A QUANTITATIVE GRAM REACTION

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At the present time there is no satisfactory procedure for estimating the degree of gram positivity although the need for a quantitative method is widely recognized. Recently Kennedy and Barbaro (1953) reported an accurate and reproducible method for determining quantitatively the adsorption of crystal violet by bacteria. In addition, the effect of the species and strain of the organism used, the method of killing the cells, and the time in contact with the dye on the quantitative adsorption of crystal violet were studied. The present report describes the application of this quantitative procedure to the gram reaction, the effect of dye concentration on the quantitative adsorption of crystal violet, and significance of the dye concentration and the counterstain in the gram reaction.

MATERIALS AND METHODS

The crystal violet used throughout the investigation was obtained from the National Aniline and Chemical Company, Incorporated, New York, New York (total dye content, 96 per cent, Certification no. NC 35, C. I. no. 681). A stock solution of crystal violet was prepared by dissolving completely 10 g of the dye in 50 ml of 95 per cent ethyl alcohol; 50 ml of M/15 phosphate buffer, pH 7.1, were added to yield a 10 per cent solution of crystal violet. The desired dye concentration was obtained by suitable dilution of the stock solution with buffer.

Eosin Y, used as a counterstain (Hucker and Conn, 1927), was obtained from the Coleman and Bell Company, Norwood, Ohio (total dye content, 91 per cent, Certification no. CE 10, C. I. no. 768). A 0.5 per cent solution of eosin Y was prepared by dissolving 0.5 g of the dye in 25 ml of 95 per cent ethyl alcohol and 75 ml of M/15 phosphate buffer (Conn, 1940). Repeated micro-Kjeldahl analyses indicated that the eosin Y solution was nitrogen-free.

The dye solutions, Gram's iodine (prepared according to Mittwer *et al.*, 1950), the decolorizing agent [80 per cent ethyl alcohol (Neide, 1904)],

and the phosphate buffer were stored at 5 C and maintained as close as possible to that temperature throughout the procedure.

The preparation of cell suspensions and the micro-Kjeldahl procedure used have been described previously (Kennedy and Barbaro, 1953).

EXPERIMENTAL PROCEDURES AND RESULTS

The successful application of quantitative methods to the gram reaction requires control of the critical decolorization step without jeopardizing the quantitative recovery of cells and decolorizer. An additional requirement of lesser importance is the ability to analyze quantitatively each step in the reaction. The use of bacterial filters seemed to be the most ideal method for the fulfillment of these requirements. Theoretically the filter would retain quantitatively the cell suspension to be tested and allow the addition and subsequent removal of each reagent under controlled conditions of time and temperature. The details of numerous experiments using this approach were in the main unsuccessful and are not reported here. Essentially, the results of the gram reaction carried out on bacterial filters indicated that quantitative recovery of the cells was not always possible with the larger filters. On the other hand, "micro-filters" could be placed in Kjeldahl flasks and their contents analyzed without loss of cells, but such filters were clogged easily with cells so that decolorization time and Kjeldahl analyses of replicates rarely agreed.

It was found that the entire gram reaction could be accomplished on cell suspensions in thick-walled pyrex test tubes and by utilizing high speed centrifugation in the cold to separate cells and reagents. Under these conditions the classical gram negative strain, *Escherichia coli*, was completely decolorized while the staphylococcus retained the primary stain as judged by Kjeldahl analysis. Tinctorially, the cells appeared gram negative and gram positive, respectively.

In preliminary experiments, the supernatant

TABLE 1

Quantitative gram reaction as determined by micro-Kjeldahl analyses

SPECIES	STRAIN	ANALYSES ON BACTERIA			ANALYSES ON SUPERNATANTS					CRYSTAL VIOLET NITROGEN RETAINED PER MG BACTERIAL NITROGEN
		Total nitrogen gram stained cells	Bacterial nitrogen unstained cells	Crystal violet nitrogen	Original nitrogen content	Nitrogen after staining	Nitrogen after decolorization	Eosin and wash	Crystal violet nitrogen	
		mg	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
Micrococcus pyogenes var. aureus	Oxford 202	0.98	0.76	0.22	0.32	0.08	0.02	0.00	0.22	0.29
Corynebacterium xerose	513	0.73	0.62	0.11	0.38	0.23	0.06	—*	0.09	0.18
Corynebacterium diphtheriae	Park 8	0.52	0.48	0.04	0.39	0.26	0.07	—*	0.06	0.08
Shigella dysenteriae (Shiga)	43-A-14	0.59	0.55	0.04	0.40	0.29	0.05	0.00	0.06	0.07
Escherichia coli	527	0.56	0.55	0.01	0.36	0.29	0.07	0.00	0.00	0.00
Salmonella typhosa	0-901	0.47	0.46	0.01	0.37	0.30	0.06	0.00	0.01	0.00

* Counterstain not used.

fluid from the primary stain, mordant, decolorizer, and counterstain, as well as the phosphate buffer washes subsequent to each step, was submitted separately to micro-Kjeldahl analyses. It was found that one wash with buffer after each step in the gram reaction was usually sufficient and that simplification of the procedure without loss of information could be accomplished by pooling all supernatant fluids obtained previous to decolorization. All supernatant fluids subsequent to decolorization were pooled also to determine the amount of primary stain removed in decolorization.

The procedure finally adopted for a typical quantitative gram reaction was as follows: One ml of a uniformly mixed, heat killed bacterial suspension was added in triplicate to centrifuge tubes and centrifuged in the cold (5 C) at approximately 6,000 rpm (rcf 4,248) for 30 minutes. The supernatant fluid was discarded, and two ml of suitably diluted crystal violet stock solution were added to the packed cells. After thorough mixing, the tubes were centrifuged in the cold for 30 minutes. The supernatant crystal violet solution was transferred to a Kjeldahl flask, and then the packed cells were washed with buffer and centrifuged again in the cold. The buffer washings were added to the Kjeldahl flask and two ml of Gram's iodine solution added to the packed cells. The cells were mixed thoroughly and centrifuged, the supernatant removed, and the packed cells washed again with buffer. Decolorization was

accomplished by suspending the packed cells in a small quantity (0.3 ml) of buffer and adding 1.6 ml of 95 per cent ethyl alcohol. The final concentration of alcohol was approximately 80 per cent. Suspension of the cells in a small quantity of buffer insured an even distribution of cells and a more uniform action of the decolorizer than would be obtained if the alcohol were added directly to the packed cells. The cells suspended in alcohol were mixed and centrifuged immediately for 5 minutes. The supernatant alcohol and the subsequent buffer wash were drained into a second Kjeldahl flask for analysis. Two ml of
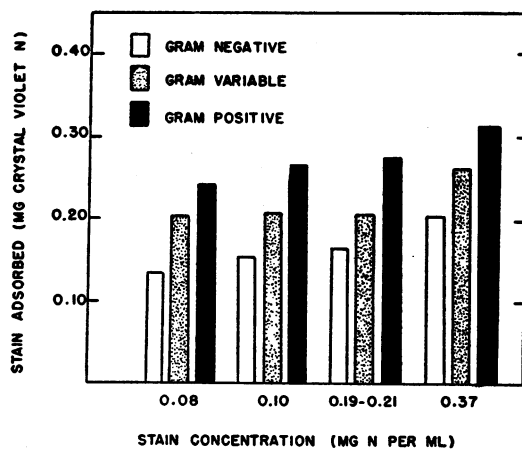

Figure 1. The effect of concentration of crystal violet on the amount of adsorbed dye.

TABLE 2

Effect of concentration of crystal violet on the gram reaction

SPECIES	STRAIN	CRYSTAL VIOLET NITROGEN CONCEN- TRATION	BACTERIAL NITROGEN UNSTAINED CELLS	DYE TO CELL RATIO (NITRO- GEN)	TOTAL NITROGEN GRAM STAINED CELLS	CRYSTAL VIOLET NITROGEN RETAINED PER MG BACTERIAL NITROGEN
		mg/ml	*mg*	*mg per ml/mg cell nitrogen*	*mg*	*mg*
Micrococcus pyogenes var. *aureus*	Oxford 202	0.08	0.40	0.20	0.45	0.12
Micrococcus pyogenes var. *aureus*	Oxford 202	0.19	0.40	0.47	0.50	0.25
Shigella dysenteriae (Shiga)	43-A-14	0.07	0.75	0.09	0.75	0.00
Shigella dysenteriae (Shiga)	43-A-14	0.19	0.53	0.36	0.57	0.07

eosin Y were added to the packed cells, mixed and centrifuged, the supernatant removed, and the cells washed with buffer. The supernatant counterstain and wash were drained into a third Kjeldahl flask for analysis. The packed cells were suspended in distilled water and transferred quantitatively to a fourth Kjeldahl flask for analysis.

Control, unstained, bacterial suspensions were added directly to Kjeldahl flasks in triplicate and analyzed.

The most significant source of error encountered in this procedure was attributed to the instability of the dye solutions on storage. The presence of a small amount of precipitate or suspended dye crystals in the primary stain manifests itself upon centrifugation in erroneously high values for adsorbed dye. This difficulty is best controlled by filtration of the dye solution just before use. As an auxiliary control, a quantitatively known gram negative strain may be analyzed simultaneously.

In table 1 are representative data obtained when the method described above was applied to known gram positive, gram variable, and gram negative organisms. With few exceptions, each nitrogen value represents the average of triplicate determinations. The precision of the method is such that triplicates usually agree within 0.02 mg of nitrogen. To facilitate the comparison of results, the values recorded in the last column of table 1 are calculated on the basis of one mg of bacterial nitrogen. The results show that two species of gram positive organisms retained 0.29 mg and 0.18 mg of crystal violet nitrogen per mg of bacterial nitrogen, respectively; two species of gram variable organisms retained 0.08 and 0.07 mg of crystal violet nitrogen per mg of bacterial nitrogen; two species of gram

negative organisms failed to retain significant amounts of crystal violet nitrogen per mg of bacterial nitrogen (i.e., more than 0.02 mg). Analyses of the counterstain supernatant fluid and the subsequent buffer wash indicated that a significant amount of primary stain was not replaced by eosin Y in the strains studied (table 1).

The use of small quantities of reagents in the procedure made it desirable to determine the effect of increased concentration of the primary stain on the amount of adsorbed dye. The uptake of crystal violet by known gram positive, gram variable, and gram negative cell suspensions is shown in figure 1. As the concentration of the dye increased there was greater adsorption of crystal violet. The results show a significantly different dye uptake among the strains studied at all concentrations.

Increasing the concentration of primary stain may also have a marked effect in the quantitative gram reaction. The values in table 2 illustrate the results obtained when quantitative gram reactions were performed on a gram positive and a gram variable strain at a concentration of 0.08 and 0.19 mg crystal violet nitrogen per ml. The relative increase in dye concentration as calculated from dye to cell ratios (mg per ml dye nitrogen per mg bacterial nitrogen) was the same in both strains. The crystal violet nitrogen per mg of bacterial nitrogen remaining after decolorization was dependent directly on the concentration of the primary stain.

DISCUSSION

The crystal violet adsorbed per mg of bacterial nitrogen was different, significantly, in the gram positive and gram negative strains studied. The amount of crystal violet adsorbed and the amount of crystal violet retained after mordant-

ing and decolorization were related directly to the dye concentration. These findings substantiate and extend a previous report (Kennedy and Barbaro, 1953). The concept that gram positive cells take up more dye than gram negative cells is usually attributed to Stearn and Stearn (1924, 1928). The latter authors presented data based on an arbitrary color intensity scale indicating that at the iso-electric point and in the range pH 3 to pH 8, stained, mordanted or buffered, and decolorized gram positive cells retained more dye than gram negative cells. Recently Finkelstein and Bartholomew (1953) have stated that their investigations failed to support the concept of Stearn and Stearn. Finkelstein and Bartholomew used colorimetric analyses to calculate uptake of dye per unit cell weight. Further details of the latter work have not been published. Regardless of the use of mordant and decolorizer, the data presented in the present report indicate that gram positive cells do adsorb and retain quantitatively more dye than gram negative cells when calculated as adsorbed dye per unit bacterial nitrogen. However, gram variable strains exist which act as intermediates between the obvious extremes.

The application of quantitative techniques to the complete gram reaction is limited, chiefly, by adequate control of the decolorization procedure. It is considered that decolorization at low temperatures, as reported in the method described, slows the decolorization process sufficiently to allow complete removal of dye from the gram negative *Escherichia coli* without over-decolorization of the gram positive staphylococcus.

The data in table 1 are not to be regarded as absolute. There appear to be many variables that influence the values obtained, such as the dye concentration, age of suspension, temperature of the reagents, and other factors. The same factors are known to influence the results obtained when a smear is stained on a glass slide. Although the values are relative, it should be emphasized that triplicate samples usually agree within 0.02 mg nitrogen. The reliability of the method is enhanced further by the fact that analyses are run not only on the stained cells but also on the supernatant fluids. In either case the results agree within the limits of error of the micro-Kjeldahl method. As a further check, it is possible to account quantitatively for all the crystal violet,

adsorbed and unadsorbed, used throughout the procedure.

The ability of one dye to replace another has been demonstrated repeatedly, and it is because of this replacement phenomenon that many investigators feel that the counterstain is a fundamental and necessary step in the gram differentiation. Stearn and Stearn (1924) and more recently Fischer and Larose (1952) omitted the use of a counterstain in investigations concerned with the gram reaction. Bartholomew and Mittwer (1950) and Bartholomew et al. (1950), after a rather extensive study of the mechanism of the gram reaction, concluded that the counterstain plays an important role in the gram procedure. Their findings established as a general phenomenon the ability of one basic dye to replace another in the bacterial cell. Eosin Y was used as a counterstain in the present investigation since it was necessary to select a counterstain that contained no detectable nitrogen. Bartholomew and Mittwer (1950) listed eosin Y among those counterstains that gave poor differentiation and offered as possible explanations either masking of the primary dye or displacement of the primary dye by the counterstain. According to the results obtained in this study, the use of eosin Y as a counterstain played little or no part in the replacement of the primary dye.

It has been suggested frequently that the difference between gram positive and gram negative organisms is quantitative rather than qualitative in nature (Dubos, 1947). The present work lends experimental support to this suggestion (figure 1, table 1). It was shown that differences in the uptake of primary dye play a vital role in the quantitative gram stain (table 2). These findings support the hypothesis that the outcome of the gram procedure is related to the amount of primary dye adsorbed. Moreover, they indicate the necessity for stating the concentration of reagents used in any procedure concerned with the gram reaction.

Bacterial species can be arranged in a continuous series with reference to their behavior in the gram stain (Kaplan and Kaplan, 1933), and it is possible that quantitative differences in staining properties can be correlated with quantitative physiological differences (Dubos, 1947). Furthermore, there are a considerable number of reports concerned with the influence of various factors on the degree of gram positivity. Although

these effects have not been studied quantitatively, there are indications that they may have a determining influence in the gram reaction. The method reported here offers a quantitative experimental approach to fundamental studies on the mechanism of the gram reaction and to studies of the effects of environment, temperature, pH, enzymes, physical and chemotherapeutic agents on gram positivity

SUMMARY

A quantitative gram reaction based on micro-Kjeldahl analyses of reagents and of cells decolorized at low temperatures is described.

Evidence is presented to indicate that bacterial species may be arranged in a continuous series with reference to their behavior in the quantitative gram stain. The crystal violet nitrogen per mg of bacterial nitrogen retained by gram positive organisms was 0.29 mg and 0.18 mg; by gram variable organisms, 0.08 mg and 0.07 mg; gram negative organisms failed to retain significant amounts of crystal violet.

The amount of crystal violet adsorbed by gram positive, gram variable, and gram negative bacteria was related directly to the concentration of the dye. The amount of crystal violet remaining in the cells after decolorization was related directly to the concentration of the primary stain.

The counterstain, eosin Y, had no significant replacement effect on the primary dye in the strains studied.

REFERENCES

BARTHOLOMEW, J. W., AND MITTWER, T. 1950 The mechanism of the gram reaction. I. The specificity of the primary dye. Stain Technol., **25**, 103–110.

BARTHOLOMEW, J. W., ROBERTS, M. A., AND EVANS, E. E. 1950 Dye exchange in bacterial cells, and the theory of staining. Stain Technol., **25**, 181–186.

CONN, H. J. 1940 *Biological stains.* 4th Edition. Biotech Publications, Geneva, N. Y.

DUBOS, R. J. 1947 *The bacterial cell.* Harvard University Press, Cambridge, Mass.

FINKELSTEIN, H., AND BARTHOLOMEW, J. W. 1953 Quantitative determination of dye uptake by bacterial cells as related to their gram reaction. Bact. Proc., **1953**, 35.

FISCHER, R., AND LAROSE, P. 1952 Mechanism of gram stain reversal. J. Bact., **64**, 435–441.

HUCKER, G. T., AND CONN, H. J. 1927 Further studies on the methods of gram staining. N. Y. (Geneva) Agr. Expt. Sta. Tech. Bull., **128**, 1–34.

KAPLAN, M. L., AND KAPLAN, L. 1933 The gram stain and differential staining. J. Bact., **25**, 309–321.

KENNEDY, E. R., AND BARBARO, J. F. 1953 Quantitative adsorption of crystal violet. J. Bact., **65**, 678–680.

MITTWER, T., BARTHOLOMEW, J. W., AND KALLMAN, B. J. 1950 The mechanism of the gram reaction. II. The function of iodine in the gram stain. Stain Technol., **25**, 169–179.

NEIDE, E. 1904 Die Alkoholentfärbung der nach Gram gefärbten Bakterien als Speciesdiagnose, in Verbindung mit einer Untersuchung der für die Gramfärbung in Betracht kommenden Faktoren. Centr. Bakteriol. Parasitenk., Abt. I, Orig., **35**, 508–521.

STEARN, A. E., AND STEARN, E. W. 1928 Studies in the physico-chemical behavior of bacteria. The University of Missouri Studies, **3**, 1–84.

STEARN, E. W., AND STEARN, A. E. 1924 The chemical mechanism of bacterial behavior. I. Behavior toward dyes—factors controlling the gram reaction. J. Bact., **9**, 463–477.