Acid Salt Derivatives of Crystal Violet in the Gram Stain and Nuclear Stain[1]

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The problem of the mechanism o the Gram stain has stimulated a considerable amount of work (1). Most of this research has centered around three basic postulated mechanisms of action: (1) the iso-electric point theory, (2) the permeability theory, and (3) the chemical theory.

Much of the work in support of the chemical theories has been directed toward extracting and replacing a gram positive compound in the bacterial cell. This approach is quite acceptable and has yielded an abundance of reliable information, but the problem has not been solved. It is believed that a study of the effect of the anion of the primary dye on the staining reaction could aid in the solution of this problem. This is a preliminary report on this approach.

The anion on the crystal violet molecule is the chloride ion. This ion was replaced by a hydroxyl ion by precipitating the crystal violet from a 1% aqueus solution with potassium hydroxide. The resulting precipitate is the color base of crystal violet. The anion to be studied was placed on the crystal violet base by reacting with an acid containing the desired anion. Enough water was added to give a 1 per cent solution. The solution of the new dye was adjusted to a *p*H of 7.5 and was used immediately since the *p*H will change on standing.

Each of the new stains was used with the following organisms under the named conditions: (1) Gram stains of *Escherichia coli*, *Bacillus subtilis*, *Bacillus cerus*, *Gaffkya tetragena*, and *Micrococcus albus* from 10 and 18 hr. nutrient agar slants, (2) Gram stains of *E. coli*, *B. subtilis*, and *G. tetragena* as 10 hr. cultures grown on phosphorus rich and phosphorus free media, and (3) nuclear stains of *G. tetragena* grown on nutrient agar.

The gram stain technique was that of Hucker and Conn (3) and the nuclear stain that of Chance (2). Both techniques were rigidly standardized so that there was only one variable, i.e., the primary stain.

Of the thirty anions tried in conjunction with the color base of crystal violet, most of them gave the same reaction as that of the chloride anion. Some observed exceptions were as follows: In making gram stains of the cells from 10 and 18 hr. agar cultures, it was found that the following

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acid salts of crystal violet stained *E. coli* gram positive: hypophosphorus, oxalic, molybdic, lactic, hydrofluoric, hydrobromic, gallic, and fumaric. Chromic, arsenous, and boric acid salt derivatives stained the gram positive organisms gram negative. When the cells were grown on phosphorus rich media, it was found that E. coli was stained gram positive by hydrobromic acid, and stannic hydroxide salts of crystal violet. Gram positive organisms (*G. tetragena, B. subtilis*) grown on phosphorus rich media were stained gram negative by the following acid salts of crystal violet: benzoic, boric, chromic, molybdic, and arsenous.

When cells were grown on phosphorus free media, it was found that *E. coli* was stained gram positive by the following acid salts: benzoic, hydrobromic, hydrofluoric, hypophosphorus, lactic, sulfuric, phosphorous, mono-chloro acetic, tri-chloro acetic, iodo-acetic, and osmic. *B. subtilis* and *G. tetragena* were stained gram negative by the boric, chromic, arsenous, fumaric, and gallic acid salt derivatives.

Nuclear stains of *G. tetragena* made with the different stains gave essentially comparable results with those found in the gram stain procedure. Cells stained with chromic, boric, and arsenous acid salts were completely decolorized (i.e., there was no differentiation between the cytoplasm and the nucleus). Cells stained with hydrobromic, stannic hydroxide, hydrofluoric, sulfuric, phosphorus, and iodo-acetic acid salts were not decolorized (the whole cell remained stained).

A review of the results shows that chromic, boric, and arsenous acid salts do not stain the gram positive organisms. Hydrofluoric, hydrobromic, phosphorous, and sulfuric acid salts stain the gram negative organisms gram positive. A search has been made to find some chemical or physical factor which is common to the acids of each group and is different between the groups. By finding a common factor, it might be possible to predict the action of other anions and possibly better explain the mechanism of the gram stain.

The factors which have been checked are molecular weight, ionization constant, solubility, valence, atomic number and atomic weight of the metal in the ion, and the electromotive forces. No correlation was found between any of these factors and the staining properties of the corresponding crystal violet salt. It is possible that the various anions act in the capacity of a mordant, even when chemically a part of the dye molecule. This possibility is now being tested and will be reported on later. An alternative explanation, which is unacceptable to us on the basis of the available information, is that some obscure factor of cellular permeability is involved, and the various staining reactions are the direct result of the different permeabilities of the various salt derivatives of the dye. The experimental proof of such a theory would be impossible with present procedures.

Although no acceptable explanation of the experimental data described here is available, one significance of this work should not be overlooked. It has been demonstrated that very minor changes in the chemical structure of a dye will greatly affect its staining characteristics. Therefore, for precise staining procedures only the purest of dyes should be used, and one should be careful to not contaminate solutions of dyes with other chemicals.

LITERATURE CITED

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