The Effect of Moisture on Gram Differentiation, and its Relation to Proposed Gram-Positive Substrates in Yeast and Other Organisms

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SUMMARY

Wet and dry Gram staining procedures have different characteristics in respect to their rates of decolorization. It has been proposed that this difference is due to a Gram-positive substrate which dissociates (becomes Gram-negative) in the presence of water. However, the differences between wet and dry Gram procedures can also be explained on the hypothesis that water influences the rate of solvent permeation through cell envelopes. Since no direct proof exists for the reality of the proposed Gram-positive substrate, and since evidence is presented here which cannot be explained by such a hypothesis, it is felt that the solvent permeation concept should be considered seriously as an explanation for the decolorization differences observed between wet and dry Gram procedures.

INTRODUCTION

The mechanism of the differentiation of bacterial cells by the Gram staining procedure is not clear. Some believe that Gram differentiation may be a permeability or permeation phenomenon (Benians, 1920; Burke & Barnes, 1929; Bartholomew, Cromwell & Finkelstein, 1959; Salton, 1963); some believe it to be due to differences in basic dye uptake (Stearn & Stearn, 1924; Barbaro & Kennedy, 1954; Lamanna & Mallette, 1954); and some believe it to be the result of some specific Gram-positive chemical component in the cell such as lipids (Schumacher, 1928), proteins (Deussen, 1921), nucleic acids (Henry & Stacey, 1943), carbohydrate (Webb, 1948), sulfhydryl groups (Fischer & Larose, 1952) and glycerophosphate (teichoic acids; Mitchell & Moyle, 1950, 1954). This variety of proposed Gram-positive components leads to the suspicion that no single component can adequately explain the phenomenon of Gram positivity.

Chelton & Jones (1959) supported the concept of a Gram-positive substrate. They observed that ruptured cells of Gram-positive bacteria and yeast would stain Gram-negative when water wet slides were subjected to a Gram procedure, but Gram-positive if a dry slide was processed. Intact cell preparations gave a Gram-positive result with either procedure. They proposed, therefore, that Gram-positive organisms contain a substrate which could form a complex with dye and iodine. This complex remained undissociated (Gram-positive) in the presence of 90 % (v/v) ethanol in water, but became dissociated (Gram-negative) in the presence of more water such as with 70 % (v/v) ethanol in water. Water wet slides would supply

sufficient water to dissociate this complex, even though a 90 % (v/v) ethanol decolorizer was used. Intact cells would not be subjected to this difference between wet and dry slides since their intact cell envelopes enabled them to control the ethanol concentration inside of the cell so that it never fell below 90 %. The mechanism of Gram-positivity was interpreted, then, as a combination of the presence of a Gram-positive component, and selective permeability to ethanol of the intact cell envelopes.

Bartholomew (1962) showed that intact cells, both Gram-positive and Gram-negative, were influenced by wet and dry Gram procedures, as well as by the water content of the decolorizer, in respect to the rate of decolorization. This presents the possibility that the differences observed by Chelton & Jones for ruptured cells can be explained without resorting to the concept of a Gram-positive component. The present paper presents the results of experiments to test this possibility.

METHODS

In the duplication of the work of Chelton & Jones (1959) all cell preparations and staining procedures were as described by them. However, since it was desired to have a more quantitative comparison of decolorization characteristics than that used by Chelton & Jones, a Gram procedure described by Bartholomew (1962) also was used. Briefly, the latter involved the substitution of Hucker's crystal violet for 0.5 % methyl violet 6B, the use of 95 % (v/v) ethanol in water as a decolorizer rather than 90 % (v/v) ethanol in water and the use of a quantitative decolorization procedure. The quantitative decolorization procedure used 3 Coplin dishes (25 × 75 mm.) each filled with 70 ml. of ethanol + water and each used for one third of the total decolorization time, which was varied in each experiment from 5 sec. to 60 min. The water content of the decolorizer was prevented from increasing by use of a dish rotation system, in which after four slides the ethanol water mixture in the first dish was discarded, the dish refilled, and placed last in the sequence.

All organisms were from 24 hr cultures grown on an enriched nutrient agar, except for *Clostridium perfringens* which was grown on a semi-solid thioglycollate medium, and *Saccharomyces cerevisiae* which was grown on Sabouraud's agar, or obtained from yeast cakes. Organisms were disintegrated in a Mickle apparatus, with cooling in ice water after every minute of treatment. The time of treatment varied from 8 to 20 min. which was sufficient time, as observed with the microscope, to produce disruption of 90–99 % of the organisms. Centrifugation was done with a Servall Centrifuge at 3000 *g* for 15 min., followed by resuspension in ice water. After two such centrifugations and resuspensions in ice water the cell fragments were recentrifuged and only the upper portion of the pellet was resuspended in ice water. This suspension was centrifuged at 3800 *g* for 15 min. and the supernatant fluid decanted. After resuspension of the pellet in ice water, the suspension was used for preparation of the slides. In the reproduction of the Chelton & Jones experiments, the suspensions were very concentrated and resulted in areas in which cell fragments and other cell debris made the slide almost opaque. In our Gram procedures, the cell fragments were diluted so that they did not overlap.

In the dry Gram procedure the smears were treated with crystal violet solution, followed by iodine, washed in water, and then blotted and air dried. Just before

passage of these slides through the decolorizer, they were lightly flamed and cooled. In the wet Gram procedure, the smears were treated with crystal violet solution, iodine, then washed in water and processed wet through the decolorizer. In some experiments it was desired to have wet smears, but with no excess of water on the surface of the slide. For this, a modified wet Gram procedure was used. This consisted of staining the slides with crystal violet, treating with iodine, washing and then thoroughly drying. Following this, the slide was exposed to a strip of No. 1 Whatman filter-paper saturated with water. This wet filter-paper was pressed to the surface of the smear to assure moisture contact, and removed just before decolorization.

A Beckman model DB automatic recording spectrophotometer was used to obtain the light extinction curves.

RESULTS

The wet and dry Gram procedures gave different results for ruptured yeast cells, as reported by Chelton & Jones (1959). The last line of results in Table 1 shows that when using their procedure (procedure II, Table 1) ruptured yeast cells were Gram-positive when a dry procedure was used, but were Gram-negative when a wet procedure was used. Almost identical results were obtained, for a 2 min. decolorization time, when a quantitative Gram procedure (procedure I, Table 1) was used. The quantitative procedure, however, showed several additional facts. When the decolorization time was reduced to 15 sec. for the wet procedure, the yeast cell fragments could then be considered to be Gram-positive (as compared with the control organisms) despite the Gram-negative results obtained with a 2 min. decolorization time. Also, despite the Gram-positive nature of the ruptured yeast cells following a dry Gram procedure and a 2 min. decolorization time, they were Gram-negative following a 30 min. decolorization time. It was thus clear that the Gram-positive or Gram-negative characteristic of ruptured yeast cells, following a wet or dry Gram procedure, was a quantitative rather than a qualitatively determined characteristic.

Chelton & Jones reported that ruptured yeast cells stained Gram-negative with a dry Gram procedure when 70 % (v/v) ethanol in water was used as a decolorizer. They suggested that the 70 % (v/v) ethanol in water supplied sufficient water to allow the dissociation of the Gram-positive complex. It can be seen in Table 2 that when a dry Gram procedure and a 2 min. decolorization time were used ruptured yeast cells were Gram-negative when 70 % (v/v) ethanol in water was used as decolorizer. However, by decreasing the decolorization time to 30 sec., the ruptured cells were Gram-positive. According to the Chelton & Jones concept, a wet Gram procedure also using 70 % (v/v) ethanol in water as decolorizer would have two exposures to water sufficient to produce a Gram-negative result for ruptured yeast cells. Yet Table 2 shows that such a procedure produced a Gram-positive result when the decolorization time was decreased to 15 sec. It would appear that Gram differentiation was not solely a function of the effect of water on the presence or absence of a Gram-positive complex, but would be at least in part a function of the proper choice of a decolorization time to compensate for factors which influence rate of decolorization.

Chelton & Jones interpreted the Gram-negative results for ruptured yeast cells stained by a wet Gram procedure as due to the dilution of the decolorizer by the

Table 1. *A comparison of the Gram decolorization characteristic of intact Gram-positive and Gram-negative organisms with ruptured yeast cells, using wet and dry Gram procedures*

The Gram stain procedures used were those of Bartholomew (1962).													
Percentage of cells (or cell fragments) retaining the primary stain when stained by the indicated Gram procedure.													
Procedure	Total time of decolorization	Dry Gram procedure						Wet Gram procedure					
		Intact Gram-positive organisms			Intact Gram-negative organisms			Intact Gram-positive organisms			Intact Gram-negative organisms		
		Sl	Bs	Y	Ruptured yeast	Ec	Sm	Sl	Bs	Y	Ruptured yeast	Ec	Sm
5 sec.	100	100	100	100	100	10	100	100	100	100	100	10	5
15 sec.	100	100	100	95	20	10	100	100	100	100	95	0	0
30 sec.	100	100	100	90	10	0	100	100	100	100	20	0	0
60 sec.	100	100	100	90	0	0	100	100	100	100	0	0	0
2 min.	100	100	100	90	0	0	95	90	100	0	0	0	0
3 min.	100	100	100	70	0	0	95	85	100	0	0	0	0
6 min.	100	100	100	50	0	0	90	85	90	0	0	0	0
15 min.	95	95	100	40	0	0	20	70	80	0	0	0	0
30 min.	70	90	100	0	0	0	10	20	70	0	0	0	0
60 min.	20	40	100	0	0	0	0	0	60	0	0	0	0
2 min.	.	.	100	80	0	.	.	.	100	105	0	.	.

I = Bartholomew (1962) Gram procedure using Hucker's crystal violet, Burke's iodine, 95 % ethyl alcohol as the decolorizer, in 3 Coplin dishes, and 0.25 % safranin as counter stain.
II = As per Chelton & Jones (1959). Jensen's Gram procedure using 0.5 % methyl violet 6B, Lugol's iodine, 90 % ethyl alcohol on the slide for 2 min. to decolorize, and 1/20 Ziehl-Neelson carbol fuchsin as the counter stain.
Sl = *Sarcina lutea*; Bs = *Bacillus subtilis*; Y = yeast, *Saccharomyces cerevisiae*; Ec = *Escherichia coli*; Sm = *Serratia marcescens*. Wet Gram procedure = wet slide processed through the decolorizer. Dry Gram procedure = slide air dried and flame dried before processing through the decolorizer.

Table 2. *The effect of water in the decolorizer on the Gram decolorization characteristic of intact and ruptured organisms as shown by wet and dry Gram procedures and using 70 %, 95 % and 100 % ethyl alcohol as the decolorizer*

The numbers shown represent the percentage of cells or cell fragments which retained the primary stain (Gram-positive).

Total time of decolorization	Dry Gram 70% ethanol			Wet Gram 70% ethanol			Dry Gram 95% ethanol			Wet Gram 95% ethanol			Modified wet Gram 95% ethanol			Modified wet Gram 100% ethanol		
	Intact yeast	Ruptured yeast	Intact <i>E. coli</i>	Intact yeast	Ruptured yeast	Intact <i>E. coli</i>	Intact yeast	Ruptured yeast	Intact <i>E. coli</i>	Intact yeast	Ruptured yeast	Intact <i>E. coli</i>	Intact yeast	Ruptured yeast	Intact <i>E. coli</i>	Intact yeast	Ruptured yeast	Intact <i>E. coli</i>
5 sec.	100	100	95	100	95	0	100	100	100	100	100	10	100	100	20	100	100	10
15 sec.	100	95	10	100	90	0	100	95	20	100	95	0	100	95	0	100	90	0
30 sec.	100	95	5	100	20	0	100	90	10	100	40	0	100	10	0	100	10	0
60 sec.	100	50	0	100	0	0	100	90	0	100	0	0	100	5	0	100	5	0
2 min.	100	20	0	70	0	0	100	90	0	100	0	0	100	0	0	100	0	0
3 min.	100	0	0	50	0	0	100	70	0	100	0	0	100	0	0	100	0	0
6 min.	100	0	0	30	0	0	100	50	0	90	0	0	95	0	0	100	0	0
15 min.	100	0	0	10	0	0	100	40	0	80	0	0	85	0	0	95	0	0
30 min.	100	0	0	10	0	0	100	10	0	80	0	0	80	0	0	85	0	0
60 min.	100	0	0	5	0	0	100	0	0	70	0	0	70	0	0	70	0	0

The wet and dry Gram procedures were the same as procedure I, Table 1. Modified wet Gram procedure was the same as for the Dry Gram procedure. However, just before decolorization the smears were exposed to water by contact with a small piece of filter paper saturated with water. After pressing to the slide the paper was removed and the slide immediately processed through the decolorizer.

Table 3. *A comparison of wet and dry Gram procedures in the demonstration of the Gram characteristic of intact cells, and cell fragments*
 95 % (v/v) ethanol in water used as the decolorizer.

Total time of decolorization	Dry Gram procedure												Wet Gram procedure											
	Intact cells						Ruptured cells						Intact cells						Ruptured cells					
	Sa	Sl	Bs	Clp	Y	Ec	Sa	Sl	Bs	Clp	Y	Ec	Sa	Sl	Bs	Clp	Y	Ec	Sa	Sl	Bs	Clp	Y	Ec
5 sec.	100	100	100	100	100	100	+	+	+	+	+	+	100	100	100	100	100	100	50	+	+	+	+	+
15 sec.	100	100	100	100	100	20	+	+	+	+	+	—	100	100	100	100	100	100	0	+	+	+	+	—
30 sec.	100	100	100	95	100	10	+	+	+	±	+	—	100	100	100	100	100	100	0	—	+	+	—	—
60 sec.	100	100	100	90	100	0	+	+	+	±	+	—	100	100	100	100	80	100	0	—	—	—	—	—
2 min	100	100	100	85	100	0	+	+	+	±	+	—	60	95	90	70	100	0	—	—	—	—	—	—
3 min.	95	100	100	50	100	0	+	+	±	—	+	—	20	95	85	40	100	0	—	—	—	—	—	—
6 min.	50	100	100	50	100	0	±	+	—	—	+	—	20	90	80	30	90	0	—	—	—	—	—	—
15 min.	5	50	80	10	100	0	—	—	—	—	±	—	0	20	50	5	80	0	—	—	—	—	—	—
30 min.	0	20	50	5	100	0	—	—	—	—	±	—	0	20	20	0	80	0	—	—	—	—	—	—
60 min.	0	0	40	0	95	0	—	—	—	—	±	—	0	10	0	0	70	0	—	—	—	—	—	—

Wet and dry Gram procedures were the same as procedure I, Table 1. Figures given represent the percentage of intact cells staining Gram-positively. + = Significant amount of Gram-positive areas as described by Chelton & Jones (1959). — = All material Gram-negative. Sa = *Staphylococcus aureus*, Sl = *Sarcina lutea*, Bs = *Bacillus subtilis*, Clp = *Clostridium perfringens*, Y = Yeast, *Saccharomyces cerevisiae*, Ec = *Escherichia coli*.

water on the slide, such dilution supposedly resulting in dissociation of the Gram-positive complex. The wet Gram procedure was therefore modified to assure that the decolorizer was in no significant way diluted by water from the slide. The slide itself was dry except for the smear area which had been exposed to wet filter-paper. The slide was then processed through three Coplin dishes, each containing 70 ml. of 95 % (v/v) ethanol in water in some experiments, and absolute ethanol in other experiments. It would have been impossible for the water in the cells to have diluted the 95 % or 100 % ethanol to any significant degree. Yet under these conditions, as shown in Table 2, the results were typical for the usual wet procedure. Certainly, the decolorization differences observed between the modified wet procedures and dry procedure cannot be explained by water dilution of the decolorizer.

Table 3 presents the results obtained with intact and ruptured organisms of many species of Gram-positive bacteria, as compared with *Escherichia coli*. With the 2 min. decolorization time of Chelton & Jones, and 95 % (v/v) ethanol in water as decolorizer, the ruptured organisms were Gram-positive with a dry Gram procedure and Gram-negative with a wet procedure, as Chelton & Jones would have predicted. However, whether ruptured organisms were Gram-positive or Gram-negative depended on the decolorization time. With the dry Gram procedure, ruptured organisms were Gram-positive with a 2 min. decolorization time, but Gram-negative with a decolorization time of 15 min. or longer. With the wet Gram procedure, ruptured organisms were Gram-negative for a 2 min. decolorization time, but Gram-positive for a 15 sec. decolorization time. It would thus appear that, as for intact and ruptured yeast cells, the length of the decolorization time was more relevant to Gram differentiation than was the presence or absence of water in the smear. Our results which show the proper Gram differentiation of ruptured Gram-positive organisms are of particular interest since it has often been reported that such ruptured organisms are Gram-negative (Benians, 1912, 1920; Burke & Barnes, 1929). It can be seen (Table 3) that such differentiation would not have been detected if a wet Gram procedure were used with a single 2 min. decolorization time. However, it was detected with either a wet or dry Gram procedure, when a quantitative decolorization procedure was used. The ability to properly differentiate ruptured organisms demonstrates the fact that the cell envelopes by themselves could be an important contributing factor to Gram differentiation.

Chelton & Jones (1959) further supported their concept of a Gram-positive substrate as part of the mechanism of Gram positivity by presenting evidence which they believed disproved the concepts involving permeability. They (also Burke, 1922) reported that ethanolic solutions of the dye iodine precipitate would stain intact Gram-positive organisms. This was interpreted as showing that the large dye iodine molecule could permeate through the envelopes of Gram-positive organisms, therefore no permeability barrier to it existed. Such a barrier to the loss of the dye iodine precipitate inside of a Gram-positive organism, therefore, could not be used to explain the mechanism of Gram positivity. While such a barrier has been proposed (Benians, 1920; Burke & Barnes, 1929), this concept has long been discounted by Stearn & Stearn (1930) who reported that ethanolic solutions of the precipitate gave identical light extinction peaks as for ethanolic solutions of the iodine and crystal violet alone. This, along with boiling point data, led them to conclude that no new molecule was produced on precipitate formation, which on redissolution in ethanol

did not revert to the original dye and iodine molecules as independent entities. Stearn & Stearn did not publish light extinction curves and therefore such curves are shown in Fig. 1. It can be seen that the extinction curves for Burke's iodine, or crystal violet alone, were identical with those obtained when the dye iodine precipitate was dissolved in ethanol. Thus, the ability of ethanolic solutions of the precipitate

Fig. 1. Extinction spectra of Burke's iodine, crystal violet, and crystal violet + Burke's iodine precipitate dissolved in 95 % (v/v) ethanol in water.

to stain intact Gram-positive organisms would not be evidence against a permeability concept since the hypothetical large dye iodine molecule does not exist in ethanolic solutions. However, the non-existence of such a large dye iodine molecule does not destroy all permeability concepts, since permeability differences could exist for the dye or iodine molecules alone, or for the solvent itself.

DISCUSSION

It is not necessary to propose a Gram-positive component with the dissociation characteristics suggested by Chelton & Jones (1959) to explain the differences in decolorization speeds of wet and dry Gram procedures. Another explanation would be that water in the cell envelopes, or in the decolorizer, could have a marked speeding effect on decolorization. That water has this effect can be well documented (Benians, 1920; Burke, 1922; Burke & Barnes, 1929; Wensinck & Boevé, 1957; Tucker & Bartholomew, 1962; Bartholomew, 1962). Thus, if a single decolorization time were used for both wet and dry Gram procedures (as Chelton & Jones) the results obtained might be misleading concerning the Gram nature of an unknown, even though comparisons were made with known organisms. Table 1 shows that when a single 2 min. decolorization time was used, ruptured yeast cells appeared to be Gram-positive with a dry procedure, and Gram-negative with a wet, as compared to known controls. However, had a 15 sec. decolorization time been used, ruptured yeast cells would have appeared to be Gram-positive, and if a 30 min. time had been used Gram-negative, with both wet and dry procedures. In both examples, although the opposite Gram results were obtained the results appeared to be valid as compared to the control organisms included in the experiments. The Gram-positive (dry) and Gram-negative (wet) results reported by Chelton & Jones for ruptured yeast cells, then, were the result of the choice of a single decolorization time for two very different Gram procedures, rather than to any real change from a Gram-positive to a Gram-negative state. The true state of affairs as to the Gram nature of ruptured yeast cells is shown in Tables 1-3. Here it can be seen that with either wet or dry procedures ruptured cells of Gram-positive organisms decolorized faster than corresponding intact cells, and slower than either the intact or ruptured cells of Gram-negative organisms. Thus, the ruptured cells were more Gram-positive than intact Gram-negative cells, but more Gram-negative than intact Gram-positive cells. This fact could not be determined from the results obtained with only one decolorization time, but could be determined only by comparing the results obtained from a series of decolorization times. The question which arises then, is what criteria can be used to evaluate the validity of results obtained with any single Gram procedure. The answer is simple if one is only concerned with intact cells and if one closely follows the ordinarily recommended Gram procedures (Bartholomew, 1962). However, if one studies ruptured organisms, or used heat in staining, or changes the decolorizer or decolorization procedure, and if at the same time one sticks to a single decolorization time, then all that emerges blue is not necessarily Gram-positive. Some of the criteria for the recognition of true Gram-positivity (in addition to rate comparisons) have been presented by Bartholomew, Mittwer & Finkelstein (1959).

A Gram-positive substrate, with the dissociation characteristics proposed by Chelton & Jones, was not confirmed by our results. Wet Gram procedures which prevented any significant water dilution of a 100 % ethanol decolorizer gave results (Table 2, modified wet Gram) as expected for wet procedures, not dry. Thus, the rapid decolorization effect of 'wet' procedures could not be due merely to the dissociation of a Gram-positive complex when exposed to ethanol (70 %, v/v) diluted by water. Furthermore if, as proposed, the Gram-positive substrate is dissociated in the presence of 70 % (v/v) ethanol in water, then when such a decolo-

rizer is used it should not be possible to obtain a Gram-positive result for ruptured yeast cells. Yet Table 2 shows that this result could be obtained for both wet (15 sec. decolorization) and dry (30 sec. decolorization) Gram procedures. Our results do not support the suggestion that intact Gram-positive organisms would not be influenced by water in the decolorizer because of their ability to control the ethanol concentration inside of the cell. If this were true then intact Gram-positive organisms should not be influenced in their decolorization rate by wet or dry Gram procedures, or by water in the ethanol decolorizer. Our results, however, showed that all organisms, intact or ruptured, Gram-positive or Gram-negative, were decolorized more rapidly by a wet than by a dry Gram procedure. A very conclusive argument against the formation of a Gram-positive substrate dye iodine complex is the fact that it has been demonstrated that cells stained with iodine alone (Bartholomew, Cromwell & Finkelstein, 1959) or dye alone (Benians, 1920; Bartholomew & Mittwer, 1950) could be properly Gram differentiated if care were taken with the decolorization procedure. Thus, the Gram-positive characteristic could not be due entirely to any such proposed complex.

If a dissociating Gram-positive complex is not responsible for the differences between wet and dry Gram procedures, how can the differences be explained? They can be explained by a concept involving the permeation rate of solvent through cell envelopes. If the dye iodine precipitate were dispersed through the whole cell or cell fragments, and if ethanol permeated the cell envelopes of Gram-positive organisms, then ruptured as well as intact Gram-positive organisms should reflect this difference in their speed of decolorization. That is, intact organisms would decolorize slower than ruptured organisms due to the lesser cell envelope surface exposed for ethanol permeation, and to the greater amounts of dye iodine precipitate present in the intact cell as compared to the fragmented envelopes. Intact or fragmented Gram-positive organisms would be slower to decolorize than intact or fragmented Gram-negative organisms since the envelopes of Gram-negative organisms possess very little resistance to ethanol permeation.

Finally, Gram differentiation is basically a rate difference in the speed of decolorization between Gram-positive and Gram-negative organisms. Therefore, under conditions where fine differences must be detected, this rate difference must be quantitatively determined. Such quantitative determinations are not possible using the usual Gram procedures with a single decolorization time. If all of the known variables of the Gram stain procedure were kept constant (Bartholomew, 1962), then a single decolorization time known to give valid differentiations could be used with a fair degree of confidence. If, however, the known variables are changed, and if determinations of the Gram nature of an unknown substrate under different conditions are to be made, then the only valid experimental procedure is to use some form of a quantitative Gram procedure.

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