells stained with Alcian Blue, also showing the third layer of the capsule. $\times 1,300$.

TABLE 1. *Cytochemical reactions of components of the Bacillus anthracis capsule*

Bacterial cell element	Metachromasia*	IEP	Reaction with Schiff reagent	Alcian Blue (pH 2.0)	Reaction for proteins	Sudan Black	Pepsin	Trypsin	Chymo-trypsin	Lyso-zyme	Hya-luro-nidase	Composition
First capsular layer (membrane-like out-line) outer fringe.....	-	Above pH 3.8	-	-	+	±	+	-	-	-	±	Peptides
Dense membrane.....	±	Below pH 2.3	±	-	+	-	+	±	±	-	±	Neutral mucopoly-saccharides or mucopeptides?
Second capsular layer (middle part of cap-sule).....	+	Below pH 3.8	-	-	-	-	†	+	+	+	+	Polysaccharide and protein complexes
Third capsular layer....	+	Below pH 2.0	-	+	-	-	-	-	-	+	-	Acid mucopoly-saccharides
Fourth, innermost cap-sular layer.....	+	Below pH 2.3	-	±	-	-	-	+	-	+	+	Acid mucopoly-saccharides
Cell wall	±		+	±	+	±	-	+	-	+	-	Mucopeptides

* First column, with thionine; second column, with Toluidine Blue.

† Slightly digested.

to reveal the stratified structure of the *B. anthracis* capsule, and showed the localization of various substances in the capsule.

Investigation of the capsule by means of electron microscopy. Investigation of capsular cells, fixed by formalin and overfixed by osmium, with the aid of an electron microscope has shown the capsule to consist of a membranelike outline, a middle part, and an inner part. The membranelike outline of the capsule is observed as a thin osmiophilic layer, but is not seen all along the cell (Fig. 14). Sometimes we observed filaments coming out from the membranelike outline of the capsule. These filaments consist of osmiophilic substance, and show an osmiophobic spiral structure (Fig. 14, 15). The main middle part of the capsule is more electron-dense than are the membranelike outline and inner part of the capsule; it often shows inclusions of less-dense material in the form of small round or angular bodies (Fig. 14), or sometimes in the form of fine transverse septa (Fig. 15).

Negative staining of capsular cells with phosphotungstic acid reveals the structure of the inner capsular layer. It consists of material with low electron density, but contains inclusions of small electron-dense particles (Fig. 16). Very thin filaments extend from this layer into the middle part of the capsule; these filaments apparently create the impression of the transverse lines seen in the capsule stained with Toluidine Blue (Fig. 11).

Thus, the studies with the electron microscope have shown that the different layers of the capsule differ in electron density, osmiophilia, and submicroscopic structure.

Immunochemical studies of the capsule. The luminescent sera obtained by immunizing rabbits with whole capsular cells labeled the capsule but not the cell wall or the protoplasm. This enabled us to give the luminescent serum the code name of capsular luminescent serum (CLS). The luminescent sera prepared from commercial precipitable sera selectively labeled the cell wall of noncapsular cells, and on this basis were given

the code name of cell-wall luminescent sera (CWLS).

Studies of CLS-treated capsular cells with the fluorescence microscope have shown the outer fringe and the middle part of the capsule (the first and second capsular layers revealed by cytological methods) to be brilliantly fluorescent. The inner part of the capsule adjacent to the cell wall (the third and fourth capsular layers) was not fluorescent (Fig. 19). CLS-treated noncapsular cells were practically nonfluorescent. The cell wall of CWLS-treated noncapsular cells also showed a green fluorescence. The cell wall of capsular cells, the thin fringe on the periphery of the capsule, and the transverse lines traversing the body of the capsule along the transverse septa of streptobacilli or between the septa fluoresced less brightly (Fig. 7, 20). As already mentioned, we observed similar transverse septa in the capsule with an electron microscope (Fig. 15).

In CLS-treated capsular cells examined electron microscopically, we observed the appearance of an osmiophilic fringe around the capsule. The middle part of the capsule acquired a delicate radial stratified structure (Fig. 17). We did not observe this picture in capsular cells treated with normal horse serum (Fig. 18).

The immunochemical studies have thus shown heterogeneity of the capsular antigens and have enabled us to localize them in the capsule. The capsule contains antigen complexes consisting of antigens revealed by CLS and homogeneously distributed in the outer and middle parts of the capsule, and antigens revealed by CWLS and localized in the membranelike outline and septa of the capsule and in separate punctate structures in the middle part of the capsule.

Action of enzymes on capsular cells. To study the action of enzymes on capsular cells of *B. anthracis*, cytological observations were made with a light microscope, and antigenic composition was studied with a fluorescence microscope. In the former, the preparations, both treated and untreated with enzymes, were stained with

FIG. 14 and 15. Capsular cells from an 18-hr culture. Formalin fixation with osmium overfixation. Membranelike outline (osmiophobic), as well as middle and inner (also osmiophobic) parts of capsules are seen. Note filaments with spiral structure extending from the membranelike outline of capsules. Electron microscope. $\times 5,500$.

FIG. 16. Capsular cells negatively stained with phosphotungstic acid. The structure of the inner layer of the capsules and thin filaments extending into the middle part of capsules is noticeable. Electron microscope. $\times 15,000$.

FIG. 17. Capsular cells after treatment with capsular serum. The middle part of the capsules has a delicate, radial, stratified structure. The membranelike outline of the capsules is somewhat swollen. Electron microscope. $\times 3,000$.

FIG. 18. Capsular cells after treatment with normal horse serum. Electron microscope. $\times 5,500$.

FIGS. 14-18

crystal violet after treatment with tannin (this stained mainly the membranelike outline and the cell wall) and with Toluidine Blue (this stained all layers of the capsule). In the latter case, the control preparations and the enzyme-treated preparations were stained with CLS (mainly the middle part and outer fringe of the capsule were labeled) and with CWLS (the cell wall, the punctate structures in the membranelike outline, and the middle part of the capsule, as well as the transverse septa of the capsule were labeled).

The action of pepsin makes the membranelike outline and, partly, the middle part of the capsule disappear, the inner part of the capsule and the cell wall remaining intact (Fig. 29, 30, Table 1). In capsular cells treated with CLS after pepsin, the capsule is unlabeled; the inner layer of the capsule is intact and appears as a dark halo (Fig. 19, 21). The cell wall of capsular cells treated with CWLS after pepsin shows the same clear fluorescence (Fig. 22) as do preparations untreated with the enzyme (Fig. 20). The fluorescent points in the membranelike outline and the transverse septa of the capsule are absent.

The action of trypsin destroys primarily the middle and inner parts of the capsule and the cell wall, especially on the side of the longitudinal septa; the membranelike outline remains intact (compare Fig. 31 with Fig. 29). In CLS-treated cells, we observed a slight fluorescence of the main part of the capsule; the fluorescence disappeared under prolonged action of trypsin (Fig. 23). In CWLS-stained preparations, it is possible to observe an intensely fluorescent cell wall, fluorescent punctate structures on the periphery and in the middle part of the capsule, and transverse lines in the capsule (Fig. 24). It is undoubtedly of some interest that, despite the visible

destruction of the cell wall under the action of trypsin, its fluorescence after treatment with CWLS does not decrease.

The action of hyaluronidase is observed to destroy both the main middle part of the capsule and its inner part. The membranelike outline of the capsule usually remains intact. The cell wall is not affected, although the streptobacilli often break up into separate cells owing to destruction of the substance which joins the cells of the streptobacilli (Fig. 32). In CLS-stained preparations, the intact part of the capsule appears as a brilliantly fluorescent rim, whereas the main part of the capsule does not fluoresce (Fig. 25). In CWLS-treated preparations, the cell wall is brilliantly fluorescent, and transverse lines and fluorescent points are seen in the middle part of the capsule (Fig. 26).

The action of lysozyme is observed to loosen the entire capsule, although, as a rule, no complete lysis of the capsule occurs because the membranelike outline of the capsule remains intact. The cell wall is also affected (Fig. 33, 34). It is interesting that lysozyme only slightly affects the cell wall of noncapsular cells. In CLS-treated preparations, the capsule is fluorescent, but is deformed and is sometimes separated from the cell body (Fig. 27). In CWLS-treated preparations, a dim punctate fluorescence of the cell wall is observed (Fig. 28). The periphery of the capsule sometimes shows a brilliantly fluorescent border which corresponds to the membranelike outline of the capsule. This border is not so clearly observed in control preparations. The transverse septa and separate fluorescent points in the middle part of the capsule are absent. Other workers also make mention of loosening of the cell wall and of lysis of the capsule under the

FIG. 19. Capsular cells from an 18-hr culture, stained with capsular serum labeled with fluorescein isothiocyanate. Punctate fluorescence of outer and middle parts of capsules is seen. Fluorescence microscope. $\times 2,000$.

FIG. 20. Capsular cells from an 18-hr culture, stained with cell-wall serum. Note fluorescence of punctate structures in membranelike outline, middle part of capsules, and in the transverse septa. Fluorescence microscope. $\times 2,000$.

FIG. 21. Capsular cells stained with CLS after treatment with pepsin. Outer and inner parts of capsules are nonfluorescent. $\times 2,000$.

FIG. 22. Capsular cells stained with CWLS after treatment with pepsin. Cell wall is fluorescent; punctate structures and septa in the capsules are nonfluorescent. $\times 2,000$.

FIG. 23. Capsular cells stained with CLS after trypsin. Middle part of capsules is dimly fluorescent. $\times 2,000$.

FIG. 24. Capsular cells stained with CWLS after trypsin. $\times 2,000$.

FIG. 25. Capsular cells stained with CLS after hyaluronidase. Only outer fringe of capsules is fluorescent. $\times 2,000$.

FIG. 26. Capsular cells stained with CWLS after hyaluronidase. $\times 2,000$.

FIG. 27. Capsular cells stained with CLS after lysozyme. $\times 2,000$.

FIG. 28. Capsular cells stained with CWLS after lysozyme. Note dim punctate fluorescence of the cell walls; transverse septa in capsules and the punctate structures in them are nonfluorescent. $\times 2,000$.

FIGS. 19-28

FIG. 29. Capsular cells from an 18-hr culture stained by Gutstein's method. Cell walls and membranelike outline of capsules are stained. Light microscope.

FIG. 30. Capsular cells stained by Gutstein's method after pepsin. Cell walls intact, outer part of capsules destroyed. $\times 1,300$.

FIG. 31. Capsular cells stained by Gutstein's method after trypsin. Destruction of inner and middle parts of capsules and of cell walls in evidence. $\times 1,300$.

FIG. 32. Capsular cells stained by Gutstein's method after hyaluronidase. Destruction of capsules and substance joining the cells into streptobacilli are seen. $\times 1,300$.

FIG. 33 and 34. Capsular cells stained by Gutstein's method after lysozyme. Capsules and cell walls destroyed. $\times 1,300$.

FIG. 35. Diagrammatic representation of the *Bacillus anthracis* capsule.

action of lysozyme and trypsin in *B. anthracis* and allied spore-bearing aerobic microorganisms (Tomcsik and Guex-Holzer, 1952; Nordberg and Thorsell, 1955).

Thus, our cytological and immunochemical studies of the action of enzymes on capsular cells have enabled us to ascertain the chemical composition of the different layers of the capsule, and to differentiate the capsular antigens according to their relations to enzymes. We have shown, for example, that the capsular antigens may be divided into at least two groups: the superficial antigens of the capsule, revealed by CLS and localized in the outer fringe of the capsule, possess selective sensitivity to pepsin; the antigens of the capsule proper, revealed by the action of CLS and CWLS and concentrated in the middle part of the capsule, are destroyed by hyaluronidase, lysozyme, and proteolytic enzymes.

DISCUSSION

The present study is one of the few attempts to investigate the structure and composition of bacterial capsules by cytological, cytochemical, and immunochemical methods. It is no mere accident that the capsule of *B. anthracis* was chosen as the model for this study. It is well known that the capsule of this microorganism has a complex composition, which includes polysaccharide as well as peptide components (Tomcsik, 1954; Nordberg and Thorsell, 1955), and that the antigens of the capsule in large measure determine the antigenic properties of the causative agent of anthrax (Tomcsik, 1956).

To investigate the capsular structure of this microorganism, we used both light and electron microscopes. Our investigations have shown the *B. anthracis* capsule to have a complex structure with a well-defined membranelike outline, which is observed by phase contrast in living cells and with electron and light microscopes in fixed preparations. This is at variance with Tomcsik's (1956) statement to the effect that the existence of the outer membranelike

outline is doubtful. We cannot fully agree with Tomcsik's (1956) and Duguid's (1951) opinions about the inferior character of the cytological studies conducted with fixed capsular bacteria. As our investigations of the capsular forms of *B. anthracis* and the investigations of the capsules of other bacteria (Klieneberger-Nobel, 1948; Katz, 1964; Katz, Solovyov, and Volkova, Zh. Microbiol. Epidemiol. i Immunobiol., *in press*) have shown, the studies of capsules in fixed preparations extend the present state of our knowledge of the structure and composition of bacterial capsules.

To study the chemical composition of the *B. anthracis* capsule, we used cytochemical methods which enabled us to ascertain the chemical nature of the capsule and to locate the various substances in it, which could not be done by chemical methods alone. It was thus shown that the *B. anthracis* capsule has a stratified structure, the various layers of the capsule differing in their IEP, sensitivity to enzymes, and, consequently, in their chemical composition. Earlier literature gave contradictory indications concerning the position of the IEP in the *B. anthracis* capsule. For example, Tomcsik and Guex-Holzer (1953), who studied the interaction of the capsule with various proteins, determined the position of the IEP at pH 2.0, whereas Ivanovics and Horvath (1953b) established its position, with the aid of stains, below pH 3. Lastly, according to electrophoresis, the IEP of the *B. anthracis* capsule is at pH 3.1 (Harden, quoted by Tomcsik and Guex-Holzer, 1954a). These data apparently pertain to different parts of the capsule, which would explain such considerable differences in IEP.

To study the antigenic composition of the capsule and cell wall of *B. anthracis* and allied species, most investigators have employed either chemical or immunochemical methods of research. However, the chemical methods of research resulted in mechanical destruction of the cell, and therefore made it impossible to locate the antigens in the intact capsular cell. The immunological investigations were conducted mainly on living capsular cells treated with "polypeptide" and "polysaccharide" sera; these studies revealed the topographical distribution of the "polysaccharide" and "polypeptide" antigens in the capsules of *B. megaterium* and *Bacillus M*, species closely related to *B. anthracis*, thereby making a definite contribution to our knowledge of the fine structure and antigenic composition of bacterial capsules (Ivanovics and Horvath, 1953a; Tomcsik and Guex-Holzer, 1954b; Baumann-Grace and Tomcsik, 1958). However, the terms "polypeptide serum" and

"polysaccharide serum" suggested by these investigators do not reflect either the morphological localization of the corresponding antigens in the cell or the chemical nature of these antigens, since the capsule and cell wall of these microorganisms contain polysaccharide and protein components. It would be more appropriate to use the terms "capsular antibody serum" and "cell-wall antibody serum," which was done in the present study.

To study the arrangement of antigens in the *B. anthracis* capsule, we used the fluorescent-antibody method; to investigate the chemical nature of antigens, we used treatment with enzymes. As is well known (Dixon and Webb, 1958), pepsin hydrolyzes only the peptide bonds of carboxyl groups which belong to aromatic amino acids, especially if glutamyl is the adjacent amino acid residue. Trypsin and chymotrypsin hydrolyze not only peptide, but also ether bonds. Hyaluronidase acts on acid mucopolysaccharides which contain hyaluronic acid and chondroitin-sulfate (Dixon and Webb, 1958). Lysozyme apparently hydrolyzes the bond between N-acetylmuramic acid and 2-acetylaminoglycose in mucopolysaccharides and mucopeptides (Report of the Commission on Enzymes of the International Union of Biochemistry, 1961). Thus, the use of enzymes makes it possible to determine the chemical nature of antigens in the intact bacterial cell. Our overall study has shown that the outer fringe of the capsule contains so-called superficial capsular antigens, revealed by CLS; these antigens are of a peptide nature and determine the immunochemical specificity of the capsule. The dense membrane of the capsule, which together with the foregoing fringe forms the membranelike outline of the capsule, contains antigens closely related in composition to the antigens of the cell wall. Like the antigens of the cell wall, they are revealed by CWLS, but differ from them in that they are insensitive to lysozyme and sensitive to pepsin. The middle part of the capsule contains antigens of the capsule proper; these antigens are revealed by both CLS and CWLS, and contain polysaccharide and protein components (digested by lysozyme and proteolytic enzymes). Lastly, the inner part of the capsule, formed by acid mucopolysaccharides, either contains no antigens at all or contains antigens which are not revealed by the methods we have used. Thus, as regards antigens, the *B. anthracis* capsule is heterogeneous and contains substances of both protein and polysaccharide nature. However, the antigenic specificity of the capsule is determined only by peptides which possess selective sensitivity to pepsin. Using immunochemical methods of studying nonfixed

bacteria, Ivanovics and Horvath (1953b), Tomcsik (1956), and others have also shown that the antigenic specificity of the *B. anthracis* and *B. megaterium* capsules is determined by glutamyl polypeptide.

Our overall immunochemical and cytochemical studies have also enabled us to obtain new data on antigenic and other properties of the cell wall of capsular and noncapsular forms of *B. anthracis*. For example, it was shown that the cell wall of capsular cells is poorly seen in dark-field illumination, compared with the cell wall of noncapsular forms, that it is more sensitive to lysozyme, and that it possesses weaker fluorescence when stained with CWLS obtained by immunization of animals with noncapsular cells of *B. anthracis*. All this warrants the assumption that there are significant differences between the chemical composition and antigenic properties of the cell wall of capsular and noncapsular forms of *B. anthracis*. It is also possible, however, that the revealed differences are due to the fact that the capsule prevents enzyme and serum from penetrating into capsular cells.

It is also of some interest that the morphological destruction of the cell wall is not always accompanied by disturbances in its antigens. Under the action of lysozyme, *B. anthracis* exhibits morphological destruction of the cell wall and a change in its antigenic properties (weakened staining when treated with CWLS). Under the action of trypsin, the morphological destruction of the cell wall is not accompanied by a change in its antigenic properties. This suggests that in *B. anthracis* the antigenic properties of the cell wall are, unlike those of the capsule, determined not by a protein, but by a polysaccharide component. Guex-Holzer and Tomcsik (1956) and Tomcsik (1956) similarly indicated that the serological specificity of the *Bacillus M* cell wall is also determined by a polysaccharide component.

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