THE CYTOLOGICAL BASIS FOR THE ROLE OF THE PRIMARY DYE IN THE GRAM STAIN[1]

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Received for publication March 15, 1954

Knowledge of the basis for the specificity of the primary dye is required for an understanding of the gram reaction. In achieving this understanding certain known facts must be taken into account. The primary basic dye must be capable of forming a colored, water insoluble lake upon mixing with a mordant solution containing iodine (Burke, 1922). Yet not all basic dyes capable of such reaction serve satisfactorily as primary dyes in the gram stain. Safranin, methylene blue, and rhodamine B are cited (Bartholomew and Mittwer, 1950) as examples of unsatisfactory primary dyes which do form lakes with iodine. Therefore, some additional and unrecognized property must account for the specificity of the useful primary dyes.

The basic triphenylmethane dyes in general and crystal violet in particular are superior to all other dyes as the primary dye reagent in the gram stain (Bartholomew and Mittwer, 1950). Also it is probable that the cell wall participates in the gram reaction (Benians, 1920; Lamanna and Mallette, 1950; Lamanna, 1951). Therefore, if the triphenylmethane dyes, unlike others, can be shown to stain the cell walls of gram positive organisms, it might be possible to impute the specificity of the primary dyes in the gram reaction to this special staining property. This communication presents evidence suggesting such a correlation.

MATERIALS AND METHODS

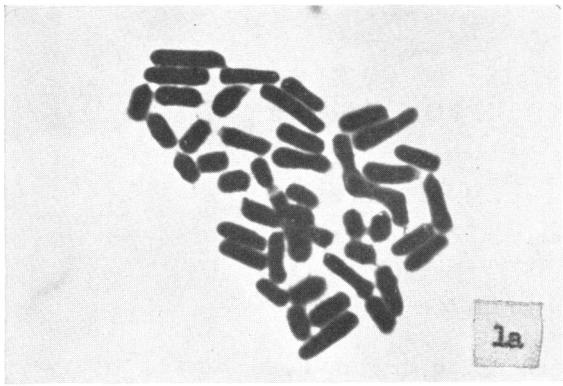
The organisms studied were all laboratory stock cultures and included the yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* and the bacteria *Bacillus cereus*, *Micrococcus pyogenes* var. *aureus*, *Sarcina lutea*, *Corynebacterium pseudodiphthiriticum*, *Escherichia coli*, and *Pseudomonas fluorescens*. The yeasts were grown on carrot slices and the bacteria on infusion agar. Capsular material was absent with the strains and conditions of growth employed.

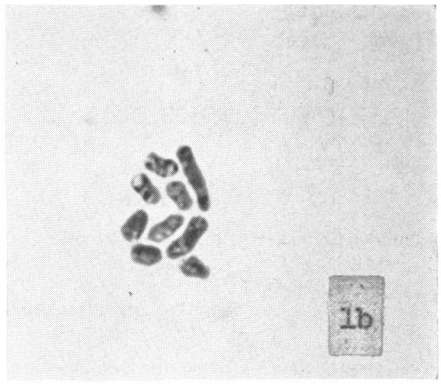
Burke's (1922) gram stain and Dyar's (1947) cell wall stain were chosen on the basis of personal experience with the merit and specificity of these procedures. Also Robinow and Murray (1953) have recently testified to the specificity of the Dyar method of staining the cell wall. Five-tenths per cent aqueous solutions of crystal violet and safranin were used in simple staining with the pH controlled as indicated for the individual experiments. The crystal violet was a certified Difco sample (ref. no. 331466; dye content, 96 per cent).

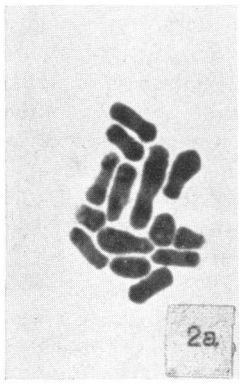
Smears were prepared from thick suspensions of organisms in distilled water and fixed by heat in the ordinary manner. By slowly air drying the smears, preparations were obtained in which most of the organisms occurred in clusters. Since all the gram positive organisms studied behaved alike, the discussion to follow is generalized and refers to all species.

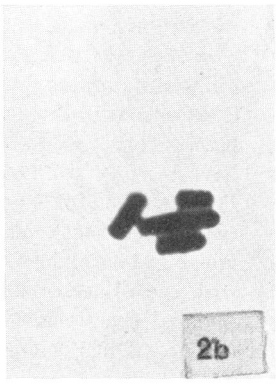
In the present investigation it was necessary to observe staining of the cell wall with crystal violet. Therefore, it was essential to decide when the stained portion of the organism included the cell wall. In the absence of capsular material the cell wall is the outer structure of the organisms studied. Thus organisms in clusters contact one another by virtue of contact by their cell walls. This situation is readily demonstrated by means of the Dyar cell wall stain. On the other hand, if the wall is unstained, the stained cytoplasmic portions of neighboring organisms in a cluster will be separated by a colorless band or area representing the unstained walls of the organisms. In such cases the subsequent application of the contrasting Dyar cell wall stain colors these areas, showing that they are indeed occupied by the cell walls. Hence the unstained spaces between the organisms in clusters cannot be at-

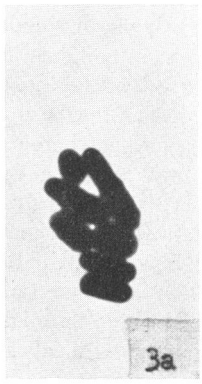
[1] This investigation was supported by a research grant, G4115, from the National Institutes of Health, U. S. Public Health Service.

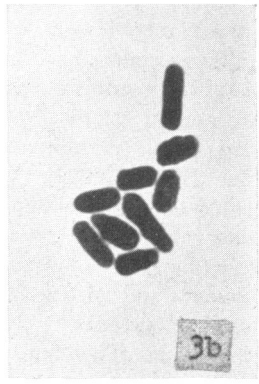


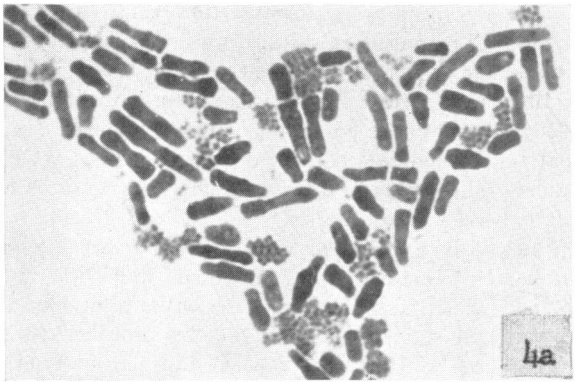


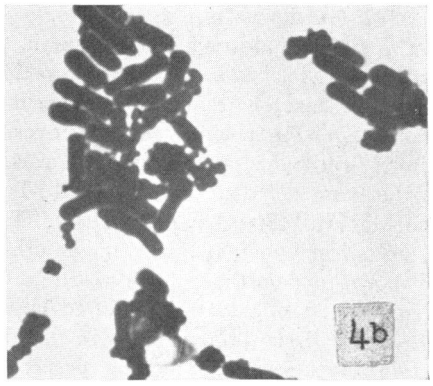


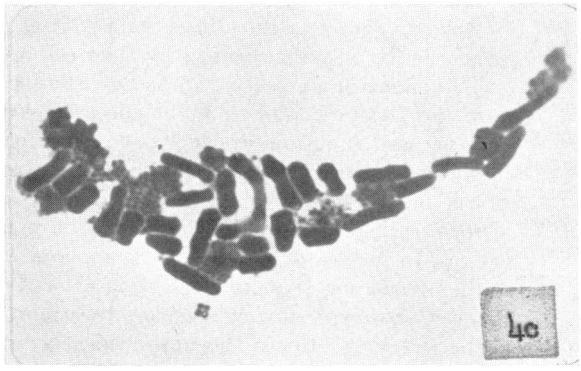


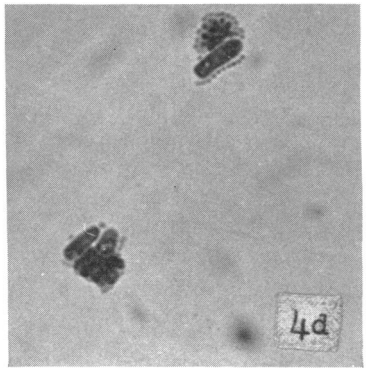












Figures 1–4

tributed to shrinkage of the whole organism and can be taken as evidence of failure to stain the cell walls.

RESULTS

The cell walls of the several species were stained by the Dyar method. In all of the preparations the walls of organisms in clusters were in contact with their neighbors. However, when similar smears of all the species were stained with safranin, the colored areas did not touch except for organisms overlapping one another in three dimensional clusters (figure 4*d*). Though the conditions of treatment with safranin were varied with respect to dye concentration, pH, and length of time of staining, the cell walls of gram positive and gram negative organisms alike did not take up the dye. Gram positive organisms stained with safranin appear to be smaller in diameter than when stained with alkaline crystal violet since Bartholomew and Mittwer (1951) have recorded a diameter of 0.74 μ with safranin and 0.90 μ with crystal violet by measurements on cells of *Bacillus cereus*.

With crystal violet the walls of the gram negative organisms did not stain just as was the case with safranin. However, the gram positive species showed a variable behavior depending upon the exact conditions of staining (see figure 1, 4*a* and 4*b*). The important conditions recognized were the pH of staining and the subsequent washing of the stained cells.

In figure 1 it is readily apparent that alkaline crystal violet stains the walls of the yeast organisms whereas acidified crystal violet does not. The other gram positive species studied exhibited the same behavior. It is also evident that much less dye is taken up at low pH even by the interior of the cells in these preparations (see also figures 4*a* and 4*b*).

When gram positive organisms stained with crystal violet at high pH are washed briefly with dilute acid or more extensively with distilled water, the walls lose the dye and preparations like those of figures 1*b* and 4*a* result. If the stained smears are washed with organic solvents in which crystal violet is soluble, the walls are rapidly decolorized. Indeed, the dye is removed by acetone, alcohol, and chloroform during exposure of the slides to these solvents for a few seconds. The cytoplasm may also be decolorized by more prolonged extraction.

The effect of the pH of staining on the mordanting step of the gram procedure is shown in figure 2. Figure 2*b* is the result of a "gram stain" employing crystal violet in sodium bicarbonate at pH 9 but omitting the decolorization and counterstaining steps. Comparison of this photograph with that obtained after staining at pH 1.5 (figure 2*a*) clearly reveals the influence of

Figure 1. a. Schizosaccharomyces pombe stained with crystal violet in a solution of sodium bicarbonate at pH 9.

b. S. pombe stained with crystal violet in 0.05 N hydrochloric acid, pH 1.5. Crystal violet in 0.125 M acetate buffer, pH 5.2, produces the same result.

Note the lack of contact between the stained organisms of *b*.

Figure 2. a. Schizosaccharomyces pombe stained with crystal violet for five minutes at pH 1.5, rinsed for a few seconds to merely remove the staining solution from the smear, treated with Burke's iodine mordant solution, and washed with tap water.

b. Same as *a* except that the crystal violet was in sodium bicarbonate solution at pH 9.

Figure 3. a. Schizosaccharomyces pombe stained by Burke's gram stain method.

b. Same as *a* except that between the staining and treatment with iodine mordant the smear was washed with tap water for one minute to remove crystal violet from the cell wall area of the organisms.

If preparations like *b* are counterstained with safranin, the lake is in part or largely lost, the organisms staining various shades of red. Counterstaining preparations of *a* do not result in either a loss of the lake or in loss of the apparent physical contact of the organisms.

Figure 4. A mixed smear of *Schizosaccharomyces pombe*, *Sarcina lutea*, and *Micrococcus aureus*.

a. Stained for five minutes with crystal violet at pH 1.5.

b. Stained for five minutes with crystal violet at pH 9.

c. Stained for five minutes with crystal violet at pH 5.2 (acetate buffer) and treated with Burke's iodine mordant and acetone. No counterstain employed.

d. Stained for five minutes with one per cent safranin at pH 7.4. Cells colored red. All other photographs shown are of organisms colored blue to black.

(Magnification of all figures × 1,330; photos taken by Dr. Katherine Schaeffer.)

the pH of staining on the uptake of crystal violet and lake formation in the cell wall.

Figure 3*a* illustrates the typical picture obtained with Burke's gram stain procedure. In figure 3*b* the yeast was stained at pH 9 with crystal violet. However, before the smear was treated with iodine and decolorizer, it was washed extensively in tap water (pH 8.3–8.5) at room temperature. Thus washing at even this relatively high pH slowly removes crystal violet from the walls of gram positive organisms. If ordinary distilled water (pH about 5.5) is used instead of tap water, the dye is more rapidly lost from the cell wall.

Bartholomew and Mittwer (1952) have recently reemphasized that the counterstaining step should be viewed as an integral part of the gram stain procedure rather than as a mere convenience in observing gram negative organisms. The counterstain helps in the differentiation by playing an active role in the displacement of the primary dye from some organisms. The best counterstains are not triphenylmethane dyes but are, according to Hucker and Conn (1927), Bismarck brown, an azo dye, pyronin, a xanthene, and safranin, an azine dye. Thus if the cell walls of gram positive organisms differ from those of gram negative organisms by an unusual capacity to take up triphenylmethane dyes, it is consistent that the best counterstains are dyes having no affinity for the cell wall and which consequently are less effective in removing the primary stain from gram positive than from gram negative organisms.

If counterstain is applied to preparations like those of figures 2*a* and 3*b*, most of the purplish-black lake is removed; the cells are colored with the counterstain and would be described as gram negative. This observation suggests that the counterstain can displace the lake formed in the cytoplasm.

The cell wall is known to be of critical importance by extending the experiments of figure 2 with gram positive organisms. When preparations like those of this figure are treated with acetone in the usual manner, a little lake is lost from the organisms stained in acid. The organisms stained at an alkaline pH value do not change in any visible way. Subsequent treatment with a counterstain (safranin) displaces the lake from the cells stained in acid solution leaving these cells colored red and "gram negative". The organisms stained in alkaline solution do not change in appearance on treatment with safranin. Moreover, when organisms stained with alkaline crystal violet are washed extensively in tap water prior to exposure to mordant and are then "decolorized" with acetone (figure 3*b*) and counterstained, the lake is largely or completely lost. This displacement occurs even though the cytoplasm is still intensely colored with lake before the counterstaining. From these observations, one may conclude that safranin cannot displace any large amount of lake from the wall of a gram positive organism. However, safranin displaces the lake from the cytoplasm of cells (including gram negative species) not containing lake in the cell wall. Perhaps safranin also displaces the lake from the cytoplasm of organisms containing it in their walls, but this process cannot be observed by the methods used herein because of the position and the dense and obscuring color of the lake in the cell wall.

DISCUSSION

It is well known that the cell walls of bacteria have little affinity for dyes. Yet Knaysi (1930, 1951) has reported that the walls of gram positive species can be stained with certain basic dyes and that basic fuchsin and the methyl violets are the most suitable dyes for the purpose. It is worth noting that these triphenylmethane dyes are among the best primary dyes in the gram stain. Although Knaysi described no studies with gram negative organisms, Robinow and Murray (1953) later were unable to stain the wall zones of gram negative species with triphenylmethane dyes that did stain these zones in gram positive organisms. These reports are in accord with and support those of the foregoing section.

The present findings correlate gram positive behavior with uptake of the primary dye by the cell wall. Three major points bear on this issue. First, the primary basic dyes do not stain the walls of gram negative species, but under suitable conditions the walls of gram positive species do take these stains. Secondly, stained organisms retain the lake against decolorization and counterstain in gram stain procedures only when there is lake in the cell walls. Finally, cytoplasm readily takes up the counterstains traditionally employed, but the cell wall has no affinity whatever for these dyes.

The role of the counterstain can now be clarified. Counterstain is readily sorbed by the cytoplasm from which it probably displaces by adsorption exchange (Bartholomew and Mittwer, 1952) the crystal violet lake. In this way any lake not lost in the decolorization step is released from the cytoplasm of the gram negative organisms which never do sorb lake in their walls. Gram positive organisms take up more crystal violet per mg of nitrogen to start with than do gram negative organisms (Kennedy and Barbaro, 1953). After treatment with iodine the lake is not lost from the walls of these heavily stained organisms to the decolorizer. Counterstain might displace the lake from the cytoplasm but not from the cell wall which has no affinity for it. Obviously counterstain must have some solvent power for the lake, but since the latter is quite insoluble *in vitro* in water, the lake in the wall is not readily lost by solution in the aqueous solutions of counterstain usually used. Counterstaining in certain organic solvents will remove the lake from the cell walls of gram positive organisms by virtue of the solubility of the lake in the organic solvent. Since this would be undesirable, the otherwise arbitrary employment of aqueous solutions of counterstains is now understandable.

Inasmuch as the primary dyes stain the walls of gram positive forms most effectively at alkaline pH values, it is not surprising that the best procedures for gram staining have provided for a high pH as the result of empirical experience. Moreover, washing steps between the application of primary dye and mordant have been generally avoided (Burke, 1922), a practice whose value is now clear.

SUMMARY

Evidence is presented for the point of view that the specificity of triphenylmethane dyes as primary dye in the gram stain rests on the unusual ability of simple solutions of these dyes to stain the cell wall of gram positive organisms. Correlatively, the gram negative character of an organism rests on the relative inability of the cell wall to sorb the primary basic dye employed in the gram stain.

Crystal violet at low pH and safranin do not stain the walls of yeast and bacteria. When organisms of a gram positive species are stained with crystal violet at a high pH and then are washed at a low pH, the dye disappears from the walls. Organisms with stained walls resist both decolorizer and counterstain after treatment with iodine and thus appear gram positive.

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