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Vital Staining of *Mycoplasma* and L-Forms with Chlorazol Black E

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Vital staining of *Mycoplasma* colonies was attempted because other dye visualization techniques kill the organisms and preclude reisolation for further studies. The lipophilic amphoteric dye Chlorazol Black E (CBE) was the most successful of 14 vital dyes tested on *Mycoplasma hominis*, *M. pharyngis*, *M. fermentans*, *M. arthritidis*, *M. salivarium*, *M. pneumoniae*, and L-forms of *Staphylococcus aureus* when used in 1:1,000 (w/v) saline dilution as the sterile suspension medium for inoculation of Hayflick's medium under both aerobic and microaerophilic (Fortner method) conditions. Colonies of all species stain homogeneously in the periphery and center portion, the latter being more refractive under positive phase contrast. All stained colonies were successfully subcultured. The most striking and promising result of the use of CBE as a tool for physiological study of *Mycoplasma* was a very significant increase in diameter of all colonies except those of *M. pneumoniae* grown with CBE: 1.5 × for *M. hominis* and 5 × for L-form *S. aureus*. This size increase in *M. hominis* is proportional to the concentration down to a 1:50,000 dilution only under microaerophilic conditions. Whether this increase in colony size is due to an increased number of cells, to larger cells, or to the adsorption of CBE on the lipid membrane is unknown at present.

The classical cytological methods of vital staining (7, 8, 9) of animal and higher plant cells have seen very limited use in microbiology (1, 3, 12). The principle of revealing living cells in situ and the relatively simple methods of vital staining appeared to be able to fill an important information gap in *Mycoplasma* cytology between killing and fixation and staining for light microscopy (4) and the elaborate procedures of electron microscopy (15). The two latter processes induce some morphological distortions and preclude subculture and direct study of the organisms. It was further hoped that differential stains might serve as diagnostic and taxonomic aids in identifying species of *Mycoplasma* on a physiological basis.

MATERIALS AND METHODS

Originally 12 vital dyes as listed by Gurr (7), neutral red, Chlorazol Black E (CBE), Brilliant Vital Red, Brilliant Cresyl Blue, Janus Green B, Safranine O, Acridine Orange, Nile Blue A, Trypan Blue, Eosin Y Extra, Benzo Purpurin 4B, and methylene blue, were tested for their ability to support the growth of visible colonies of *Mycoplasma pneumoniae* Bru strain, *M. pharyngis* Patt strain, and *M. arthritidis* in the following manner. Petri dishes containing 20 ml of solidified Hayflick's medium (10) were flooded with 2 ml of sterile 1:1000 (w/v) dilutions of each tester dye (Harleco) and allowed to dry overnight. Duplicate plates were then inoculated by the push-block method, one plate grown aerobically at 37 C and another under microaerophilic conditions with *Serratia marcescens* (Fortner Method; 5) also at 37 C. Observations were made at 2-day intervals for 10 days, and viability was determined by subculturing any visible growth after 4 and 10 days.

These preliminary experiments indicated that CBE merited further study, and CBE was then tested by a somewhat different method on the three species previously mentioned as well as on *M. hominis*, *M. fermentans*, *M. salivarium*, and on L-form *Staphylococcus aureus*. CBE (7, 8) is an amphoteric tris-azo dye, with a molecular weight of 782 and an empiric formula $C_{34}H_{25}N_9O_7S_2Na_2$ (*p*H 9.05), also known as Pontamine Black E, Erie Black, Direct Black, and Renol Black, which has been used as a fat stain as well as a vital stain (3).

Stock cultures were subcultured under microaerophilic conditions, except for *M. pneumoniae* and L-form *S. aureus*, on Hayflick's medium for 3 days at 37 C, blocks of which were suspended in 3 ml of sterile saline and 3 ml of CBE in a 1:1000 (w/v) sterile solution and were homogenized. The resulting suspension was then streaked on half of a divided petri dish (Bi-plate, Falcon Plastics) with a 2-mm layer of agar, and a similar suspension in saline with-

out CBE was streaked on the other half as a control. L-forms were grown on L-form medium (13). Incubation either aerobically or microaerophilically was at 37 C. After 4 days, thin blocks 1-cm square were mounted on slides and covered with cover slips. These were observed on a Zeiss microscope with medium phase-contrast objectives, as well as with bright light. Photographs were taken on Polaroid P-52 film. For 30 random colonies, maximal diameters were measured for each experimental condition on contiguous agar blocks stained with Dienes' stain (13). Material from all plates stained with CBE was subcultured, and viability was ascertained after 4 days.

Additional series of experiments were conducted on 3-day-old colonies of *M. hominis*, *M. arthritidis*, *M. salivarium*, and *M. pharyngis* grown microaerophilically on divided petri dishes to study the effects of various killing procedures on membrane permeability of dead cells to CBE. One series was killed by flooding the petri dish with 70% ethyl alcohol for 1 hr, another by exposure to 50 C in a water bath for 1 hr, and three by exposure to 40% Formalin vapor for 8, 24, and 48 hr as described by Dienes (4). After the various killing times, one side of the plates was flooded with 1:1000 CBE solution. Observations within 3 hr were made on stained and unstained colonies.

RESULTS

Preliminary experiments showed that Brilliant Cresyl Blue and Acridine Orange completely inhibited growth at the dilution used, whereas the other 10 dyes permitted unrestricted growth; only Chlorazol Black E was actually taken up by the colonies and stained the colonies.

Measurement of the colonies showed that the consistent size increase due to CBE at the 1:1,000 dilutions (except in *M. pneumoniae*) ranged from 1.1 times in aerobically grown *M. pharyngis* to 4.6 times in L-form *S. aureus* regardless of the growth conditions (Table 1). Similar relative increases in colony size were observed at CBE concentrations as low as 1:50,000 (w/v) when the organisms were grown microaerophilically with *Serratia*. When organisms were grown without *Serratia*, there was virtually no size difference at the 1:5,000 dilution. The increase in size was consistent for all colonies of a given species except in the case of *M. arthritidis* in which both large and small colonies were found side by side (Fig. 11 and 12). There was no overlapping or obvious crowding and very little juxtaposition of colonies. In *M. hominis* we consistently observed colony-associated artifacts of the same general colony size (Fig. 10) which did not stain with Dienes' stain. These were not measured in size evaluations even though they might have been colonies of vacuolized bodies (11) or, less likely, "pseudocolonies" (2).

Stained colonies took up the dye uniformly, and this could be visibly ascertained by focusing up and down on the deeper center portion and on the surface periphery but was not always demonstrable in one focal plane with phase contrast (Fig. 9, 13, 14). Observation with phase contrast greatly enhanced visibility and colony morphology even in unstained preparations (Fig. 1, 2).

The various killing procedures did not yield any additional information on the mode of action of CBE but were nevertheless of comparative interest. Heating at 50 C for 1 hr tended to make individual organisms separate and distinctly visible (Fig. 19) with loss of phase-contrast refractivity of the entire colony. Ethyl alcohol preserved colony morphology with the same loss of phase-contrast refractivity as the heat treatment (Fig. 22). Colonies killed with Formalin vapor for 20 to 48 hr usually had a broad halo which stained clearly with CBE (Fig. 20) and appeared to be acellular. There was no loss of refractivity in Formalin-killed cells.

DISCUSSION

Vital staining of *Mycoplasma* colonies with CBE indicates great promise as a tool for direct visual studies on living colonies. We can only

TABLE 1. *Average and standard deviation of 30 randomly counted colony diameters of Mycoplasma species and L-forms of S. aureus observed after 3 days*

Organism	Colony diameter			
	Grown with CBE		Grown without CBE	
	Aerobically	Fortner plate	Aerobically	Fortner plate
	μm	μm	μm	μm
M. hominis	98 ± 10.7	102 ± 12.9	73 ± 15.1	66 ± 12.7
M. arthritidis	38 ± 5.4	31 ± 6.6	31 ± 5.6	26 ± 5.5
M. pharyngis	26 ± 4.4	27 ± 3.3	24 ± 4.3	22 ± 3.3
M. fermentans	49 ± 12.3	34 ± 5.3	27 ± 7.3	26 ± 7.2
M. salivarium	79 ± 20.1	47 ± 11.8	38 ± 10.5	37 ± 8.1
M. pneumoniae	21 ± 5.2		36 ± 6.4	
L-form of *S. aureus*	3,292 ± 61.2	2,240 ± 54	715 ± 30.4	583 ± 13.8

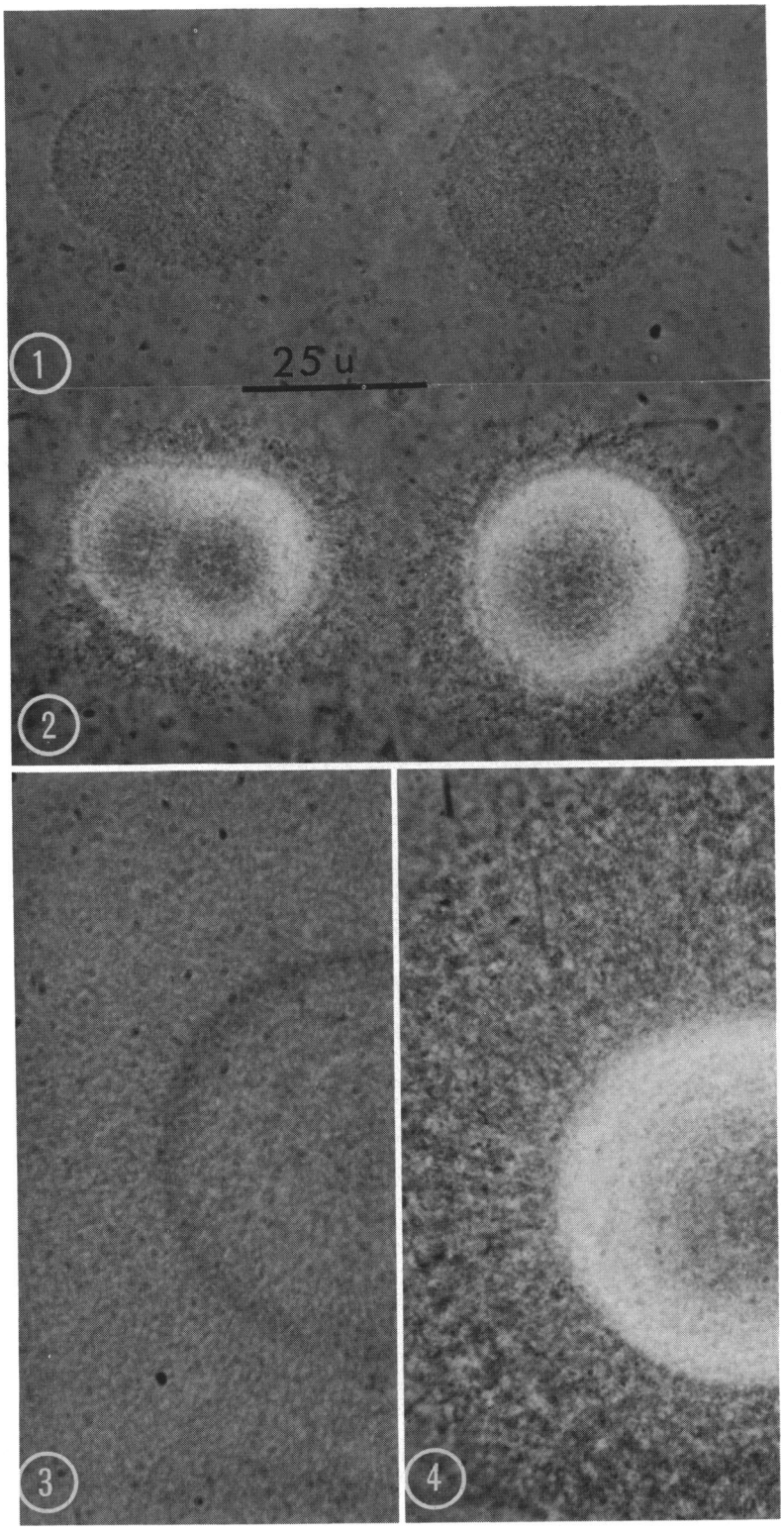

FIG. 1. *M. salivarium colonies grown without Serratia and without CBE, bright light.* × *1,000.*
FIG. 2. *Same as Fig. 1, phase contrast. Colonies have larger visible diameter.* × *1,000.*
FIG. 3. *M. salivarium colony grown with Serratia and CBE, bright light.* × *1,000.*
FIG. 4. *Same as Fig. 3, phase contrast.* × *1,000.*

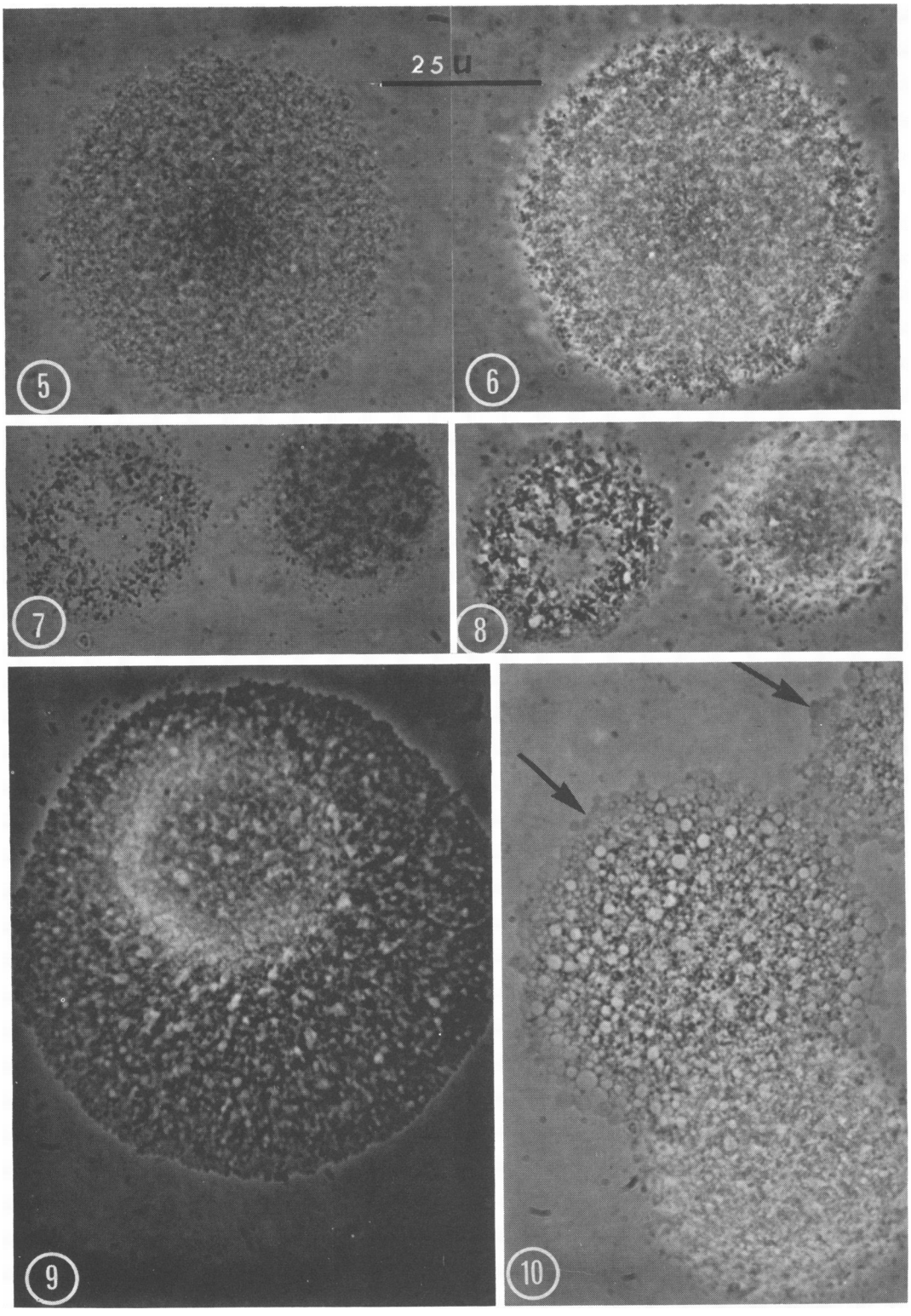

FIG. 5. *Mycoplasma hominis grown with Serratia and CBE, bright light.* × *1,000.*

FIG. 6. *Same as Fig. 5, phase contrast.* × *1,000.*

FIG. 7. *M. hominis grown with Serratia, no CBE, bright light.* × *1,000. Colony on the left is lacking a center portion.*

FIG. 8. *Same as Fig. 7, phase contrast.* × *1,000.*

FIG. 9. *M. hominis grown without Serratia but with CBE. Notice smooth colony margin, phase contrast.* × *1,000.*

FIG. 10. *M. hominis grown without Serratia and without CBE. Arrows indicate two colony-associated artifacts which did not stain with Dienes' stain, phase contrast.* × *1,000.*

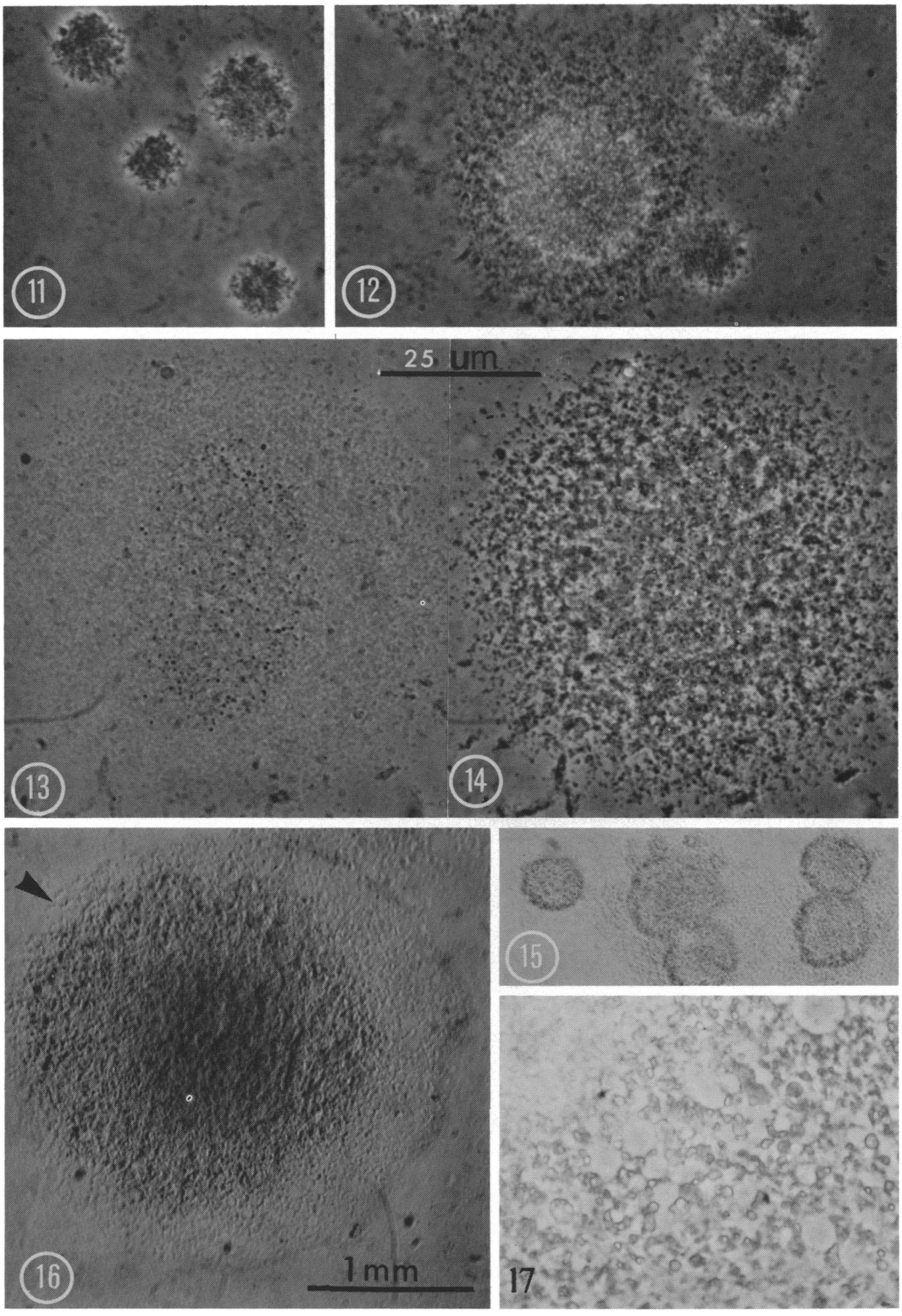

FIG. 11. *Mycoplasma arthritidis grown without Serratia and without CBE, phase contrast.* × *1,000.*

FIG. 12. *M. arthritidis grown without Serratia but with CBE. Large and small colonies, phase contrast.* × *1,000.*

FIG. 13. *M. fermentans grown with Serratia and with CBE. Only central portion of colony is visible, bright light.* × *1,000.*

FIG. 14. *Same as Fig. 13. Phase contrast makes entire colony visible.* × *1,000.*

FIG. 15. *Colonies of L-forms of S. aureus grown without CBE, bright light.* × *140.*

FIG. 16. *L-forms of S. aureus colony grown with Serratia and with CBE. Note crater-like edges upper left of colony (arrow), bright light.* × *140.*

FIG. 17. *Edge of colony in Fig. 15, phase contrast.* × *1,000.*

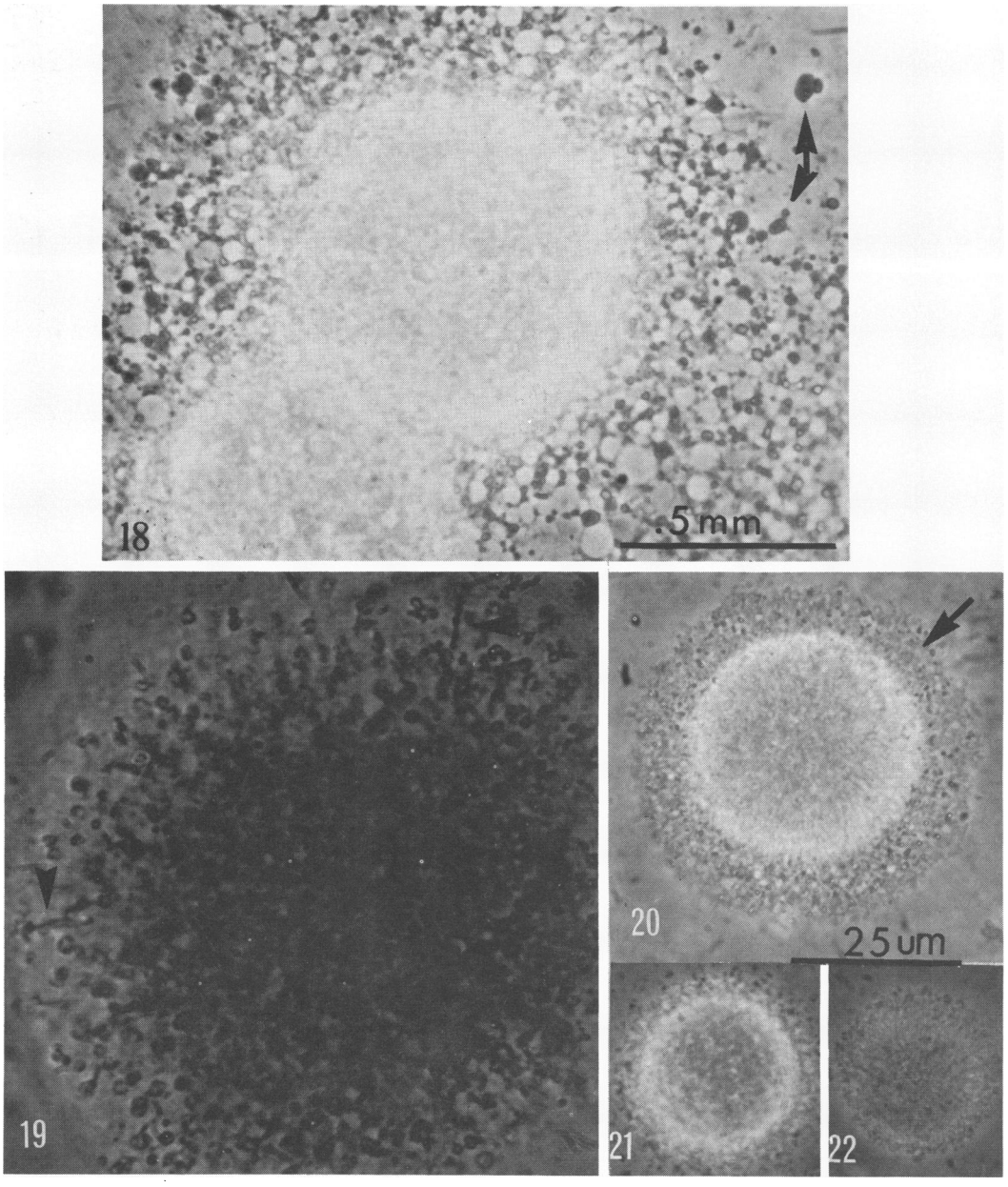

FIG. 18. *Edge of L-form S. aureus small colony grown with Serratia and with CBE similar to Fig. 16, phase contrast. × 300. Arrows indicate clearly stained dividing cells and cells in chains which were approximately twice as large as those in Fig. 17.*

FIG. 19. *M. hominis. Colony was killed by heating at 50 C for 1 hr and was stained with CBE, phase contrast. × 1,400. Arrows indicate string and beadlike forms.*

FIG. 20. *M. pharyngis killed with Formalin vapor for 48 hr and stained with CBE, phase contrast. × 1,000. Arrow indicates stained halo surrounding highly refractive colony.*

FIG. 21. *M. pharyngis grown with Serratia and CBE for comparison with Fig. 20, phase contrast. × 1,000.*

FIG. 22. *M. pharyngis killed with 70% ethyl alcohol for 1 hr and stained with CBE, phase contrast. × 1,000. Loss of phase refractivity and no stainable halo comparable to Fig. 20.*

speculate on the mode of action of CBE, particularly as it affects colony size, an effect which might be due to an actual increased number of cells, to larger cells, or to the adsorption of CBE on the lipoprotein limiting membrane. The latter appears to be likeliest because CBE, like the known lipophilic dyes Sudan III, Sudan IV (3), and Oil Red O (Del Giudice and Carski, Bacteriol. Proc., p. 67, 1968), is also an azo dye capable of coloring by a strictly physical process since it is fat-soluble. The lack of effect on *M. pneumoniae* colonies may be due to the usual lack of a conspicuous periphery which makes the dye less available. Nevertheless, many other factors besides lipid solubility enter into dye permeability of living membranes, including dye particle size and the presence of electrolytes in the surrounding medium. Gordon and Chambers (6) did show that vital staining of membrane-bound animal cells was due to a simple physical diffusion of the dyes, unrelated to aerobic or anaerobic growth conditions. Only by electron microscopy will we be able to determine if there is a larger lipid membrane which in turn would increase the amount of usable substrate transported across the membrane (10) and thus perhaps yield a larger individual cell. CBE might also be providing physical or structural support to these osmotically fragile cells and thus might be an aid in primary isolations from mixed samples.

It must be remembered that lipid composition of the membrane depends on the composition of the growth medium (15). Since all were grown on the same medium, differences in the nature and amount of lipid in the membrane of different species may account for differences in the extent of colony growth stimulation (14).

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