THE CYTOLOGY OF A STRAIN OF CORYNEBACTERIUM DIPHTHERIAE¹

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This is a study of the cytological organization of a strain of *Corynebacterium diphtheriae* under several conditions of culture. Oxidation-reduction indicators, differential stains, and electron microscopy were used as means of investigation.

MATERIALS AND METHODS

Organism studied. *Corynebacterium diphtheriae*, type *mitis*, strain A9255. Growth media included M. and E. (Morton and Engley, 1945) broth and agar, with and without enrichment by whole blood, plasma or the lysate from human blood cells subjected to freezing and thawing. Culture was usually at 37 C; occasionally, at room temperature.

Stained preparations. Cells in broth culture were placed on agar blocks, fixed for 1 minute in the vapors of a 2 per cent aqueous solution of osmium tetroxide, partially dried, and impressed onto coverslips. Cells from agar grown cultures were smeared directly onto coverslips and similarly fixed. Preparations were stained by a variety of oxidation-reduction indicators and differential stains as indicated under "Results". Mounting was usually in Farrant's (aqueous) medium or H.S.R. (Harleco Synthetic Resin). Examination was with a Bausch and Lomb research microscope with bal-coated lenses, a 15 × eyepiece (12.5 for photography), and 97 × achromatic oil immersion objective of n.a. 1.25. Photographs were taken with a Bausch and Lomb L camera. Final magnification of prints was 4,850 ×.

Preparations for electron microscopy. Cells were suspended in distilled water, placed on collodion films overlying agar, and dried (Hillier *et al.*, 1948). Films bearing the cells were cut out, fixed in the vapors over 2 per cent OsO₄ solution, floated in distilled water for an hour to dialyze away undesirable deposits, and picked up on 200

mesh copper screen. After drying overnight, preparations were stored in a desiccator until the time of observation. Examination was with an RCA electron microscope, model EMU, 2A. Final magnification of prints was 16,000 ×.

RESULTS

Some effects of growth conditions. Rods or coccoid cells, with or without metachromatic granules, were obtainable by appropriate control of growth conditions. Rods with prominent polar or subpolar metachromatic granules were obtained from growth on 5 per cent blood agar slants, good growth appearing within 14 hours (figures 16, 18, and 20). When rods were inoculated from mineral oil-covered M. and E. agar or blood agar stock cultures into blood broth, the resulting cells were usually nonmetachromatic; and in time, during the stationary phase, were characterized by strongly basophilic, localized areas often located near the center of the cells (figure 11). These cells rapidly became metachromatic when placed in certain modified environments, e.g., M. and E. agar. In time, these cells were nonmetachromatic again and possessed a generalized basophilia. In some instances blood broth cultures also contained a high percentage of metachromatic cells.

Coccoid colonies appeared on the surfaces of old blood agar slant cultures about 14 hours after most of the cells had been washed off with saline; they were also obtained on M. and E. agar containing 5 per cent human blood cell lysate.

A growth curve based upon both plate counts and Klett turbidity readings was made of an M. and E. broth culture containing 5 per cent horse serum. The cultures reached a stationary growth period by 11 hours when counts were approximately 6×10^7 cells per ml. However, most cells remained viable even after 24 hours, as shown by their ability to form metachromatic granules when transferred to M. and E. agar. Cells from blood broth cultures were usually used

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in the stationary phase, at which time they showed a localized basophilia rather than the generalized basophilia seen in younger cells.

Diagrammatic drawings representing the essential features of the nonmetachromatic rod, the metachromatic rod, and metachromatic coccoid cells are shown in Diagram I.

Cell walls and septa. Cell walls and septa were stained by the methods of (1) Robinow (1945), (2) Robinow and Murray (1953), and (3) Hale (1953).

(1) Preparations were mordanted in 5 per cent tannic acid solution at 60 C for 30 seconds, or at room temperature for several hours, and then

Diagram I

Figures 1-62. *Corynebacterium diphtheriae* type *mitis*, strain A9255.

Figure 1. Cell walls and septa of nonmetachromatic rod forms from blood broth culture stained according to Hale using unextracted methyl green; a and b are from same preparation.

Figure 2. Cell walls and septa of metachromatic rods from blood agar culture stained as in figure 1. Metachromatic granules are not stained.

Figure 3. Formazan granules in nonmetachromatic rods from blood broth culture stained with 0.1 per cent triphenyltetrazolium chloride for 30 minutes.

placed for a minute or more in a solution of either 0.1 per cent aqueous basic fuchsin or 0.2 per cent aqueous crystal violet. Mounting was in either H.S.R. or Farrant's medium.

(2) Osmium fixed cells were placed 20 minutes in a saturated solution of mercuric chloride, rinsed, stained 30 seconds with a 0.05 per cent aqueous solution of Victoria blue B, rinsed, blotted, and mounted in Farrant's medium.

(3) Unfixed air dried coverslip impression smears were placed 5 minutes in a 1 per cent phosphomolybdic acid solution, rinsed, and placed in a 1 per cent solution of either methyl green or chloroform extracted methyl green. Following rinsing and blotting, mounting was in Farrant's medium.

When cell walls and septa were prominent, all these stains were quite satisfactory, but when the septa were poorly defined, the choice of staining procedure became critical. Nonmetachromatic rods with localized basophilia frequently had a prominent central septum (figure 1). The septa of metachromatic rods were often absent or difficult to stain although occasional cells showed distinct multiple septation (figure 2). Most coccoid forms

were either undivided or possessed a single septum though occasional cells were divided into 4 sections (figure 5).

Following mordanting with tannic acid, crystal violet was better than basic fuchsin for showing the poorly staining septa of metachromatic rods. In Hale's procedure employing unextracted methyl green, structures first stained green and then purple. Chloroform extracted methyl green stained cell walls and septa a pure green but with such poor intensity that it could only be used on avidly staining coccoid cells.

Tetrazolium chlorides. Solutions of these salts were added to M. and E. broth cultures or agar cultures suspended in broth; neotetrazolium chloride (NT) and triphenyltetrazolium chloride (TPT) were used at a concentration of 0.1 per cent, and blue tetrazolium chloride (BT) usually at a concentration of 0.05 per cent. Cells were usually mounted in H.S.R.

The reduction of TPT revealed at first discrete intracellular granules, but later the formazan became indiscriminately distributed throughout the cells. Rods from 10–14 hour old cultures reduced TPT within an hour. Rods from older

Figure 4. Formazan granules in nonmetachromatic rods from same blood broth culture as in figure 3 stained with 0.05 per cent blue tetrazolium chloride for 30 minutes.

Figure 5. Cell walls and septa of metachromatic coccoid forms from blood agar stained according to Hale using unextracted methyl green. Metachromatic granules are not stained.

Figure 6. Formazan granules in nonmetachromatic coccoid forms from M. and E. agar culture stained with 0.1 per cent neotetrazolium chloride for 10 minutes and then stained for cell walls with tannic acid-basic fuchsin.

Figure 7. Formazan granules in coccoid forms from blood agar culture stained with 0.15 per cent triphenyltetrazolium chloride and then stained for cell walls and septa according to Hale, using chloroform extracted methyl green. Note cell wall and septum. TPT = triphenyltetrazolium chloride stained granule; S = septum.

Figure 8. Colored granules in metachromatic rod from blood agar culture stained with 0.001 per cent Janus green B agar for 2 hours.

Figure 9. Colored granules in metachromatic rods stained with 0.05 per cent Janus green B agar for 1 hour.

Figure 10. Staining of metachromatic rods with 0.005 per cent Janus green B broth for 30 minutes.

Figure 11. Localized basophilic areas in nonmetachromatic rods from blood broth culture stained with old Loeffler's methylene blue.

Figure 12. Localized basophilic areas in nonmetachromatic rods from blood broth culture stained with Neisser's acidified methylene blue; a and b are from same preparation.

Figure 13. Centrally located basophilic areas bordered by metachromatic granules in rods from blood broth culture. Basophilic areas stained with malachite green. Metachromatic granules stained with crystal violet-citric acid. MG = metachromatic granule.

Figure 14. Metachromatic granules and basophilic bodies (nuclei) in rods originally grown in blood broth (nonmetachromatic), then placed on M. and E. agar for 1 hour (becoming metachromatic). Stained with Neisser's solution. MG = metachromatic granule; N = nucleus indicated by closely associated ribonucleic acid.

Figure 15. Generalized basophilic staining in nonmetachromatic (excepting one) rods originally grown in blood broth, then placed on M. and E. agar for 3½ hours.

Figure 16. Polar metachromatic granules and faint basophilic areas in metachromatic rods from blood agar culture. Stained with Neisser's solution.

Figure 17. Scarlet staining cell walls, septa, and metachromatic granules of coccoid forms from blood agar culture stained with Neisser's solution. MG = metachromatic granule; S = septum; a and b are from same preparation.

Figure 18. Polar metachromatic granules in metachromatic rods from blood agar culture stained with azure A-citric acid and counterstained with fast green.

cultures exhibited little or no reduction. However, coccoid forms from a 20 hour culture showed noticeable reduction within 5 minutes. Satisfactory reduction by rods usually revealed 1 to several polar granules although sometimes numerous peripheral granules stained as well (figure 3). In the coccoid forms 1 to several granules were observed (figure 7).

The reduction of BT and NT produced a more extensive staining pattern than did TPT in most rods but not in the coccoid forms (figures 4 and 6). This staining pattern was similar to that of Sudan black B.

Formazan has been shown to be lipid soluble (Tynen and Underhill, 1950; Seligman and Rutenburg, 1951; Shelton and Schneider, 1952). Therefore a test for nonvital staining of lipid was made by placing unfixed rods from a 24 hour blood agar culture in a 0.1 per cent aqueous solution of formazan produced by hydrosulfite reduction of either TPT or NT. No staining occurred within an hour. Similar cells fixed with osmium tetroxide remained unstained even after overnight treatment.

Janus green B (Lazarow and Cooperstein,

1953). Mitochondria in cells 12 to 24 hours old could be successfully stained by the dye used in M. and E. agar at concentrations as low as 0.005 to 0.0025 per cent, and even with 0.001 per cent when the medium was diluted 1:1 with phosphate buffer and contained an additional 5 per cent of human blood plasma. Using 0.001 per cent Janus green B agar, most cells were observed to contain 1-2 green granules, often polar, within an hour (figure 8) although some cells contained numerous faintly stained granules. After 2 hours many of these granules were red, and later they became colorless. Cells stained with 0.05 per cent Janus green B in M. and E. agar possessed numerous dark bluish-green granules resembling granules stained either by NT or by Sudan black B (figure 9). Cells stained with varying concentrations (0.05 to 0.0005 per cent) of Janus green B in M. and E. broth had stained areas of varying intensity which correspond to basophilic areas demonstrable with basic stains (figure 10) rather than to discrete granules.

The Nadi reagent. The procedure used was that of Hawk *et al.* (1947). The results were negative.

Staining of metachromatic granules. Neisser's

Figure 19. Dispersed metachromatic material in metachromatic rods from blood agar culture, first hydrolyzed 1 minute in 1 N HCl at 60 C, then stained as in figure 18.

Figure 20. Similar to figure 18 only metachromatic granules were stained with crystal violet-citric acid.

Figure 21. Metachromatic granules stained with crystal violet-citric acid and cell walls stained according to Hale (using extracted methyl green) of metachromatic coccoid forms from blood agar culture.

Figure 22. Discrete gram positive intracellular areas and gram negative areas of nonmetachromatic rods from blood broth. Gram stained.

Figure 23. Extended gram positive areas of rods originally from blood broth (nonmetachromatic) and then placed for 1 hour on M. and E. agar (producing subpolar metachromatic granules). Gram stained.

Figure 24. Gram positive metachromatic rods with polar metachromatic granules.

Figure 25. Gram positive metachromatic coccoid forms from blood agar culture.

Figure 26. Lead sulfide stained metachromatic coccoid forms and metachromatic rods from mixed culture on blood agar.

Figure 27. Nuclei of nonmetachromatic rods from blood broth culture stained according to DeLamater; cell walls stained with tannic acid-basic fuchsin.

Figure 28. Nuclei stained according to DeLamater in nonmetachromatic rods from blood broth culture. Counterstained with fast green.

Figure 29. Nuclei stained according to DeLamater in rods originally from blood broth culture, placed on M. and E. agar for 5 hours.

Figure 30. Underhydrolyzed DeLamater nuclear stain of metachromatic coccoid forms from blood agar culture. Cells were only hydrolyzed 4 minutes before staining. Note stained cell walls and septa.

Figure 31. Satisfactory DeLamater nuclear stain of nonmetachromatic coccoid forms from M. and E. agar culture. Cell walls then stained with tannic acid-basic fuchsin. Hydrolysis was for 8 minutes before nuclear staining.

Figure 32. Lipid bodies in nonmetachromatic rods from blood broth culture stained with Sudan black B-citric acid.

Figure 33. Lipid staining of coccoid forms and rods from blood agar culture using Sudan black B-citric acid.

acidified methylene blue stain was used for demonstrating metachromatic granules. For staining granules with crystal violet, cells were placed for 10 seconds in a 0.2 per cent aqueous solution and destained for 1 minute in 5 per cent citric acid. For malachite green staining, cells were suspended in 1 N HCl, spread on a coverslip, fixed with heat, and stained for 20 seconds in a 0.25 per cent aqueous solution. The cells were partially destained for 1 minute in 5 per cent citric acid. Metachromatic granules were also stained with lead sulfide (Wachstein and Pisano, 1950). Osmium fixed impression smears were placed 1 minute in a 10 per cent lead nitrate solution, rinsed well, placed 1 minute in a 0.1 per cent aqueous solution of ammonium sulfide, rinsed, dipped briefly in saturated aqueous safranin, rinsed, blotted, and mounted.

Most rods grown on blood agar had 2 prominent polar granules (figures 16, 18). When non-metachromatic rods with discrete strongly basophilic intracellular areas (figure 12) were placed on M. and E. agar, the cells became metachromatic, the majority of the granules first appearing on either side of these areas (figure 13); in time these granules had a much more polar location (figure 14). Eventually such cells became non-metachromatic again and possessed a generalized basophilia (figure 15). Cultures of coccoid forms were frequently nonmetachromatic, particularly when grown in the absence of blood. Metachromatically stained coccoid forms usually averaged 1-2 granules per cell (figure 17). Metachromatic stains ordinarily failed to reveal numerous small granules demonstrable in rods stained either with tetrazoles or Sudan black B.

In cells treated with crystal violet-citric acid, only the metachromatic granules were stained; but in cells stained with the nonacidified gram stain, basophilic areas as well as metachromatic granules were colored. Nonmetachromatic cells with localized basophilia are shown in figure 22, cells with generalized basophilia and subpolar metachromatic granules in figure 23, and cells with generalized basophilia and polar metachromatic granules in figure 24. Coccoid cells were gram positive (figure 25).

When rod forms were stained with lead sulfide and mounted in H.S.R., the metachromatic granules appeared dark brown or black, whereas they appeared red when these cells were mounted

in Farrant's medium (figure 26). In the coccoid cells these granules were far more infrequent than metachromatically stained granules. Walls and septa of the majority of coccoid forms stained brown.

Staining for ribonucleic acid. Neisser's acidified methylene blue was used although nonacidified methylene blue was initially used for comparative purposes. The series of metachromatic dyes used also stained ribonucleic acid; following treatment with ribonuclease, such staining was absent. The cytoplasm of the coccoid forms was stained blue in varying intensities. The walls and septa were sometimes colorless and sometimes stained a metachromatic scarlet (figure 17).

Nuclear staining. Both the Feulgen stain, utilizing a Schiff reagent prepared according to Winterscheid and Mudd (1953), and DeLamater's stain (1951), employing azure A and thionyl chloride, were used.

Feulgen and DeLamater stains presented a similar nuclear picture (figures 27-31). Rods and coccoid forms usually had 1-2 nuclei per cell. Underhydrolyzed coccoid forms (figure 30) showed stained cell walls and septa rather than nuclei (figure 31).

Sudan black B staining for lipid. The method of Burdon (1946) for free lipid and that of Davis *et al.* (1953) for bound lipid were used.

In nonmetachromatic rods numerous relatively small granules stained (figure 32), while in metachromatic rods the larger metachromatic granules stained as well and were easily recognized because of their limited number, location, and relatively large size (figures 34 and 35). Coccoid forms (figure 33) from different cultures varied in staining properties. In some the septa and cell walls were stained a definite brown, while in others these structures were almost imperceptible. Stained granules in coccoid cells were either absent, faintly colored, or intensely black; in the last case they seemed to correspond to metachromatic granules.

Baker's (1946) stain for phospholipids. Neither rods nor coccoid forms showed the dark blue staining regarded by Baker as specific for phospholipid. Rods showed a dark brown refractive staining of the cell surface, of large polar bodies, and sometimes of tiny peripheral bodies (figure 36). Coccoid forms were similarly stained on their outer surfaces and on the surfaces of intracellular

bodies. Attempts to demonstrate phospholipids by the procedure described by Menschik (1953) were negative.

Hotchkiss' (1948) stain for polysaccharide. Non-metachromatic rods from blood broth cultures, which showed localized basophilia, were surface stained an intense dark red except where the basophilia was intense, this location remaining unstained (figure 37). When these cells were placed in certain other environments, e.g., on M. and E. agar, the staining changed to a light even diffuse red or pink (lower cells of figure 38). The temporary appearance of metachromatic granules was not accompanied by any increase in polysaccharide staining. Indeed, strongly metachromatic rods usually stained poorly for polysaccharide when compared to the nonmetachromatic cells and only occasionally showed fairly intense staining at the poles or sites of cell division, where undoubtedly there were metachromatic granules (figure 39). Metachromatic and nonmetachromatic coccoid forms stained uniformly and were faint pink.

Protein stain of Mazia, Brewer, and Alfert (1953). Rods with localized basophilia and with generalized basophilia stained for protein in the basophilic areas (figures 40 and 41). The basophilic areas of coccoid forms also stained while their cell walls and septa were unaffected (figure 42). Metachromatic granules remained colorless (figure 43).

Harman's (1950) stain. This stain was used on nonmetachromatic cells with localized basophilia. It had no visible effect on the numerous mitochondria throughout the cell; the strongly basophilic areas were stained rather poorly and other areas even less (figure 44). Staining was the same after preliminary treatment with ribonuclease.

Brachet's (1953) methyl green/pyronin stain. Cells were stained with methyl green/pyronin according to Brachet's procedure. Destaining for 1 minute in ethanol removed excess pyronin which obscured staining by methyl green.

Pyronin stained the cytoplasm, metachromatic granules, and possibly the nuclei. Subsequent extraction of pyronin with ethanol was necessary for revealing any green nuclear staining in non-metachromatic rods (figure 45) or in metachromatic rods with pyronin stained metachromatic granules (figure 46). Methyl green staining was irregular in intensity and not evident in every

rod. Nonmetachromatic coccoid forms destained with ethanol had red cell walls and 1 to 2 faded green granules suggestive of nuclei.

Tronnier's (1953b) azure-eosin stain for nuclei. This stain was used as indicated. Some preparations were partially destained for 1 minute in a 5 per cent citric acid solution.

The blue staining component of the stain apparently affected ribonucleic acid since areas that stained blue did not occur after preliminary treatment of the cells with ribonuclease or subsequent treatment with citric acid. The red component stained metachromatic granules, other intracellular bodies, and areas revealed after removing the blue component with citric acid. In figure 47 nonmetachromatic rods have localized areas containing concentrations of both components. In the stained metachromatic rods of figure 48a the blue dye bound to ribonucleic acid masks the other red stained structures clearly seen after destaining with citric acid (figure 48b). This strain was not considered to be reliable for showing nuclei since the red stained material within the cells was often diffuse rather than localized.

Observations on staining combinations. Non-metachromatic mitochondria possessed redox activity and lipid as shown by first staining non-metachromatic cells with NT and then with Sudan black B. Such mitochondria could also be distinguished from metachromatic granules in metachromatic cells (figures 50 and 54). That metachromatic granules also possess redox activity is suggested in figure 49, showing a cell with a formazan granule adjacent to a polar metachromatic granule, and by examining numerous metachromatic cells first stained with tetrazoles for redox activity and then stained for metachromatic granules. Such associated activity was difficult to prove since its apparent existence, seen in some of the granules, could have been due to an inability to resolve adjacent structures.

The mitochondria were distinct from the nuclei as shown in figures 51 and 52, as were the metachromatic granules (figure 14). Furthermore, the mitochondria could also easily be distinguished from cell walls and septa (figure 7) as could metachromatic granules (figures 17a, 55, 56). Mitochondria could be differentiated from localized concentrations of basophilic ribonucleic acid

Figure 34. Lipid staining of prominent metachromatic granules (somewhat central in location) and faint nonmetachromatic granules in rods originally in blood broth culture, then placed on M. and E. agar for 1 hour. Stained with Sudan black B-citric acid; a, b, and c are from same preparation.

Figure 35. Lipid staining of prominent polar metachromatic granules and faint nonmetachromatic granules in metachromatic rods from blood agar culture. Stained with Sudan black B-citric acid.

Figure 36. Undetermined stained material in nonmetachromatic rods from blood agar culture shown using Baker's stain for phospholipid.

(figure 53) although they frequently bordered such concentrations.

Metachromatic granules were red and non-metachromatic mitochondria were black (figure 54) when metachromatic cells were first stained with azure A-citric acid and then with Sudan black B-citric acid. However, the metachromatic granules were black using the reverse order of staining or using Sudan black B alone. In order to determine whether the sudanophilic material of

the granules was lipid or not, metachromatic cells were extracted 19 hours with either 95 per cent ethanol, glacial acetic acid (both at room temperature), or pyridine (at 60 C). When these extracted cells were stained with Sudan black B, they were completely colorless except for small reddish granules located where the metachromatic granules are usually seen. Apparently both lipid and metachromatic material stains in these granules.

Figure 37. Polysaccharide staining of nonmetachromatic rods from blood broth culture. Stained according to Hotchkiss; a and b are from similar preparations.

Figure 38. Faint, diffuse, polysaccharide staining in majority of rods originally grown in blood broth, then placed on M. and E. agar for 4 hours. Stained according to Hotchkiss.

Figure 39. Polysaccharide staining of metachromatic rods with polar metachromatic granules from blood agar culture. Stained according to Hotchkiss.

Figure 40. Strong protein staining of localized intracellular areas in nonmetachromatic rods from blood broth culture. Stained with mercuric bromphenol blue as described.

Figure 41. Protein staining extended throughout rods originally grown in blood broth, then placed on M. and E. agar for 3 hours. Stained as in figure 40.

Figure 42. Protein staining of nonmetachromatic coccoid forms from M. and E. agar culture. Stained as in figure 40.

Figure 43. Protein staining of metachromatic rods from blood agar culture. Stained as in figure 40. MG = unstained metachromatic granule; a and b are from same preparation.

Figure 44. Faint Harman staining of nonmetachromatic rods from blood broth culture. Mitochondria unstained. Central, poorly stained intracellular areas correspond to similar areas stained for protein in figure 40.

Figure 45. Central, methyl green stained nuclei in nonmetachromatic rods from blood broth culture. Stained with methyl green/pyronin according to Brachet and partially destained in ethanol.

Figure 46. Pyronin stained metachromatic granules (MG) and methyl green stained nuclei (N) in a metachromatic rod from a blood agar culture. Partially destained in ethanol.

Figure 47. Central, localized blue and red staining in nonmetachromatic rods from blood broth stained with Tronnier's azure-eosin solution.

Figure 48a. Generalized basic staining in metachromatic rods from blood agar stained with Tronnier's azure-eosin solution.

Figure 48b. Same as in figure 48a only stained cells subsequently partially destained in citric acid.

Figure 49. Formazan granule adjacent to polar metachromatic granule in metachromatic rod from blood agar culture. Cell first stained with 0.1 per cent triphenyltetrazolium chloride, then with Neisser's solution. TPT = triphenyltetrazolium chloride stained granule; MG = metachromatic granule.

Figure 50. Formazan granules (NT) resulting from neotetrazolium chloride reduction and metachromatic granules (MG) stained with malachite green-citric acid in a metachromatic rod from blood agar culture.

Figure 51. Formazan granules (NT) resulting from neotetrazolium chloride reduction and nuclei (N) stained according to DeLamater in a nonmetachromatic rod from a blood broth culture.

Figure 52. Formazan granules (BT) resulting from blue tetrazolium chloride reduction and nucleus (N) stained using the Feulgen reaction in a nonmetachromatic cell from a blood broth culture.

Figure 53. Formazan granules resulting from blue tetrazolium chloride reduction, and ribonucleic acid containing intracellular bodies (RNA) stained with Neisser's solution in nonmetachromatic rods from blood broth culture.

Figure 54. Metachromatic granules (MG) stained red with azure A-citric acid and nonmetachromatic granules stained black with Sudan black B-citric acid in a metachromatic rod from a blood agar culture.

Figure 55. A metachromatic granule (MG) stained with crystal violet-citric acid and cell wall and septa (S) stained with tannic acid-crystal violet in a metachromatic rod from a blood agar culture.

Figure 56. A metachromatic granule (MG) stained with azure A-citric acid and a septum (S) and faint cell wall stained according to Robinow's method.

Figure 57. An electron micrograph showing localized dense areas in nonmetachromatic rods from a blood broth culture.

Figure 58. Similar to figure 57, only a few small dark peripheral granules are also visible. These are probably mitochondria.

Figure 59. An electron micrograph of nonmetachromatic rods from an anaerobically grown *M. and E.* agar culture originally inoculated with nonmetachromatic rods.

Figures 60 and 61. Electron micrographs of metachromatic rods grown on blood agar.

Figure 62. An electron micrograph of metachromatic coccoid forms grown on blood agar.

Electron microscopic observations. The strongly basophilic areas of certain nonmetachromatic rods were shown to have a high content of nucleic acid and protein, which were very opaque to electrons (figure 57). Small dark peripheral granules were observed in some of these cells (figure 58) which were probably mitochondria. Nonmetachromatic rods with diffuse basophilia showed a corresponding opacity (figure 59). In metachromatic rods, the metachromatic granules were extremely dense, the cytoplasmic material

was less so, and the areas of septation were relatively transparent (figures 60 and 61). The high density of metachromatic and nonmetachromatic coccoid forms made differentiation of internal structure impossible (figure 62).

Physical, chemical, and enzymatic treatment. Metachromatic granules were subjected to hot distilled water at 80 C for 5 minutes or at 100 C for 1 minute, to 1 N HCl at 60 C for 1 minute, to ribonuclease at 37 C at a concentration of 4 mg per 100 ml of distilled water (pH 6.5) for as long

as 12 hours, and to freshly prepared desoxyribonuclease at room temperature, using the same concentration with an additional trace of magnesium sulfate, for 1 hour (Murray and Whitfield, 1953). Results were checked with Neisser's stain.

Metachromatic material was extracted by both hot water treatments. The originally polar metachromatic material of metachromatic rods from blood agar slants, when hydrolyzed for a minute, became more centrally located (compare figures 18 and 19). However, the centrally located metachromatic granules, produced by placing broth grown nonmetachromatic cells on agar, were completely absent after a minute of treatment. Metachromasia was markedly reduced by 4 hours of ribonuclease treatment and was completely removed after 12 hours of treatment, indicating that ribonucleic acid is a component of the granules. Metachromatic granules of cells treated with desoxyribonuclease remained unchanged.

Metachromasia under aerobic and anaerobic conditions. When nonmetachromatic rods were inoculated onto blood agar, the resulting cells were almost all nonmetachromatic when grown anaerobically but became strongly metachromatic when grown aerobically. Similarly, when nonmetachromatic coccoid forms were inoculated onto blood agar and incubated, the production of metachromatic granules in the resulting culture was much more limited under anaerobic conditions than under aerobic conditions. However, strongly metachromatic rods were obtained from an anaerobic blood agar culture initially inoculated with strongly metachromatic rods. It was further shown that rods could multiply extensively in blood broth (on the bottom of a petri dish) in the presence of oxygen without becoming metachromatic.

Nutritional factors affecting metachromatic granules. When nonmetachromatic rods with localized basophilia were transferred from blood broth culture onto M. and E. agar, they became strongly metachromatic after approximately 2 hours and became nonmetachromatic again after approximately 4 hours. During this process the cells grew out on agar; fixed cells, of course, showed no changes. Unfixed, freshly prepared, moist coverslip impression smears from such cultures placed in distilled water remained nonmetachromatic, whereas the cells in preparations placed in saline or phosphate buffer became temporarily metachromatic, as had the cells

placed on M. and E. agar. In a similar fashion, strongly metachromatic cells from blood agar cultures slowly lost their metachromasia in physiological saline but remained unchanged in distilled water. Thus apparently only metabolizing cells undergo changes in metachromasy.

DISCUSSION

Conditions favoring the production and stability of coccoid forms have been described by Parish (1927) and Morton (1940a,b). The present demonstration by staining of the infrequency of cross-septa agrees with observations of Hewitt (1951) who viewed living *mitis* strains by phase-contrast microscopy. However, some strains of this organism have numerous prominent cross-septa (Bisset, 1949; Hewitt, 1951; Bisset and Hale, 1953).

We have demonstrated granules with reductive activity in the diphtheria organism using tetrazolium chlorides. Hewitt (1951) showed that the reduction of either tellurite or selenite resulted in the formation of localized deposits in diphtheria bacilli. Wachstein (1949) stained *C. diphtheriae* with both potassium tellurite and NT and compared the two types of resulting inclusions. He found that "the ones following treatment with 'neotetrazolium' were larger and coarser, but their general shape and distribution was alike with both preparations."

We interpreted Janus green B staining in the diphtheria bacillus as indicative of enzymatically active mitochondria only when colored granules were obtained within a colorless cytoplasm and they underwent the expected sequence of color changes.

Our failure to obtain a positive Nadi reaction is not surprising since Pappenheimer and Hendee (1947) showed that phenylenediamine is slowly oxidized by cell-free extracts of the diphtheria bacillus but not by intact cells, presumably because they are impermeable to such a substrate. They also showed that the oxidative system involved was present in trace amounts.

While Bisset (1949) contended that the metachromatic granules of *C. diphtheriae* are "the shrunken contents of the enlarged terminal cells", Schmager and Kludas (1950), using phase-contrast microscopy, observed metachromatic granules in living diphtheria bacilli. In addition, the present work has shown that metachromatic granules appeared in potentially metachromatic

cells only when they were viable. Also, the particular location, number, and size of these granules were primarily the result of environmental influences on living cells and were not the result of preparatory drying.

The metachromatic granules of the diphtheria bacillus contain metaphosphate and calcium ions (König and Winkler, 1948; Ebel, 1949) and require phosphate and a metabolizable substrate for their formation (Wiame, 1947; the present authors). Mudd (1954) suggests that the storage of metaphosphate in many microorganisms may represent "an energy accumulator mechanism".

From the present work it appears likely that the metachromatic granules of the diphtheria bacillus have associated redox activity. Krieg (1954a) believes this to be so after studying the effect of tetrazolium chlorides and metachromatic stains on this organism.

Lewis (1941) stated that metachromatic granules did not stain with fat dyes, but we found them to be sudanophilic. Metachromatic granules probably possess ribonucleic acid since we as well as Minck and Minck (1949) observed that these granules lose their metachromatic properties following ribonuclease treatment, due presumably to a loss in closely associated metaphosphate.

Wherry (1917) and Winkler (1953) reported that diphtheria bacilli produce metachromatic granules only under aerobic conditions. It is possible that the particular inoculum used by Winkler consisted of nonmetachromatic cells which we have shown characteristically to produce nonmetachromatic offspring under anaerobic conditions. Furthermore, Megrail (1922) showed that the particular medium used can affect the anaerobic production of granules.

The use of regular or acidified methylene blue as a stain for ribonucleic acid in this organism seemed justified in view of the findings of Brachet (1953) who used the closely related toluidine blue in conjunction with ribonuclease, and of Michaelis (1947) who attributed nonmetachromatic basic staining in cells to their nucleic acid content.

A positive Feulgen reaction on diphtheria bacilli was obtained by Preuner and v. Prittwitz und Gaffron (1951) and in this study. Diphtherial nuclei have also been demonstrated by Giemsa staining (Parvis, 1949; Bisset, 1949; Tronnier, 1953a) and DeLamater's nuclear stain. Nuclei of coccoid forms have been stained by Bisset (1949, figure 6). A distinction between metachromatic

granules and nuclei was made by Tronnier (1953a) and Krieg (1954b).

The presence of stainable polysaccharide in the cell wall of the diphtheria bacillus was to be expected since Holdsworth (1952) showed that most of the carbohydrate of the organism is in a protein-oligosaccharide complex of the cell wall. The metachromatic granules of *C. diphtheriae* have been strongly stained for polysaccharide by Dr. C. H. Winkler (McManus, 1948) using the Hotchkiss procedure.

The stain for protein appears to be specific as discussed by Mazia *et al.* (1953). It was to be expected that this stain would not affect the metachromatic granules since Grimme (1902) and Meyer (1912) found that trypsin and pepsin did not digest such granules.

Our failure to color mitochondria of the diphtheria bacillus with Harman's stain is in accord with the findings of Mudd *et al.* (1951a,b) who tried the stain on several kinds of bacteria of which only mycobacterial cells showed colored granules.

The difficulties involved in the use of a methyl green-pyronin stain for demonstrating bacterial nuclei and cytoplasm were indicated by Bringmann (1951) who showed that both staining components color metachromatic granules, and by Tronnier (1953a) who believes that the final staining results depend on the basic properties of the dyes and basophilia of the cells.

SUMMARY

Study of the rod and coccoid variations of a *mitis* strain of *Corynebacterium diphtheriae* with the aid of redox indicators, differential stains, and electron microscopy yielded the following: Mitochondria showed redox activity with tetrazoles and Janus green B but not with Nadi reagent, were sudanophilic and nonbasophilic, and could be stained differentially from nuclei, cell walls, and septa. Metachromatic granules were sudanophilic, were removable with ribonuclease but not with desoxyribonuclease, were not invariably associated with stainable polysaccharide, and may have contained redox activity. These granules were also distinct from nuclei, cell walls, and septa. When nonmetachromatic cells were used as inocula onto solid medium, the resulting cells were usually nonmetachromatic when grown anaerobically and metachromatic under aerobic growth conditions. However, when metachro-

matic cells were used as inocula onto similar medium, the resulting cells were metachromatic under either anaerobic or aerobic growth conditions. Cells at times stained strongly for polysaccharide, and had concentrations of ribonucleic acid, gram positive material, protein, and electron scattering material which could be localized or generalized in distribution depending upon environmental conditions. The coccoid forms were electron opaque.

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