CRYSTAL VIOLET BINDING CAPACITY AND THE GRAM REACTION OF BACTERIAL CELLS

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Kennedy and Barbaro (1953) presented data suggesting that crystal violet adsorption values correlated directly with the gram staining character of bacterial cells, and implied that such quantitative dye uptake studies might prove a basis for a much needed quantitative index of gram positivity. Their results are consistent with a popular theory of the mechanism of gram differentiation presented by Stearn and Stearn (1926).

In a previous paper, the authors (Finkelstein and Bartholomew, 1953) presented a colorimetric method for measuring the maximum dye uptake of bacterial cells. This method then was used to determine whether or not a correlation existed between the gram reaction and dye uptake. The conclusions to be presented here, however, differ from those of Kennedy and Barbaro (1953).

METHODS

Dye uptake was determined quantitatively by a colorimetric method, the details of which have been reported previously (Finkelstein and Bartholomew, 1953). Briefly, the procedure was to add to a series of test tubes a known concentration of bacterial cells in aqueous suspension plus varied but known concentrations of aqueous crystal violet. After centrifugation the concentration of the supernatant dye solution was determined with a Klett-Summerson photoelectric colorimeter. The difference between the original dye concentration and the concentration of the supernatant dye solution represented the dye uptake by the cells. Plotting the original dye concentrations against the amounts of dye taken up by the cells resulted in a curve, the top of which did not parallel the base line but continued to rise slightly. Curves of this type were used to obtain very close approximations of the maximum dye uptake ability of the cells. In most cases the concentration of the cells at the saturation point was about 2 mg dry weight, and the dye concentration was 0.4 to 0.6 mg, in a total volume of 15 ml. This resulted in very favorable dye-cell ratios for dye saturation of the cells. The pH of the dye-bacterial systems varied from 6.0 to 6.5. Cellular nitrogen determinations were made with a micro-Kjeldahl method, and the results reported are the averages of four determinations. Crystal violet nitrogen was calculated from its chemical formula. All cultures were grown on nutrient agar slants and harvested after 24 hours' incubation, except for *Bacillus subtilis* which was harvested after 16 hours.

RESULTS AND DISCUSSION

Table 1 presents the results obtained in the determination of dye uptake for four species of gram negative and four species of gram positive organisms. The values represent as nearly as possible the dye saturation level of the cells. It can be seen that there was no correlation between the crystal violet uptake and the gram characteristic of the organism.

On first thought, one might conclude that these results are in experimental disagreement with those presented by Kennedy and Barbaro. However, on inspection of the methods used several differences in procedure are apparent which possibly could explain the different results.

The minor differences in methods, such as the temperature used for killing the cells, and the use of different culture media possibly could influence the amount of dye taken up. However, these factors should not affect the quantitative relationship between dye uptake and gram differentiation since methods of ordinary heat fixation and normal differences in culture media are known not to affect seriously the gram character of a microorganism (Bartholomew and Mittwer, 1952). The first major difference, however, is the fact that this method carefully avoided the use of competing ions, whereas Kennedy and Barbaro used M/15 phosphate buffer at pH 7.1 as a suspension medium both before and during exposure to dye and also as

TABLE 1

Crystal violet uptake at dye saturation levels of several gram positive and gram negative organisms

	SPECIES	AVERAGE PER CENT CELLULAR N	MG CRYSTAL VIOLET UPTAKE PER MG CELL WEIGHT	MG CRYSTAL VIOLET UPTAKE PER MG CELLULAR N*
Gram negative	*Acetobacter pasteurianum*	13.9	0.09	0.65
	Escherichia coli	13.1	0.19	1.45
	Serratia marcescens	12.2	0.19	1.56
	Neisseria catarrhalis	13.2	0.25	1.90
Gram positive	*Saccharomyces cerevisiae*	8.7	0.07	0.80
	Bacillus subtilis	12.3	0.10	0.81
	Micrococcus pyogenes var. *aureus*	12.6	0.18	1.43
	Sarcina lutea	11.9	0.36	3.02

* Ten per cent of the values in this column are equivalent to the "mg crystal violet N per mg of bacterial N" values of Kennedy and Barbaro (1953).

a final double wash following dye exposure. This is fundamentally similar to the procedure as used by Stearn and Stearn (1926) in their isoelectric point determinations. Stearn and Stearn applied the dye and followed it with exposure to a buffer.

The consequences of the use of buffer ions are great. First, maximum dye uptake will not be achieved due to the presence of the competitive ions. We have found (*unpublished data*) that monovalent cations, such as in sodium chloride solutions, greatly reduce dye uptake. The effect of divalent cations is even greater and may reduce dye uptake up to 70 per cent. Secondly, the buffer wash will serve as a decolorizer. This has been shown by the work of Bartholomew *et al.* (1950) who demonstrated dye replacement by cations, and Coudray (1929) who actually used salts as a substitute for the decolorizer in the gram procedure. The procedure used by Kennedy and Barbaro, therefore, possibly measured dye retention rather than dye uptake, while our method actually measured dye uptake. Thus, the Kennedy and Barbaro procedure would be analogous to the earlier work of Stearn and Stearn and possibly would confirm the Stearns' conclusion that the dye retention powers of gram positive bacteria are greater than those for gram negative bacteria. Thus, the differences between our results and those of Kennedy and Barbaro probably result from differences in procedure rather than being in experimental disagreement.

The present authors would like to emphasize, however, that the implied conclusion of both Kennedy and Barbaro and Stearn and Stearn, that gram positive bacteria possess a greater dye uptake ability than gram negative bacteria, is contrary to our experimental determinations. Several possible explanations exist as to why dye retention correlates with the gram character whereas dye uptake capacity does not. First, Nakanishi (1952) has shown that lead acetate selectively competes for dye binding sites in that the lead acetate had little effect on gram negative bacteria but considerable effect on gram positive bacteria. Thus, the effect of competing ions might be selective rather than equal, for both gram positive and gram negative bacteria. Secondly, permeability differences are known to exist. For example, Mittwer *et al.* (1950) showed that gram positive organisms were less permeable to iodine in alcoholic solution than gram negative organisms. Thus, it is entirely possible that permeability differences result in the correlation of dye retention with the gram character, either directly by influencing the effects of competitive ions for dye sites, or indirectly by influencing cell constituent losses during the suspension of cells in a liquid medium. The authors are conducting extensive tests to determine the exact effect of competing ions on the dye content of gram positive and gram negative cells.

The work of Stearn and Stearn (1926) is used most often to support the contention that dye uptake ability should correlate with gram character. Stearn and Stearn explained the mechanism of gram differentiation on the basis of differences between the isoelectric points of the protoplasm of bacterial cells. Gram positive organisms were interpreted as having lower isoelectric points and stronger dye retention properties than the gram negative organisms. Although the Stearns measured only dye retention ability, it is clear by their statements that they thought that increased retention was related to increased dye uptake. It is true that if the experiments of Stearn and Stearn are repeated, similar data can be obtained. However, the present authors believe that such data merely show the effect of

pH and competitive ions on dye retention, and that they represent very little as far as the mechanism of gram differentiation by the gram stain method is concerned. Several experimental factors indicate that this is true. First, the iodine cannot precede the dye in the gram procedure, and this should be possible by the Stearn and Stearn concept (Burke and Barnes, 1929). Secondly, all oxidizing agents cannot replace iodine as claimed by Stearn and Stearn (Mittwer *et al.*, 1950). Thirdly, the isoelectric point differences claimed by the Stearns lack validity since they were obtained only when acetone was used as the decolorizer (Stearn and Stearn, 1928), and yet we know that many other decolorizers can be used with good gram differentiation (Bartholomew and Mittwer, 1952). Fourthly, by the Stearn and Stearn concept any protein with a low isoelectric point should stain gram positively, yet Mittwer (1953) has stained a mucoprotein with an isoelectric point of about 1.8 and it was gram negative. Lastly, the Stearn and Stearn concept fails to explain why the rupture of the cell wall will change a gram positive cell into a gram negative state (Burke and Barnes, 1929). It is obvious then that the Stearn and Stearn concept of the mechanism of gram differentiation leaves much to be desired and possibly totally lacks validity.

The last significant difference between our procedure and that of Kennedy and Barbaro which might have contributed to the difference in results obtained was the selection of test organisms. An apparent correlation of gram character with dye uptake ability would have occurred if we had compared only *Sarcina lutea* with *Escherichia coli* or *Micrococcus pyogenes* var. *aureus* with *Acetobacter pasteurianum*. However, this correlation would have reversed itself if we had compared only *Saccharomyces cerevisiae* with *Neisseria catarrhalis* or *Bacillus subtilis* with *Serratia marcescens*. This emphasizes the fact that if one wishes to correlate a characteristic with the gram state of a cell, it is essential that several species of both gram positive and gram negative organisms be used rather than a single representative species for each.

The results presented in this paper show clearly that dye uptake ability does not correlate with the gram character of a cell. It has been suggested also that dye retention ability, which other workers have indicated as correlating with the gram character, has often been confused with dye uptake ability.

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SUMMARY

Quantitative determinations of crystal violet uptake by bacterial cells failed to show any correlation with their gram character. This fact argues against the Stearn and Stearn concept of the mechanism of gram differentiation. The results presented by Kennedy and Barbaro showing a correlation between gram character and cellular dye content represent measurements of dye retention rather than dye uptake.

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