QUANTITATIVE STUDIES OF DIFFERENTIAL STAINING REACTIONS

I. The Effect of pH on the Quantity of Dye Retained by Bacteria and the Apparent Isoelectric Point[1]

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Received for publication March 15, 1956

It has been shown previously (Kennedy and Barbaro, 1953) that the amount of crystal violet adsorbed by bacteria can be measured quantitatively by either one of two procedures. One procedure involved micro-Kjeldahl analyses of bacterial cells before and after staining. The increase in total nitrogen in the stained cells represented the dye nitrogen adsorbed. The second procedure was based on micro-Kjeldahl analyses of the dye nitrogen in the stain solution before staining and in the supernatant fluids after staining and washing with M/15 phosphate buffer (pH 7.1). The decrease in dye nitrogen in the supernatants represented the dye nitrogen adsorbed by the cells. The simultaneous use of both procedures provided added credence to the quantitative dye adsorption values obtained. Application of these procedures to the complete gram stain led to the development of a quantitative gram reaction (Barbaro and Kennedy, 1954). The investigations of Kennedy and Barbaro (1953), Barbaro and Kennedy (1954), and Barbaro (1954) on 13 species of bacteria showed that gram positive cells adsorbed and retained more crystal violet than gram negative cells and that gram variable species adsorbed an amount intermediate between the two.

Bartholomew and Finkelstein (1954) studied the dye uptake of 7 species of bacteria and 1 species of *Saccharomyces* by colorimetrically analyzing the dye content of a dilute stain solution before and after staining the organisms. Their cells were suspended in distilled water and stained at pH 6.0 to 6.5. Bartholomew and Finkelstein were unable to find a correlation between dye uptake ability and the gram stain.

[1] Presented in part at the 55th General Meeting of the Society of American Bacteriologists, New York City, 1955.

MATERIALS AND METHODS

The crystal violet used was Certification No. NC 20, C. I. No. 681, with a total dye content of 96 per cent. The crystal violet stock solution was made by dissolving 10 g of the dye in 50 ml of 95 per cent ethyl alcohol and adding distilled water to give a final concentration of approximately 10 per cent. The stock staining solution was diluted to approximately 0.25 per cent dye content (0.19 to 0.21 mg crystal violet nitrogen per ml) for use. This solution had a pH of about 5.2.

The acid fuchsin used was Certification No. NR 21, C. I. No. 692, with a total dye content of 51 per cent. The acid fuchsin solution was made by dissolving 5 g of the dye in the required amount of distilled water to give 0.50 mg of acid fuchsin nitrogen per ml (approximately 1.35 per cent).

All pH determinations were made electrometrically with a Beckman pH meter. Sørensons citrate-hydrochloric acid and Sørensons phosphate buffer mixtures were used for pH values of 1 to 5 and 6 to 8 respectively.

Micrococcus pyogenes var. *aureus* Oxford 202 strain, *Corynebacterium diphtheriae* Park 8 strain, and *Escherichia coli* strain 527 were cultivated at 30 C on a modified tryptose agar. After 18- to 24-hr incubation the cells were harvested with unbuffered 0.85 per cent saline, killed by heating in a 99 C water bath for 30 min and washed with unbuffered 0.85 per cent saline until the supernatant fluids yielded no detectable nitrogen within the limits of error of the micro-Kjeldahl method ($\pm$0.02 mg N). The killed and washed cells were made up into a homogeneous suspension which was adjusted to the desired concentration of cells. One ml of the suspension was added to each of 16 centrifuge tubes and to Kjeldahl flasks in duplicate to serve as controls.

The tubes were centrifuged in the cold (5 C) at about 6000 rpm (rcf 2250) for 30 min. The supernatant was discarded and 2 ml of unbuffered dye solution was added to the packed cells and thoroughly mixed with a glass rod. The tubes were centrifuged for 30 min, the supernatant dye was collected in Kjeldahl flasks and the stained organisms were washed twice with 2 ml of the appropriate buffer solution. The washings were added to the original dye supernatant and analyzed for nitrogen content by the micro-Kjeldahl method. The stained cells were transferred quantitatively to Kjeldahl flasks and analysed. Two ml of the dye solution were added directly to Kjeldahl flasks in duplicate and the dye nitrogen determined.

RESULTS

The variable decolorizing action of buffer is demonstrated when the mg of crystal violet nitrogen retained per mg of bacterial nitrogen is plotted against the pH of the buffer used to wash the stained cells. Figure 1 shows the results with *M. pyogenes* var. *aureus*, *C. diphtheriae* and *E. coli*. The curves shown illustrate that under these conditions the gram negative organism retains more dye than the gram positive organisms at all pH values from 1 to 8. The term "retention" or "retained dye" is used to indicate cellular dye adsorption values after treatment with a decolorizing agent. The agent may be buffer, as in figures 1, 2 and 3, or it may be alcohol in the complete gram reaction (Barbaro and Kennedy, 1954).

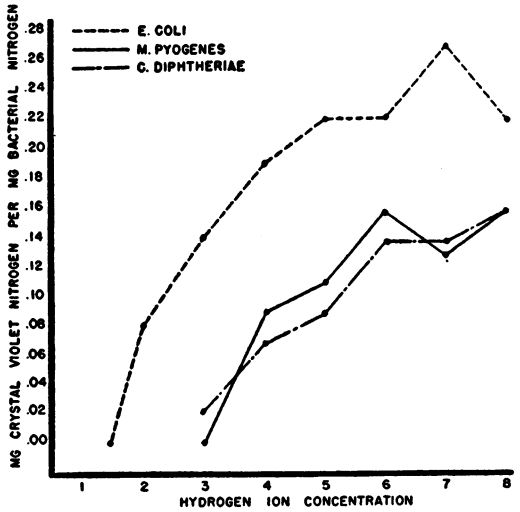

Figure 1. Retention of crystal violet, pH 5.2 by *Micrococcus pyogenes* var. *aureus*, *Escherichia coli*, and *Corynebacterium diphtheriae*.

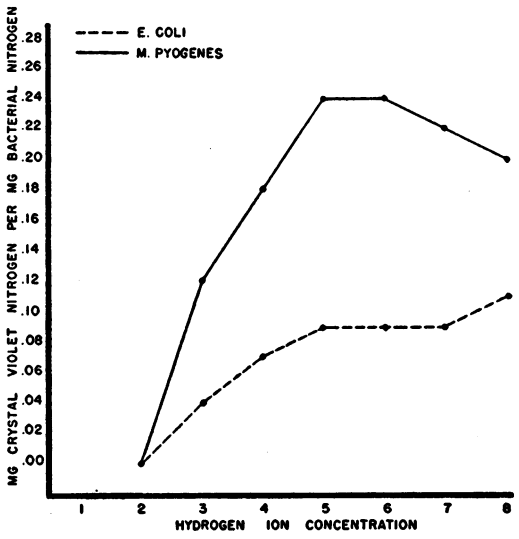

Figure 2. Retention of crystal violet, pH 7.5, by *Micrococcus pyogenes* var. *aureus* and *Escherichia coli*.

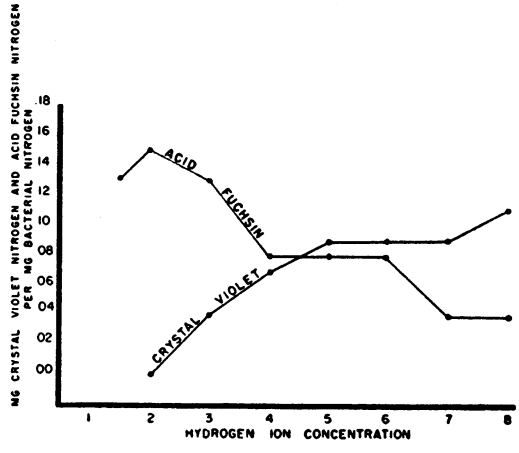

Figure 3. Retention of acid fuchsin, pH 1.8, and crystal violet, pH 7.5, by *Escherichia coli*.

If the pH of the dye solution is adjusted, without buffer, to 7.5 before staining and the cells washed with buffer after staining, as previously, the gram positive organism retains more dye than the gram negative organism at all pH values (figure 2). The results also show that the decolorizing action of buffer is minimal at pH 5 to 7. Quantitative gram reactions (Barbaro and Kennedy, 1954) and dye adsorption studies

(Kennedy and Barbaro, 1953) in the micro-Kjeldahl method were accomplished with crystal violet in phosphate buffer at pH 7.1. The first buffer wash after staining at this pH contains a small amount of dye which we believe represents excess dye nonspecifically held in the packed cells and on the walls of the centrifuge tube. Some justification of this belief is obtained by an analysis of the second and third buffer wash in which there is no dye detectable by the micro-Kjeldahl method.

Similar experiments were performed with the same organisms and with acid fuchsin at pH 1.8 Repeated trial runs with staphylococci showed gradation of acid-dye retention as determined by visual observation of the packed cells in the centrifuge tubes. Micro-Kjeldahl analyses of the cells and supernatant fluids consistently yielded dye retention values of not more than 0.02 mg N. This value is the limit of error in the method. When *E. coli* was tested, sufficient acid-dye was retained in the cells to be measured in the micro-Kjeldahl method. The values obtained are shown in figure 3, together with corresponding values for basic-dye retention. The point of linear intersection represents, theoretically, the isoelectric point of *E. coli* quantitatively determined by the dye method. The results with staphylococci and *E. coli* support quantitatively the old and well-known contention of Eisenberg (1912) that gram negative organisms have a greater tendency to retain acid-dyes than do gram positive organisms. Not so obvious, however, is the effect of the pH of the crystal violet staining solution on the apparent isoelectric point of *E. coli* (figures 1 and 3). If the values for crystal violet retention by *E. coli* shown in figure 1 are used instead of the values in figure 3 the apparent isoelectric zone becomes 2 to 3 rather than 4 to 5 as indicated by figure 3.

DISCUSSION

Bartholomew and Finkelstein (1954) believe 1) that the use of buffer would prevent maximum dye uptake, and 2) the buffer would serve as a decolorizer. Any competition for dye attributable to phosphate buffer at pH 7.1 must be minimal since dye adsorption values obtained by the micro-Kjeldahl method (Kennedy and Barbaro, 1953) are the same as, or greater than, the dye uptake values obtained colorimetrically by Bartholomew and Finkelstein when the same species of bacteria were compared. Moreover, the values for dye adsorption and retention obtained in previous work (Kennedy and Barbaro, 1953; Barbaro and Kennedy, 1954) are greater, not less, than the values in the present work (figure 2) where staining occurred in the same pH range but in the absence of buffer. This is interpreted as additional evidence that phosphate buffer at pH 7.1 does not prevent maximum dye adsorption.

The results presented here fully support the contention that buffer will serve as a decolorizer and they also show that the capacity to act in this manner is a function of the pH of the buffer and dependent upon the pH of the dye solution used to stain the cells. This capacity is minimal in the optimum pH range for the gram stain as shown in figure 2. Moreover, if the buffer was an effective decolorizer in this pH range, in previous quantitative studies conducted in this laboratory the second and third buffer washing of the cells would be expected to contain measurable amounts of dye. This was not the case. These results may be expressed in another way; at pH 7.0 to 8.0 the retention of basic dye closely approached the adsorption of dye.

It has been suggested by Bartholomew and Finkelstein that the differences between their results and ours may be due to "differences in procedure rather than to experimental disagreement." That this suggestion is correct is supported in the present report by the demonstration that the hypothesis that "gram positive organisms adsorb more basic dye than gram negative organisms" reverses itself when the pH of the crystal violet solution is 5.2. Finkelstein and Bartholomew's colorimetric method for measuring dye uptake uses cells suspended in distilled water at an acid pH (6.1 to 6.3) and cells which, according to their published procedure (1953), have not been washed after heating. In addition to the known deleterious effect of distilled water and low pH the use of an unwashed heated cell suspension would offer a complex menstruum whose action is more difficult to interpret than phosphate buffer. In the quantitative gram reaction we believe it is better to use thoroughly washed cells in a system in which the kinds of ions are known and the pH controlled at what is considered the optimum for gram stains. [In a personal communication Finkelstein (1955) stated that the cells used in his experiments were washed after heating. The

possible effects of the use of unwashed cells are considered here to avoid any repetition of an erroneous impression given by the published (Finkelstein and Bartholomew, 1953) procedure.]

The quantitative approach offered by the micro-Kjeldahl method to studies of bacteria-dye relationships becomes limited when acid-dyes are used. The present work indicates that the limiting factor is the dye adsorption capacity of the organisms selected for study rather than the acid-dye itself.

Harden and Harris (1953) have concluded that isoelectric points of bacterial cells with the dye method are determined by the cytoplasmic membrane and isoelectric points determined electrophoretically are governed by the cell wall. Electrophoretic measurements of *E. coli* cells by Winslow and Upton in 1926 place the isoelectric point at pH 2.5. Stearn and Stearn (1924) used an arbitrary color-intensity scale in the dye method and their curves indicate an isoelectric point of pH 5.5 for *E. coli*. The quantitative determination of the curves for the isoelectric zone obtained in figure 3 of the present work indicates an isoelectric point of about pH 4.5. It is considered that a more significant conclusion apparent from quantitative studies is that the results obtained are relative rather than absolute and a number of factors and conditions may significantly alter the end point.

SUMMARY

The amount of crystal violet retained per mg of bacterial nitrogen in *Micrococcus pyogenes* var. *aureus* was greater than that retained by *Escherichia coli* when the pH of the staining solution was 7.5. This relationship was reversed when the pH of the staining solution was 5.2.

M. pyogenes var. *aureus* and *E. coli* retained less dye when stained in the absence of M/15 phosphate buffer at pH 7.1 than they retained in previous studies when they were stained in the presence of buffer.

Phosphate buffer will serve as a decolorizer of stained cells. The decolorizing capacity is minimal at a pH of about 7.0.

The quantity of acid fuchsin retained by *E. coli* can be measured by micro-Kjeldahl analyses. Staphylococci retained less than 0.02 mg of acid fuchsin nitrogen per mg of bacterial nitrogen.

The isoelectric zone of *E. coli* as determined by the dye method can be made quantitative by micro-Kjeldahl analyses of stained cells and of staining and buffer solutions. The values obtained are relative rather than absolute.

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