STUDIES ON THE CYTOCHEMISTRY OF BACTERIA[1]

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Since bacterial cells are basically similar to other living cells (Mudd, 1954; DeLamater, 1954) they are also susceptible to the histochemical analyses performed on higher cells. The present study, employing histochemical techniques, may help to resolve some of the problems and areas of disagreement posed by previous investigations in bacterial cytology (Bisset, 1951; Robinow and Hannay, 1953; Vendrely, 1953; Bergersen, 1953; DeLamater, 1954; Mudd, 1954; Knaysi, 1955).

MATERIALS AND METHODS

Strains used. Bacillus megaterium (Robinow's strain), a wild type *Bacillus mycoides*, and a wild type *Escherichia coli* were used. *B. megaterium* and *B. mycoides* were grown on corn meal agar (Difco), and incubated at 30 C. *E. coli* was grown on Sabouraud's agar (Difco), containing 50 units of penicillin and 50 units of streptomycin per milliliter of medium, and was incubated at 37 C as well as at 30 C. The foregoing media were used since the wild type organisms were originally isolated from them. At the desired times impression smears by the agar block method were taken onto no. 1 coverslips and fixed while moist. Smears were also obtained by picking up the bacteria with a cotton swab and streaking over coverslips. The various staining procedures were run in Columbia dishes.

Observations and photographs were made with a Bausch & Lomb research microscope fitted with a 90× (1.30 NA) apochromatic oil immersion objective, 12.5× compensating oculars, and a 1.4 NA aplanatic condenser. A Bausch & Lomb ribbon filament lamp was used. Photographs were taken on 35-mm Kodak microfilm with a primary magnification of 1125 or 2250 diameters. These magnifications were raised two to four times in printing to give final magnifications up to 4500 diameters.

CYTOCHEMICAL METHODS

Periodic acid-Schiff (PAS) (for 1,2-glycol group, its oxidation product, equivalent amino or alkylamino derivatives). It should be pointed out that the nature of the PAS-positive material must be differentiated, since substances such as ATP, free lipids and lipoprotein complexes as well as polysaccharides may also be PAS-positive (Pearse, 1953). Smears were fixed for 2 min in 2 per cent osmium tetroxide vapors. They were then oxidized at room temperature in 1 per cent periodic acid for 3 min, and repeatedly washed in distilled water to remove all traces of the reagent. After 10 min in Coleman's modification of Schiff reagent (or 2 hr in azure A-SO_2), the smears were rinsed in sulfurous acid solution (10 per cent potassium metabisulfite, 5 ml; N HCl, 5 ml; water, 90 ml), then rinsed in water and mounted on slides in Farrant's medium (Coleman and Bell). No difference in final results was achieved through the use of a buffered alcoholic solution of periodic acid. Positive-staining structures could be abolished by pretreatment in dry pyridine: acetic anhydride (1:1) for 1 hr at 60 C, and restored by treatment with 1 per cent KOH for 1 hr at room temperature.

Differentiation of periodic acid-Schiff reaction. A battery of various tests was used to differentiate PAS-positive materials in the cell (Pearse, 1953).

Tests for protein and protein constituents were employed as described below.

1. Coupled tetrazonium reaction. Osmium fixed smears were placed in freshly prepared 0.1 per cent tetrazotized benzidine (Pearse, 1953), or

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0.1 per cent tetradiorthodianisidine (TDA) in pH 9.2 veronal acetate buffer for 15 min at 4 C. After washing in water and then in several changes of cold buffer solution (pH 9.2), the smears were immersed in a saturated solution of beta-naphthol in the veronal buffer for 15 min. They were then washed in water and mounted in Farrant's medium.

2. Ninhydrin-Schiff (NS) reaction (for proteins and amino acids). Osmium-fixed smears were incubated at 37 C in a 0.5 per cent ninhydrin in ethanol solution for 24 hr. After thorough washing, they were placed in Schiff reagent for 10 min, carried through an SO_2 rinse, a water rinse, and then were mounted in Farrant's medium.

3. —SH groups. Smears were fixed in 1 per cent trichloracetic acid in 80 per cent ethanol. After being washed in 70 per cent alcohol and then in water, they were held for 1 hr at 50 C in a solution made up of 25 mg 2,2-dihydroxy-6,6-dinaphthyldisulfide (DDD) in 15 ml of ethanol plus 35 ml of 0.1 M veronal acetate buffer at pH 8.5. The smears were then cooled for 10 minutes and washed with water. Then followed several 2-min rinses in water acidified to pH 4 with a few drops of acetic acid, after which the smears were again washed in water. Dehydration to ether through graded alcohols was carried out, and then rehydration was performed by reversing the schedule. This procedure removed unwanted organic by-products. The smears were then placed in a freshly prepared 0.1 per cent solution of TDA in 0.1 M phosphate buffer at pH 7.4 for 2 min. They were then rinsed in water and mounted in Farrant's medium.

Lipids. Several methods for identifying fats were tested. Although no difference in staining results was apparent among the several methods using Sudan black B or oil red 0, 0.25 per cent acetylated Sudan black B in ethylene glycol was used in this study. Using leukocytes as test objects, Lillie (1954) has pointed out that acetylated Sudan black B will discriminate true fats from chemically sudanophilic substances. Smears were immersed in this solution for 2 hr. Differentiation was performed by rinsing briefly in 70 per cent ethanol. The smears were mounted in Farrant's medium after being washed in water.

"Masked" lipids. Smears were dipped in freezing (−50 C) methanol, then fixed in a mixture of 40 per cent formalin:10 per cent $CaCl_2$ (anhydride):2 per cent $Co(NO_3)_2$:H_2O (1:1:1:7) for about 12 hr, washed well, then placed in 5 per cent citric acid:Sudan black B in ethylene glycol (sat. sol.) (1:1) for 2 hr at 60 C. Differentiation was carried out in 70 per cent ethanol. The smears were then mounted in Farrant's medium after washing.

Glycogen. Digestion with ptyalin in saliva for 30 min at room temperature failed to abolish the PAS-positive structures in the bacterial cells.

Metachromasy. In order to study metachromasy, osmium-fixed smears were placed in 0.5 per cent aqueous toluidine blue for 15 min, rinsed in water, then wet-mounted in the adhering wash water by means of a paraffin seal. Mounts in glycerine jelly caused excessive fading of the dye after several weeks.

Methylene blue extinction (MBE) (to differentiate acid mucopolysaccharides from mucoproteins) (Dempsey and Singer, 1946). Veronal acetate buffer solutions of the following pH values were prepared: 2.62, 3.62, 4.66, 5.32, 6.12, 7.25, and 8.18. Of each solution, 5 ml were mixed with 5 ml of aqueous methylene blue (0.001 M) in each of 7 separate Columbia dishes. Osmium-fixed smears were then immersed in these buffered methylene blue solutions for 24 hr, after which they were rinsed in water and wet-mounted with a paraffin seal. This test is most specific in the absence of the nucleic acids. Since the walls of gram positive organisms and the poles of coliform organisms are charged with RNA, results achieved through the application of this test are inconclusive when they are taken by themselves. It was, therefore, necessary to run the test after extraction with ribonuclease.[3] According to Kaufman *et al.* (1951), 0.5 per cent ribonuclease in water at pH 6 was used.

Acid mucopolysaccharides by Alcian blue staining. Osmium- and Carnoy-fixed smears were stained for 5 min in a 1 per cent solution of Alcian blue 8GN150, rinsed in water, then mounted with Farrant's medium.

Acid mucopolysaccharides by the dialysed iron method (Hale, 1946). Carnoy-fixed smears were immersed in dialysed iron (5 per cent Fe_2O_3):2 M acetic acid (1:1) for 10 min. After being washed well, they were placed in acid ferrocyanide solution (0.02 M potassium ferrocyanide:0.14 M HCl, 1:1) for 10 min. They were then washed again and mounted in Farrant's medium.

[3] Ribonuclease (crystalline), Nutritional Biochemicals Corp., Cleveland, Ohio.

Hyaluronidase extraction. A 1:5 aqueous dilution of hyaluronidase, Wydase,[4] (1500 TR units/ml) at 37 C for 3 hr failed to abolish PAS-staining in Carnoy-fixed smears. This test does not exclude the presence of endocellular hyaluronidase-labile substances.

Plasmal reaction. Unfixed smears and smears fixed for 10 min in 7 per cent mercuric chloride failed to give a positive reaction in Schiff reagent.

A double-stain, using the Feulgen and PAS technique, was developed along the lines indicated by DeLamater *et al.* (1955). A simple PAS-Sudan black B double stain technique was also developed. These techniques are considered in the next section.

EXPERIMENTAL RESULTS AND DISCUSSION

In studying the cytochemistry of the progressively aging cell, it soon becomes apparent that several indicators of the aging process are available. The progressive development of lipids and PAS-positive materials within the cell are exemplary.

B. megaterium. The spore coat of *B. megaterium* by routine procedures has a single homogeneous appearance, but at least two components were found by Knaysi and Hillier (1949), using the electron microscope, and at least two components are distinguishable cytochemically. A lipoprotein component is in evidence, since both the tetrazonium reaction for histidine, tryptophan, and tyrosine, and the citric-Sudan test for masked lipids (figures 12 and 13) are positive. There is no question of solubility of the tetrazonium reagents in fat since the spore wall is refractory to Sudan black alone. The second component appears to be an acid mucopolysaccharide since the spore wall also takes Alcian blue, specific for insoluble carbohydrates at neutral or alkaline pH (McKinney, 1953), and also stains blue after the Hale technique for acid mucopolysaccharides (figure 14). The latter method is admittedly of low specificity; it depends upon the combination of iron with acid polysaccharides, but not with neutral polysaccharides or proteins, and is detected by the subsequent formation of Prussian blue. It is now generally agreed that the spore nucleus is centrally located.

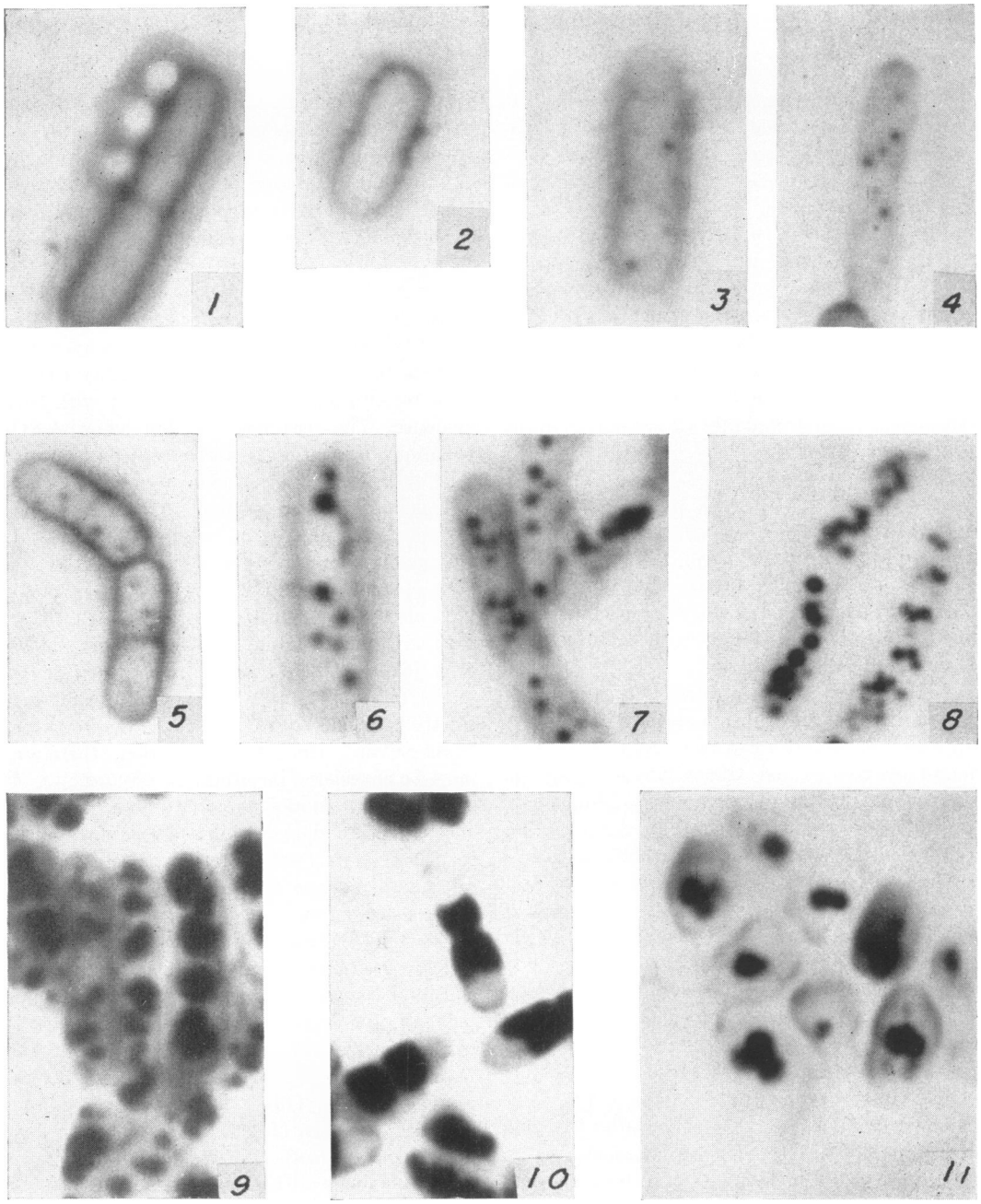
The germinating cell is Sudan-negative until about the third hour after erupting through the spore coat. At that time occasional fat granules appear along the cell wall; very delicate granules also appear in the cytoplasm (figures 1 to 5). During the next 3 hr, fat granules appear throughout the cell in progressively heavier concentrations (figures 6 to 8)—apparently by confluence of the smaller granules (figures 9 to 11). Claims that the nuclear configurations produced by the azure A-SO_2 technique are the result of the displacement of surrounding basophilic structures by fat (Robinow and Hannay, 1953) do not stand up to analysis, nor, it should be noted, have these authors supported their contentions with any evidence. The usefulness of the azure A-SO_2 technique has been shown in fat-free bacterial cells, such as *E. coli* and *Salmonella typhosa*, as well as in *B. megaterium* (figure 31) and pyridine extracted *B. megaterium* (figures 32 and 33). Characteristic nuclei in the spores and in the young fat-free germinating cells of *B. megaterium*, have also been demonstrated (DeLamater, 1951). Further, temporary mounts of double-stained cells (azure A-SO_2 and oil red O) show the nuclei and the fats to be separately and distinctly stained, each with a distinctive color. Robinow and Hannay's contention, therefore, must be considered incorrect.

Temporary mounts of double-stained cells may be obtained by dehydrating azure A-SO_2 stained cells in freezing (−50 C) ethyl phosphate saturated with oil red O. Mounts are sealed with Vaspar after a quick rinse in water.

The lipid inclusions of *B. megaterium* are extremely resistant to removal by the usual fat solvents, even at elevated temperatures. Solubility of oil red O and acetylated Sudan black B in these inclusions indicates the presence of true lipids and not merely basophilic staining bodies. Lemoigne *et al.* (1944) has shown these lipid granules to consist of beta-hydroxy-butyric acid. The Nile blue method of Cain (1947) indicates that these lipids are acidic in nature, but this test does not exclude the presence of neutral lipids. Besides free fat, the young cell is heavily laden with combined lipids, as evidenced by the citric-Sudan test.

The resting spores of *B. megaterium* are hyaline and PAS-negative. Since the spore wall stains with Alcian blue (figure 15) and the Hale technique, an acid mucopolysaccharide with substituted 1,2-glycol groups is indicated. Acid mucopolysaccharide, as bound in the resting

[4] Kindly supplied by Wyeth, Inc., Philadelphia, Pa.



Figures 1–11. All photographs are of *Bacillus megaterium* at a magnification of 4500 ×. Cells stained with acetylated Sudan black B in ethylene glycol.

Figure 1. Spores and 2-hr cells. No free fat is present in the spores.

Figure 2. One-hour cells emerging from spore (spore outlines at 1 o'clock). Fat granules appear along the cell wall.

Figures 3–5. Appearance of small granules of free fat in the cytoplasm. Figure 3, 2-hr cells; figures 4 and 5, 3-hr cells.

Figures 6–8. Four- to 6-hr cells. Heavier concentrations of soluble lipid due to confluence of smaller granules.

Figure 9. Thirteen-hour culture. Maximum concentration of free fat.

Figure 10. Spores (22–24-hrs) are terminal with contiguous fat in adjoining cells.

Figure 11. Five-day-old cells undergoing fatty degeneration.

spore wall, does not exhibit metachromasy, but subsequent hydration during germination reveals γ-metachromasy (red) when a dilute solution of toluidine blue is applied.

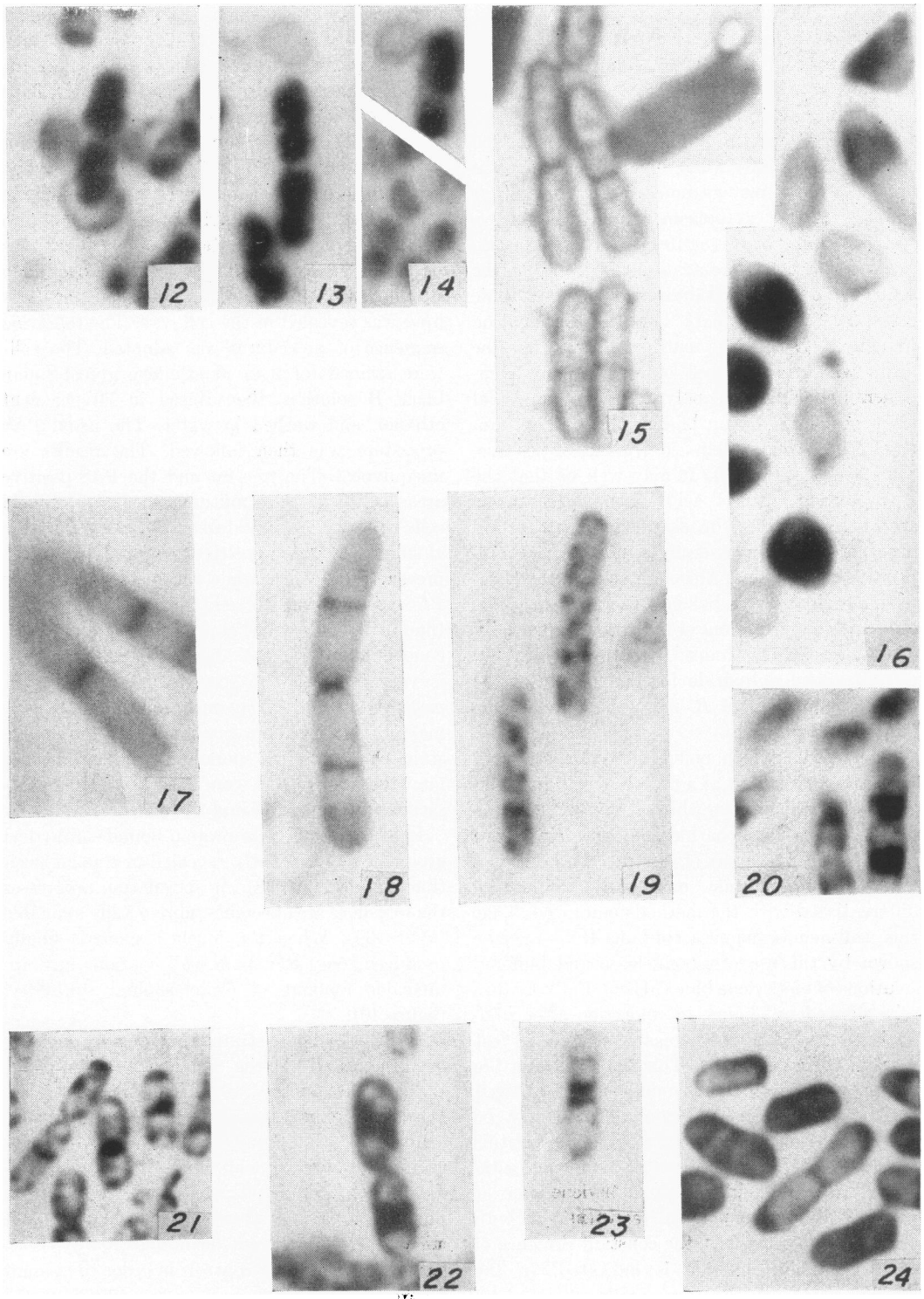
In young germinating cells (ca 3-hr) the cell walls are a uniform pink and the septa red (figures 17 and 18) when the PAS technique is applied. Tiny red granules begin to appear throughout the cytoplasm, giving the cell a speckled appearance (figure 19). There are also areas of heavier concentration, especially at the septum line of incipient division, which are visible after the fifth and sixth hours of germination (figures 20 to 23). The mature cell wall, like the spore wall, is composed of at least two components: acid mucopolysaccharide material (PAS-positive, Alcain blue-positive, Hale-positive) and lipoprotein (citric-Sudan-positive, tretrazonium-positive). It may well be that the PAS-positive sites of acid mucopolysaccharide are bound, like the "masked" lipids, to certain basic proteins, since equivalent sites are also positive for proteins. Although acetic anhydride-pyridine treatment abolishes the PAS reaction, either reagent used alone or both used sequentially do not abolish it. Indeed, treatment with hot (60 C) acetic anhydride for one hr causes the resting spore wall of *B. megaterium* to become faintly PAS-positive.

The picture which emerges from a study of *B. megaterium* is that of a protein framework and ground substance upon which PAS-positive areas (acid mucopolysaccharides, lipoproteins) are bound, enclosing areas of free fat. The cell wall and the cytoplasmic membrane cannot be differentiated with the methods employed. That this wall-membrane area contains RNA may be shown by the use of ribonuclease and buffered solutions of methylene blue (MBE). Fully mature cells showed no change in coloration after ribonuclease treatment, whereas young cells lost most of their basophilia. This indicates that the wall-membrane locus of young cells is charged with RNA, whereas this component cannot be detected in fully mature cells by the use of this method. Since the wall-membrane area continued to stain lightly with methylene blue at pH 2.9 after ribonuclease treatment in both young and mature cells, the constant presence of acid mucopolysaccharide is indicated. In the mature cell, in the areas normally obscured by free fat, protein granules are seen (by —SH test) reminiscent of the condensed chromosome phases described by DeLamater (1951) (figures 34 and 35), suggesting the presence of protein in the chromosomes. Keigler and Smith (1954) have made analogous findings in *Bacillus cereus*.

Since the free fat of *B. megaterium* is so resistant to extraction, it is possible to combine certain procedures on the same cells. Sudan staining followed by 1 per cent periodic acid was found to be equivalent to Sudan staining alone. When the periodic acid hydrolysis was followed by Sudan staining the result differed only in that "masked" lipid was revealed in the cell wall. The following sequence of procedures was adopted. The cells were stained for 2 hr in ethylene glycol-Sudan black B solution, then rinsed in 70 per cent ethanol, and washed in water. The usual PAS procedure was then followed. The results are unequivocal. The free fat and the PAS-positive areas of the cell are mutually exclusive. In old cells undergoing fatty degeneration several large globules of Sudan-positive material are often present (figure 11), while the rest of the cell is PAS-positive due to acid mucopolysaccharides (figure 16). Sulfhydryl staining in these old cells, concomitant with the absence of bound lipids, is very intense. The spore-forming cells of *B. megaterium* and *B. mycoides* appear to have a marked polarity when examined with the double stain. One end of the sporangial cell contains free fat; the other end contains acid mucopolysaccharide (figures 10 and 24 to 26).

In sporulating cells protein-bound sulfhydryl groups are prominently revealed in a polar position (figures 36 to 38). As sporulation progresses the forespore area becomes more readily stainable (figure 39). When the finished spore is finally produced, only the spore wall contains any appreciable amount of protein-bound sulfhydryl (figure 40).

It has been demonstrated that azure A-SO_2 may be substituted in the Feulgen procedure for staining the nucleus of various types of cells. It was, therefore, thought feasible to substitute azure A-SO_2 for Schiff's reagent in other procedures (periodic acid-Schiff, peracetic acid-Schiff, ninhydrin-Schiff) where a positive reaction might be faint or absent. In developing substituted Schiff's reagent techniques it was necessary to run a series of controls in order to account for excess unreacted azure A. In addition to the test run (acid hydrolysis followed by 2 hr in



Figures 12–24

azure A-SO_2), four controls were run as follows: azure A alone; azure A-SO_2 alone; hydrolysis followed by azure A; hydrolysis followed by an aldehyde block (sodium bisulfite, saturated aqueous solution, 60 C/2 hr) followed by azure A-SO_2. The second and fourth listed controls were found to yield equivalent results. Freeze-drying, alcohol dehydration, and water mounting were all possible with the above procedures. Generalized basophilia due to RNA in the cytoplasm and especially the cell wall and septa is apparent when azure A or azure A-SO_2 is used without preliminary hydrolysis (figures 27 to 30). Adequately controlled, however, the periodic acid-azure A-SO_2 technique was found to give excellent comparative results on bacteria (figure 22).

The development of a Feulgen-PAS (azure A-SO_2 substituted) double stain proved to be impractical with bacteria, since the weakly staining chromatin (by Feulgen technique) faded entirely upon exposure to periodic acid. The azure A-SO_2 substituted Fuelgen procedure followed by the normal PAS technique was more successful with bacteria. The simultaneous demonstration of nuclear components, cell walls, and septa by chemically specific methods invalidates another criticism (Bisset, 1952) of the DeLamater nuclear technique as applied to bacteria. Since the bacteria investigated contained no free aldehydes, the principles of the technique (DeLamater *et al.*, 1955) were applied as follows: The cells were hydrolyzed in N HCl at 60 C for 4 to 6 min, washed, then stained in azure A-SO_2 for 2 hr. 1,2-Glycol groups were oxidized, and excess dye was simultaneously and selectively removed from cell walls and septa by controlled rinsing in 0.25 per cent periodic acid. Schiff reagent was then applied for 15 min, followed by the usual SO_2 and water rinses before freeze-drying or mounting in Farrant's medium. Nuclear components are stained blue, whereas cell wall components stain red.

Peracetic acid oxidation may be used to show the presence of unsaturated lipids (Lillie, 1954; Bangle, 1954) or disulfide groups if lipids are not present (Pearse, 1953). The presence of unsaturated lipid could not be demonstrated in *B. megaterium* with either the peracetic acid-Schiff technique or the peracetic acid-azure A-SO_2 substitution. In *E. coli*, however, the entire cell stained a uniform pale pink or blue, depending on which staining reagent was used. Similar results were obtained when azure A-SO_2 was substituted for Schiff reagent in the ninhydrin-Schiff technique. The presence of protein-bound sulfur groups in the wall of *E. coli* is indicated, although Salton (1953) states that the amino acid cystine is absent from all bacterial cell walls. The equivalence of azure A-SO_2 with Schiff reagent is demonstrable when the appropriate controls are run.

E. coli. Compared to the gram positive aerobic spore-formers, the internal organization of *E. coli* is relatively less complex. The slime layer of mucoid strains of this organism stains with Alcian blue, indicating the presence of acid

Figures 12–24. All photographs are of *Bacillus megaterium* at a magnification of 4500×.

Figures 12 and 13. Citric-Sudan stain on spores and young cells. Spore coats and cell walls are positively stained for bound lipid.

Figure 14. Resting spores; Hale mucopolysaccharide technique.

Figure 15. Alcian blue; staining of spore and cell walls demonstrates presence of acid mucopolysaccharide; old degenerate cell (non-living?) takes up dye in protoplast.

Figure 16. Periodic acid-Schiff (PAS) technique on old degenerate cells; faint pink coloration throughout cells due to acid mucopolysaccharide; sporangial cells in center show PAS-positive granules in polar position.

Figures 17–22. PAS technique applied to germinating and dividing cells.

Figures 17 and 18. Two- and 3-hr cells; cell walls are pink and septa are red.

Figure 19. Five-hr cells; red granules appear scattered throughout the cytoplasm.

Figures 20–23. Six- and 7-hr cells; progressively heavier concentrations of PAS-positive material accumulate at the cell septa.

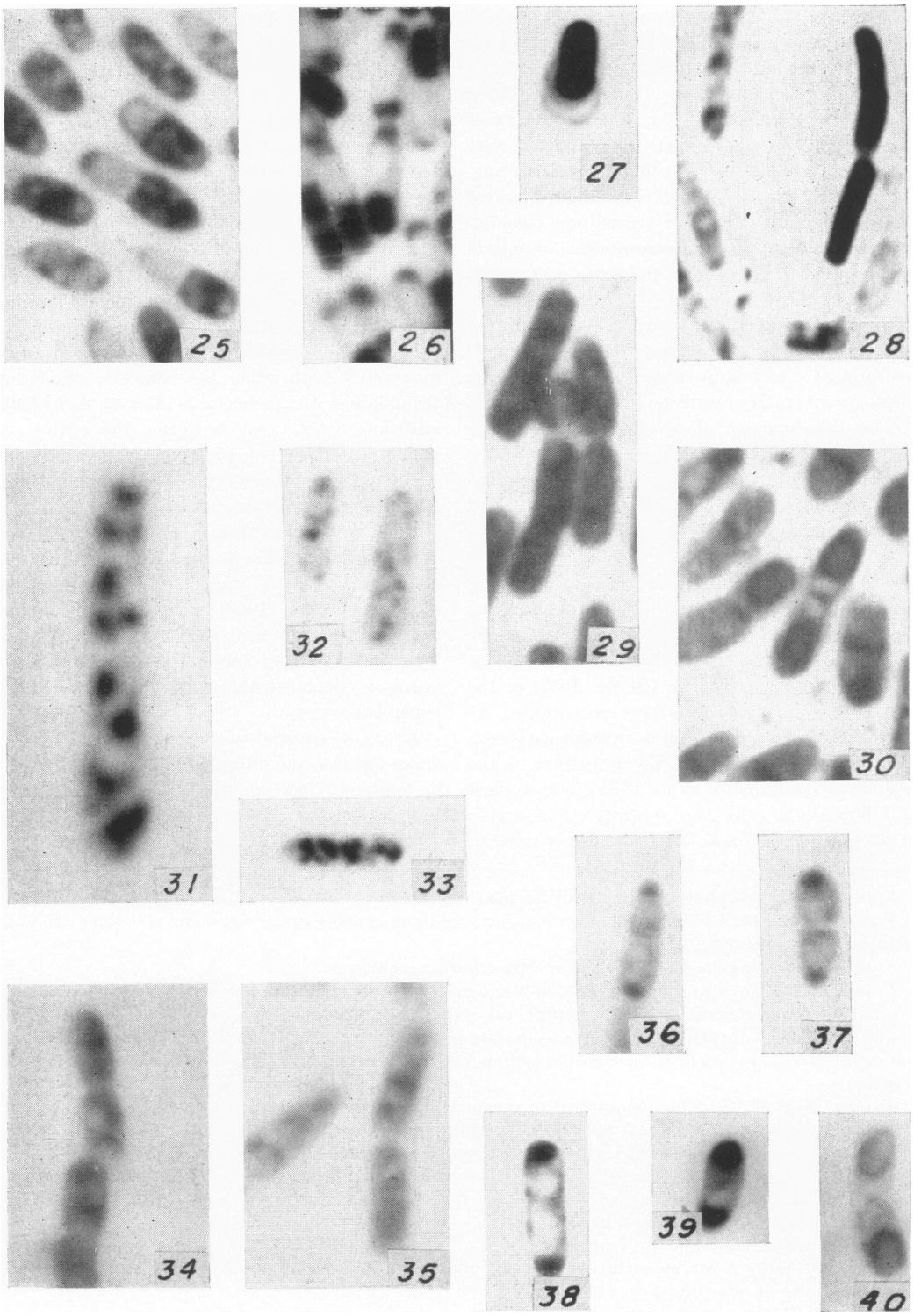
Figure 20. PAS technique on 6-hr cells.

Figure 21. PAS technique on 7-hr cells.

Figure 22. Azure A-SO_2 substitution for Schiff reagent in PAS technique. 7-hr cells, showing concentrations at site of next division.

Figure 23. PAS technique on 7-hr divided cells.

Figure 24. Twenty-two-hr spores in sporangial cells; polar areas and septa are PAS-positive.



Figures 25–40

mucopolysaccharides. The cell walls of both mucoid and smooth strains likewise stain with Alcian blue, are PAS-positive, are colored blue by Hale's technique, and resist the action of hyaluronidase. The cell wall also stains for protein by the tetrazonium, ninhydrin-Schiff, and —SH group techniques. In the present study, application of the citric-Sudan technique results in the cell wall staining only a faint brown. Weidel (1953), through the use of phage adsorption techniques, has shown the basic framework of the cell wall to be lipoprotein. He has also shown a second component to be embedded in this basic framework. Salton (1953), through fractionation and chemical analysis, has shown the wall of *E. coli* to be essentially lipoprotein with "a small carbohydrate moiety or residue." It may be concluded, therefore, that the cell wall of *E. coli* consists of at least two components, viz., lipoprotein and acid mucopolysaccharide.

The uni- or bipolar granules of *E. coli* (figures 41 and 42) and other coliform bacteria have been the cause of much discussion and controversy—so much so that it seems to at least one reviewer (Vendrely, 1953) that the authors do not speak the same language. The present study reveals these granules to be lipoprotein and to contain a greater concentration of —SH positive protein than the remainder of the cell. "Masked" lipids of the nitrogen and phosphorus-containing type, such as lecithin, are generally found in mitochondria. Since Potter and duBois (1943) have postulated the presence of sulfhydryl groups in the enzyme succinic dehydrogenase, the finding of sulfhydryl group concentrations in the polar granules of *E. coli* tends to substantiate the belief that these polar areas may be the sites of mitochondrial activity. A comparison of these polar granules with isolated liver mitochondria showed similar staining reactions between the two. Both were Sudan-negative, PAS-positive, citric-Sudan-positive, and positive by the protein tests.

Since Alcian blue apparently does not penetrate the cell wall (McKinney, 1953), its usefulness with unsectioned bacterial cells is limited. In order to help differentiate the internal PAS-positive material of the cell, resort was made to the Hale technique for acid mucopolysaccharides. This showed the cell wall and cytoplasm to contain acid mucopolysaccharide material, whereas the polar granules did not stain.

The techniques for staining proteins failed to reveal the nucleus of *E. coli*. This result may be due to masking materials in the wall or perhaps to some intrinsic difference in nuclear structure from that of *B. megaterium*. Further work is needed. Hartman and Payne (1954) have demonstrated DNA-protein in the cells of *E. coli* by means of the May-Grunwald-Giemsa technique. The chemical basis for this procedure, however, is obscure. Davis and Mudd (1955) have applied a protein stain to what is probably the nucleus of *Corynebacterium diphtheriae*.

E. coli large bodies were subjected to preliminary examination. No free fat was found in these structures, but bound lipid was present in abundance (figure 49). Large quantities of PAS-positive material and chromatin were found (figures 43 to 47 and 50 to 53) also. Thick cell

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Figures 25–40. All photographs are of *Bacillus megaterium* except Figure 25. Figures 25–30 are at a magnification of 4500×.

Figure 25. Periodic acid-Schiff (PAS) technique; the spore coats of resting spores do not stain; the polar location of PAS-positive material in the sporangial cell is illustrated here.

Figure 26. Sudan-PAS technique; *Bacillus mycoides*, 24-hr sporangial cells showing polarity of soluble fat and acid mucopolysaccharide.

Figures 27–30. Azure A used alone to stain cells 1, 3, 7, and 22 hr after seeding spores on fresh media. The intense basophilia of the young cells may be abolished by ribonuclease treatment.

Figure 31. B. megaterium, 4500×; multinucleate cell at 6 hr; HCl-Azure A-SO_2 nuclear stain. (Metaphase?)

Figure 32. 2250×; same treatment as figure 31 after pyridine extraction. (Interphase?)

Figure 33. 2250×; same treatment as figure 31 after pyridine extraction. (Metaphase?)

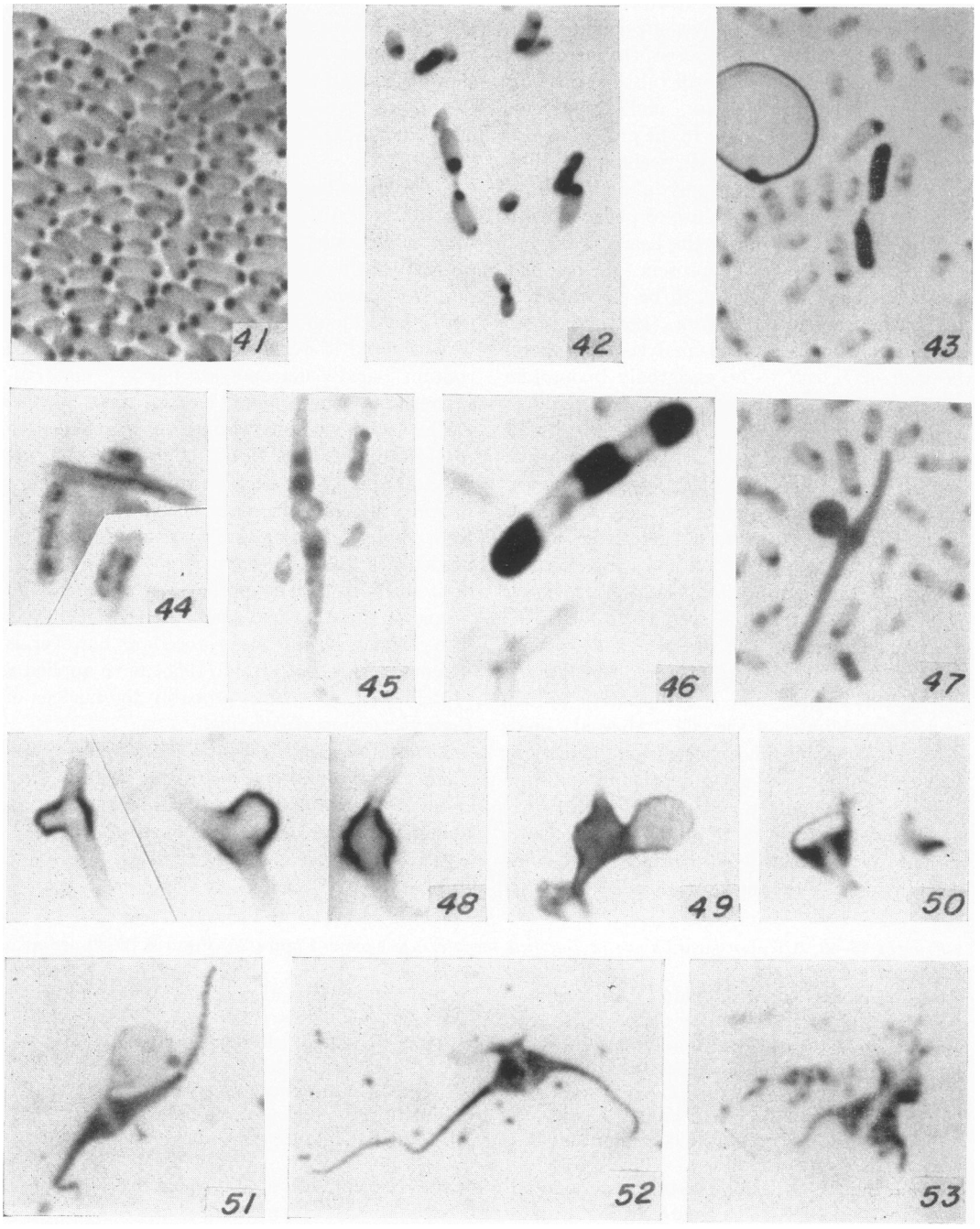
Figures 34–40. Magnification of 3375×; —SH technique of Barrnett.

Figures 34 and 35. B. megaterium, 6-hr cells; walls and nuclear sites are stained; new septa (PAS-positive) are unstained.

Figures 36–38. Polar caps are illustrated in sporulating cells.

Figure 39. Sporulating cell showing darkened forespore area.

Figure 40. Finished spores. Spore coat is positive for protein-bound sulfhydryl groups.



Figures 41–53

walls were evident in large bodies stained by Dyar's (1947) method (figure 48). The large bodies often gave rise to sac-like membranous structures, apparently contiguous with the cell membrane, which were seen to contain chromatin granules (figures 49 and 51). The granules are apparently released upon rupture of the sac (figure 53).

Issue has been taken with the concepts of organization and behavior of the bacterial cell as presented by DeLamater (1951, 1954) and Mudd (1953, 1954). The nuclei and mitochondria described by these respective authors have been lumped into the classification of nuclei, either "primary," "compound," or "complex primary" by Knaysi (1955). The basis of Knaysi's classification is a cytological study involving the use of non-specific methods for nuclei, i. e., DNA-containing structures as conventionally understood. One method involves the use of acidified methylene blue, equivalent to one phase of the methylene blue extinction technique. This may also be used to demonstrate (Davis and Mudd, 1955) RNA or polysaccharide substances in the absence of nucleic acids (Pearse, 1953). Another method makes use of partially reduced neotetrazolium (soluble in fat) or Janus green, the classic use of which has been the demonstration of mitochondria. A final unevaluated method involves the decolorization of thionin by acidified methylene blue. Discussion of nuclear structure or behavior based on the above methods would be fruitless; conclusions derived from the use of these methods would be specious. The multiplicity of terms proposed by Knaysi likewise serves but to cause further confusion.

The recognition of mitochondria in bacteria and the relationship of certain enzymatically active particles to cell structure are current problems. In view of the diverse functions and forms which mitochondria may assume, criteria for the recognition of these organelles must be broad enough to encompass the classic findings on higher cell forms and the newer findings on bacteria.

Not all the enzymes of the tricarboxylic acid cycle are localized in particulate fractions of the cell (Alexander and Wilson, 1955). Only the cytochrome system is consistently associated with the particulate fraction (Smith, 1954; Weibull, 1953*a*; Stanier *et al.*, 1953; Billen and Volkin, 1954; Alexander and Wilson, 1955); thus succinic dehydrogenase activity may logically be employed as a measure of the oxidative phosphorylation activity of various particulate fractions. Succinoxidase activity has been so used classically. Billen and Volkin (1954), however, have demonstrated qualitative differences within the succinoxidase active fractions of *E. coli*.

A distinct group of criteria for mitochondria based on available data from higher cell forms and bacteria would include the following: Mitochondria are particulate, electron opaque, and visible with the light microscope (at least in the aggregate) (Selby, 1955; Wilson and Wilson, 1955), containing proteins of the —SH type, RNA, and bound lipids of the phosphorus- and nitrogen-containing type; no DNA is present. The particles show succinic dehydrogenase activity and contain all the cytochrome fractions which may exist in the cell. The particles show a characteristic sequence of color changes when vitally stained with Janus green B. When

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Figures 41–53. All photographs are of *Escherichia coli* at a magnification of 4500×.

Figure 41. Periodic acid-Schiff (PAS) technique; normal polar and bipolar appearance of stained cells.

Figures 42 and 43. PAS technique; normal cells (before large body formation) sometimes show a connecting link with a deeply staining central granule.

Figure 44. Nuclear stain; HCl-Azure A-SO_2 technique; enlarged cells preceding large body formation.

Figures 45–47. PAS technique; different types of large bodies containing large quantities of PAS-positive material.

Figure 48. Cell wall stain; Dyar technique.

Figure 49. Citric-Sudan stain; a membranous structure containing bound lipids is present; sacculation from the main part of large body is evident.

Figures 50–53. Nuclear stain; HCl-Azure A-SO_2 technique; main part of large body is filled with chromatin granules; a central cavity or channel is apparent in all the cells.

Figure 53. Nuclear stain as above; release of chromatin granules following rupture of membranous sac

properly controlled, tetrazolium techniques may indicate the presence of reducing groups (—SH, reduced flavoprotein) in mitochondria. Techniques depending on the presence of phospholipid in these granules have long been classic procedures.

Using the above criteria as a guide, it is seen that the polar granules of *E. coli* and those of related gram negative species are the major sites of the kind of activity associated with mitochondria. It is not surprising, therefore, to find several different types of material accumulating about these active chemical centers. Among these may be mentioned RNA (Hartman and Payne, 1954) and the acid mucopolysaccharides of the growing cell wall. The hypothesis that metaphosphate may accumulate about these centers has also been advanced (Mudd, 1954; Davis and Mudd, 1955). Alkaline phosphatase activity has likewise been located primarily at these polar loci with fainter staining of the cytoplasm and cell wall (Schaechter *et al.*, 1954).

Claims as to the location of mitochondria in *B. megaterium* should be carefully re-evaluated in the light of more recent findings on the use of the tetrazolium dyes (Weibull, 1953*a*; Hartman and Liu, 1954; Novikoff, 1955; Gonse and Yotsuyanagi, 1955) and the location of the cytochrome system in the cytoplasmic membrane of this organism (Weibull, 1953*b*, *c*). Alkaline phosphatase activity in *B. megaterium* was irregularly located in the cytoplasm and the cell wall by Schaechter *et al.* (1954). Bergersen (1953) has provided evidence that the granules in *B. megaterium* which show oxidative activity are associated with the cell membrane. He further intimates a relationship between these granules and the so-called "growing points" of coliform organisms. The densely staining "growing points" revealed by HCl-Giemsa (Bergersen, 1953) (plate 1, figure 3) are clearly to be equated with the acid mucopolysaccharide found at the same site in this study (figures 22 and 23). It would thus seem that "growing points" are polysaccharide accumulations in the cell wall area whereas "peripheral bodies" (Chapman and Hillier, 1953), the oxidase-positive granules observed by Bergersen, and the "mitochondria" of Mudd (1954) are identical structures.

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SUMMARY

This study is concerned with the application of histochemical techniques to bacteria. Through the use of these techniques it has been possible to characterize some of the key structures and inclusions of *Bacillus megaterium* and *Escherichia coli*. The cell wall and cytoplasmic membrane were not differentiated by the methods employed. However, it may be concluded on the basis of these studies that the cell wall-membrane area of *B. megaterium* contains acid mucopolysaccharide, lipoprotein, and especially in young cells, an abundance of ribonucleic acid. A ground protein and bound lipids are stainable throughout the life cycle of the cell. Discrete concentrations of soluble fat and acid mucopolysaccharide are stainable several hours after spore germination. The nuclear apparatus of *B. megaterium* appears to contain —SH protein in addition to deoxyribonucleic acid. Several combination stains were developed for fat, nuclei, and 1,2-glycol groups.

The cell wall-membrane of *E. coli* is a lipoprotein-acid mucopolysaccharide complex. The polar granules are sites of ribonucleic acid, lipoprotein, and protein bound —SH group concentrations. Nuclear protein could not be demonstrated in this organism with the techniques employed. Large bodies of *E. coli* were also subjected to preliminary examination.

The significance of this study in relation to current controversy in the field of bacterial cytology is discussed.

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