THE RELATION OF THE GRAM STAIN TO THE CELL WALL AND THE RIBONUCLEIC ACID CONTENT OF THE CELL

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The problem of the nature and location in the cell of the substrate responsible for the gram reaction has received a great deal of attention. Recent work has indicated the chemical nature of the gram-positive substrate to be nucleoprotein containing magnesium ribonucleate, and has lent support to the theory associated with Churchman (1927) which supposes the substrate to be in a special outer layer or "cortex" of the gram-positive organism (Henry and Stacey, 1943, 1946; Bartholomew and Umbreit, 1944). In the language of present-day cytology (Knaysi, 1944) this would mean that the gram-positive-staining nucleoprotein is present in the cell wall, since the cell wall is considered to be the ever-present outermost boundary of the living bacterial and yeast cell.

The observations locating the presence of gram-positive material in the cell wall have been of an indirect nature. For the most part, comparisons of sizes and appearances of gram-positive cells with gram-negative cells derived from them have been made. No attempt to stain the cell wall and gram-positive material of the same cell differentially has been recorded. Yet this would seem to be a highly desirable undertaking since it is a direct cytological approach, the results of which could lead to unequivocal conclusions. The obvious difficulty with such a direct approach has been the unavailability of techniques that would permit the superimposition of one staining procedure on another without washing out the reagents first applied. We have been happy to discover that the latest cell-wall-staining technique developed by Dyar (1947) admirably lends itself to overcoming this difficulty. By first applying the gram stain to a smear, it is then possible to apply to the same smear the Dyar cell wall technique without loss of the gram-positive elements. The use of counterstains is neglected in this application.

DIFFERENTIAL STAINING OF THE GRAM-POSITIVE MATERIAL AND CELL WALL

The Burke (1922) gram stain method has been employed. With the Dyar cell wall procedure two reagents are used, cetyl pyridinium chloride as a cationic mordant and Congo red as the dye. The fact that the cell wall stain could be imposed on a gram stain was determined by studying the appearance of *Bacillus cereus* and *Clostridium botulinum*, two organisms from which are easily obtained cultures and smears each containing a mixture of gram-positive, gram-negative, and irregularly staining cells. With these organisms it was noted that the cells staining gram-positive did not show a red-colored cell wall and that they were of the same size as cells in the same chain staining gram-negative. The latter gram-negative cells showed the red-staining cell wall characteristic of the Dyar

method. The cells staining irregularly appeared with a red-colored cell wall that was interrupted by black gram-positive granules, with these granules located at the periphery of the cell. These observations could only be interpreted to mean that, in a cell taking the gram stain reagents, the presence of these reagents in the cell wall prevented the visible uptake of cell wall stain but did not prevent any gram-negative portions of the cell wall from taking the cell wall stain. These observations, therefore, are evidence for the presence of material in the cell wall responsible for the gram reaction. However, they cannot be construed as indicating that the gram-staining material is exclusively limited to the cell wall. Our impression is that the material is not limited to the cell wall.

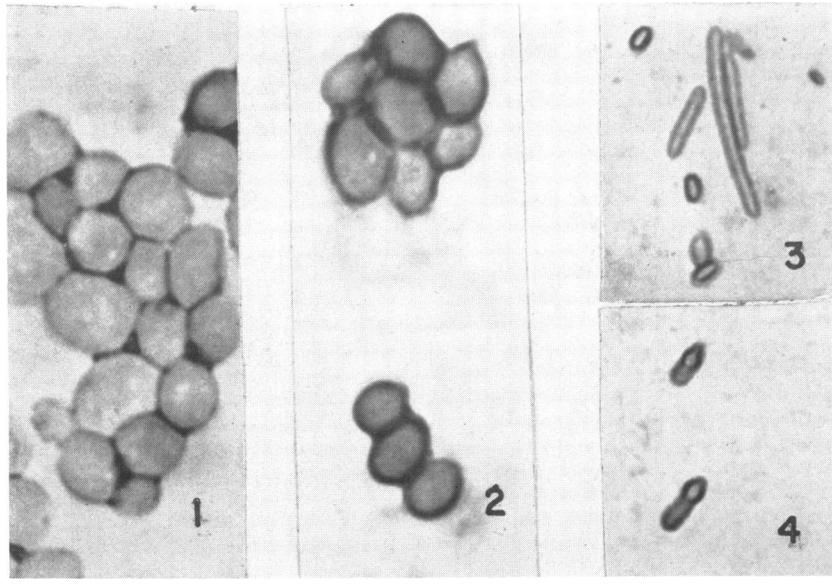

Figure 1. Cell wall of baker's yeast and bacterial spores stained by Dyar's method. 1. Normal yeast cells. × 2,200. 2. Yeast cells alkali-extracted by method of Chantrenne. × 2,200. 3. Spores and vegetative cells of *Bacillus brevis*. × 1,750. 4. Spores within vegetative cell. × 1,750.

When the cell wall stain was applied to bacterial endospores, all species studied showed an outer coat or cell wall taking the stain (figure 1). These results were obtained with spores still within the mother cell as well as with free spores. When the gram stain was followed by the cell wall stain, the bacterial spores stained only with the cell wall stain, a fact that was not unexpected. After the spores of *B. cereus* were autoclaved, it was found that the interior of the spores stained gram-positive while the spore coat continued to take the Dyar stain. The same result was obtained when germinating spores were studied. In both these cases, granules or the entire interior of the spore stained gram-positive while the coat took the Congo red. When the emerging vegetative cell became visible, the remnants of the spore coat continued to stain red, and the cell itself, if gram-positive,

showed no red rim, which would have been indicative of gram-positive material lying beneath rather than within the cell wall. Ascospores of the yeast *Schizosaccharomyces pombe* were also stained, and proved to contain a gram-positive interior surrounded by a thick cell-wall-staining material.

These results demonstrate that in spores the gram-positive substrate is not associated with the coat. Therefore, it is necessary to conclude that in nature the gram reaction is not invariably limited to materials deposited in the cell walls. Incidentally, the technique of using the gram stain followed by the cell wall stain would seem to offer new possibilities for the demonstration and study of the characteristics of spores during the various stages of their life cycle.

Vegetative cells of baker's yeast and *S. pombe* were also studied by the combined gram and cell wall stains. These cells stained intensely black with Burke's gram stain and showed no obvious cell wall stain. Thus the experience with bacteria was repeated, namely, that the Congo red of the cell wall stain is not visibly taken up when the gram reagents are already present in the cell wall. A variable number of cells, irrespective of the age of the yeasts, often showed a barely perceptible red rim, this appearance being especially evident at the ends of the cells. The appearance may indicate that the outermost edge of the cell wall was taking the cell wall stain. This does not contradict the conclusion that gram-staining material is present in the cell wall and, when specifically stained, interferes with the cell wall stain. The outermost molecular layer of the cell wall is probably of a complex chemical nature and includes molecules responsible for the characteristic staining properties of the cell wall as a whole. It is also pertinent in assessing the suggested significance of this variable and thin line of Congo red to consider that Dyar (1947) has found "the relatively thick cell wall of yeasts . . . to be accentuated by a red precipitate of dye-surface-active agent on its surface."

From the data presented it is obvious that, with cells stained gram-positive, exposure to the cell wall stain does not reveal a cell wall. If the correct conclusion from this type of observation is that the initial presence of the gram reagents prevents or conceals the uptake of the cell wall stain, then it should be possible to remove the gram stain reagents and simultaneously replace them by the cell-wall-staining reagents. This would constitute cytological evidence of the most direct sort for the presence of gram-positive-staining material in the cell wall. By a simple modification of the Dyar procedure it was possible to make the indicated observation.

In the typical cell-wall-staining procedure the mordant is added and followed by the Congo red in a rapid time sequence. But if smears of yeasts are subjected to the gram stain and then exposed to the cetyl pyridinium chloride mordant of the cell wall stain for varying lengths of time, it is found that the gram stain reagents are gradually washed away. Thus at any desired time Congo red can be applied to the smear already flooded with cetyl pyridinium chloride to see whether it restains the areas of the cell from which the gram stain reagents are washed out.

When this was done it was noted that the periphery of the cells lost the gram stain first and then was stainable with the Congo red. Furthermore, the interior of the yeast cell gave up the gram reaction more slowly than the edges; for the

most part it remained a faint blue color long after the periphery of the cell had lost the methyl violet, and it did not take up obvious quantities of Congo red. In a few cases the gram stain reagents washed out of certain areas in the edges of the cells in an irregular manner, so that the resultant red-colored cell wall had black spots or segments embedded in it. In many cell interiors there were present deeply stained gram-positive granules not easily decolorized by the procedure used. The appearances recorded can only lead to the conclusion that gram-staining material does constitute part of the structure of the cell wall, though it is not exclusively limited to the cell wall.

For additional information, commercially prepared baker's yeast was extracted with cold strong alkali at 5 C according to the method of Chantrenne (1946) for the extraction of ribose nucleic acid. Unstained treated cells appeared smaller than normal control cells. They stained as a mixture of gram-positive and gram-negative cells. By the Dyar method the treated cells appeared of undiminished size, the cell walls being intact and taking the Congo red.

After the alkali extraction the yeast cells were found to release further nucleic acid when exposed at 60 C to 2 per cent sodium desoxycholate. Of cells so treated almost all stained gram-negative. With the cell wall stain the cells appeared intact and of normal size, the only difference being that the treated cells seemed to stain more heavily and to present thicker cell walls (figure 1). Such an impression, if true, suggests that the extraction of materials from the periphery of the cell makes possible a greater uptake of Congo red by the cell wall.

LOSS OF RIBONUCLEIC ACID BY THE CELL WITHOUT LOSS OF THE GRAM REACTION

The work of Henry and Stacey (1943, 1946) and Bartholomew and Umbreit (1944) has shown the cellular substrate of the gram reaction to be nucleoprotein containing magnesium ribonucleate. The findings of these authors raise and leave unanswered the question as to whether all or only a fraction of the total ribose nucleic acid present in gram-positive organisms is associated with the gram reaction. An answer to the question should be forthcoming from experimental findings in attempts to extract ribonucleic acid from cells without loss of the gram reaction. If some nucleic acid can be removed from cells without loss of gram-positiveness, then a possible deduction is that ribose nucleic acid in gram-positive cells is not all bound with the nucleoprotein acting as the substrate for the gram reaction. Commercially prepared baker's yeast, which is known to be rich in ribonucleic acid and poor in desoxyribonucleic acid, was selected as a proper object for the type of study indicated.

Extraction of nucleic acid by recorded methods such as treatment with a bile salt (Henry and Stacey, 1946), alkali (Chantrenne, 1946), or ribonuclease (Bartholomew and Umbreit, 1944) is unsuitable since a complete or partial loss of gram-positiveness takes place. Therefore other methods were sought, and an extremely simple one was found. By merely shaking living cells with chloroform, ribose nucleic acid is released into the suspending fluid without any evidence of

the cells' loss of capacity to take the gram stain. Control batches of cells shaken under the same conditions except for the absence of chloroform did not release nucleic acids. The method was as follows:

Forty-five grams of moist baker's yeast cake free of any binder was added to 220 ml of distilled water with 6 grams of sodium chloride. Added salt in the range indicated improved the removal of nucleic acid. The acidity of the resultant yeast suspension fell in the range of pH 5.6 to 6.0. With 150 ml of reagent grade chloroform added, the suspension was stirred in a Waring blender for 5 minutes and then centrifuged. The cells collected as a compact gel at the bottom of the aqueous supernate that contained the extracted components of the cell including nucleic acid. The nucleic acid was precipitated from solution after the addition of 2 or 3 volumes of 95 per cent ethyl alcohol and adjustment to pH 3. The cells in the gel were resuspended and reshaken with chloroform as often as desired without any microscopic evidence of mechanical rupturing of the cell wall. The nucleic acid

TABLE 1

Yield of nucleic acid on successive shakings of baker's yeast with chloroform

SHAKING SAMPLES POOLED	WEIGHT OF ALCOHOL-PRECIPITABLE EXTRACT	ESTIMATED WEIGHT OF NUCLEIC ACID EXTRACTED	PER CENT PURITY OF NUCLEIC ACID EXTRACTED	YIELD OF NUCLEIC ACID PER SHAKING
	mg			*mg*
1	70	17	24	17
3, 4	100	85	85	43
5, 6, 7, 8	160	154	96	39
9, 10	50	41	82	20

These data were obtained on shaking 45 grams of commercially prepared baker's yeast cake. The alcohol-precipitated extract was air-dried and the quantitative estimate of nucleic acid was calculated from extinction coefficients determined with a Beckmann spectrophotometer at 258 mμ.

obtained was relatively free of impurities, and in some extractions of the order of 95 per cent pure based on calculations from absorbancy data obtained by spectral analysis.

Microscopic examination in a wet mount revealed that the cells shaken with chloroform appeared smaller than control untreated cells. The greenish-hued refractile cytoplasm was somewhat shrunken, and in some cells irregular in outline. By the cell wall stain the cell wall did not differ in appearance or size from normal cells, nor had the cells lost their capacity to take the gram stain in spite of the loss of nucleic acid. These observations were made after the first shaking, and did not change in any visible way with further shakings.

Table 1 lists data on the quality and amount of nucleic acid isolated by the shaking procedure. The first extract collected was always yellow in color, and contained less nucleic acid than the third and subsequent extractions. The second shaking yielded so little solid extract that in the experiment of table 1 it was not collected for study. Subsequent shakings yielded water-clear colorless extracts. The amount of acid-alcohol-precipitable material collected by successive shak-

ings eventually diminished progressively. In one experiment the fifteenth shaking and beyond yielded minimal amounts of nucleic acid.

What is the significance of these observations? The data, of course, show that the gram stain does not become negative with the loss from the total nucleic acid content of the yeast cell of the portion of nucleic acid released by chloroform shaking. But do these results mean that the ribonucleic acid extracted by chloroform shaking is not involved as substrate for the gram reaction? The only test available for noting loss of gram-positiveness is to stain the cell, a method that suffers from the serious disadvantage of being strictly qualitative. Loss of nucleic acid with a persistent positive gram stain might only mean that the amount of nucleic acid remaining was more than the sensitivity of the qualitative test, rather than the alternative that the nucleic acid removed was not involved in the gram reaction. The residence of the gram-positive character in the cell wall suggests that the nucleic acid associated with this property might be accessible to extraction by chloroform shaking. By the nature of the method one would expect that, if any cellular elements resist chloroform shaking by reason of location, it would most likely be those situated in the interior of the cell. With repeated shakings it should be possible eventually to deplete, at the least, the gram-positive-staining nucleic acid near the cell surface. This does not happen, nor do we have an explanation for the resistance of the gram-staining nucleoprotein. Repeated shaking eventually releases only about a third of the nucleic acid content of the cell, while leaving the gram-staining ability unaffected. There is, therefore, some experimental justification for the concept that cytologically the ribonucleic acid in the yeast cell has a plural nature. Since the cytological picture might well be a reflection of differences in the nature of the ribonucleic acids at the molecular level, future studies should be directed toward comparison of the physical and chemical properties of the ribonucleic acids extractable with and without the loss of the gram-positive character. The chloroform shaking method might well serve as one technique for supplying the raw materials of such a study.

SUMMARY

By combining the gram stain and the cell-wall-staining technique of Dyar, it was shown that the cellular substrate responsible for the gram reaction is partly located in the cell walls of vegetative cells. In the case of the bacterial endospore and yeast ascospore, the cell wall is free of gram-staining material even when the interior of the spore stains gram-positive.

Not all the ribonucleic acid of yeast cells is associated with the gram reaction. By shaking normal yeast cells with chloroform it is possible to extract nucleic acid without loss of the gram reaction. This raises a question as to the chemical similarity of the ribonucleic acids of the gram-positive cell.

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