The Gram Stain after More than a Century

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ABSTRACT. The Gram stain, the most important stain in microbiology, was described more than a century ago. Only within the past decade, however, has an understanding of its mechanism emerged. It now seems clear that the cell wall of Gram-positive microorganisms is responsible for retention of a crystal violet:iodine complex. In Gram-negative cells, the staining procedures damage the cell surface resulting in loss of dye complexes. Gram-positive microorganisms require a relatively thick cell wall, irrespective of composition, to retain the dye. Therefore, Gram-stainability is a function of the cell wall and is not related to chemistry of cell constituents. This review provides a chronology of the Gram stain and discusses its recently discovered mechanism.

Key words: Gram stain, crystal violet, iodine, gentian violet

The Gram stain was first described by Carl Friedlander, not by Christian Gram (Friedlander 1883). Friedlander was a colleague of Gram in a Berlin hospital and alluded to the now famous stain of his co-worker in a paper concerned with pneumococci. Just a year later, a complete description of the stain was published (Gram 1884). Gram must have shared his observations generously with his fellow worker prior to publication. The original Gram stain has been improved, but retains most of its early features (Burke and Ashenfelter 1926, Kopeloff and Cohen 1928). Basically, a clinical specimen (sputum, spinal fluid, etc.) or a microbial culture is gently heat-fixed (Churchman 1928) on a microscope slide. A few drops of crystal violet (Fig. 1) are then added to the specimen (the original stain used by Gram was gentian violet, a mixture of dyes, the main component of which was crystal violet). The unbound dye is then washed away with a water rinse and a source of iodide is added (KI, MgI_2, I_2:I-mixtures etc.). After a suitable period of time, usually 0.5–1.0 min, the slide is rinsed again with water. A solution of ethanol (95% in H_2O) or acetone (30% in absolute ethanol) is then added to the slide to remove unbound dye. The decolorizing solvent removes the dye from tissues and from some bacteria. The bacteria which retain the crystal violet stain are referred to as Gram-positive, whereas the cells which lose the dye are Gram-negative. Gram employed Bismarck brown as a counterstain to observe the Gram-negative cells. Safranin, which stains pink to nearly red, is the most commonly used counterstain today.

Factors Influencing the Gram Stain

Many factors influence the Gram stain (Table 1). Among these are the physical condition of the microorganisms (autolyzed bacteria tend to be Gram-negative), the nature of primary stains and counterstains, the nature of the iodide and/or other counterions, the temperature and time of fixation, the decolorizing solvent, and endogenous enzymes. The reviews of Biswas et al. (1970), Shugar (1962) and Bartholomew and Mittwer (1952) provide comprehensive discussions of the factors influencing the Gram stain.

The principal dye used in the Gram stain is crystal violet (Fig. 1), a basic (i.e., positively charged) dye. In the Gram stain a variety of basic dyes can be used, but none are superior to crystal violet (Bartholomew and Mittwer 1950, 1951). The stability of the crystal violet in solution is known to be enhanced by oxalate. In fact, Hucker (1921) used ammonium oxalate, a salt commonly used today, to increase the stability of crystal violet solutions. The oxalate does not, however, change the specificity of crystal violet.

The iodine:iodide in the Gram stain serves as a mordant. Basically, a mordant forms a complex with the primary stain and is typically

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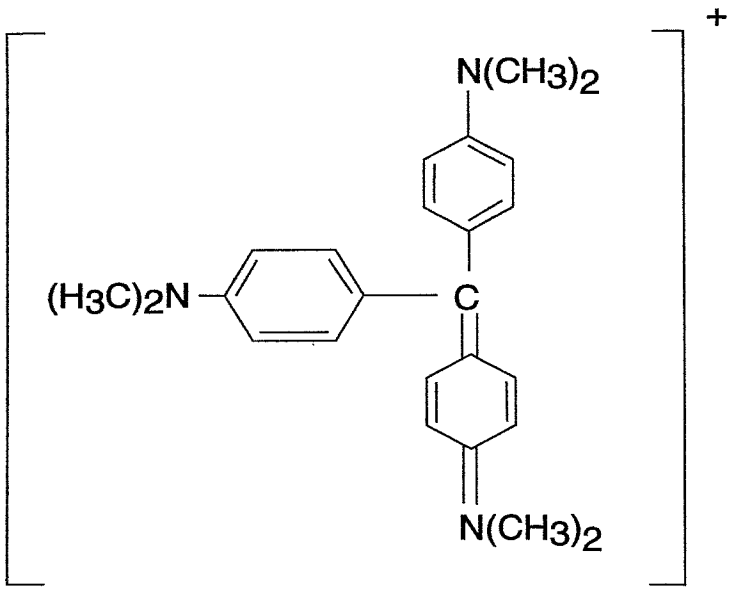

Fig. 1. Structure of crystal violet, the principal dye of the Gram stain. The stain may precipitate with various anions, including I_2 and organometal anions.

added to the stain prior to its application to cells or tissues. In the case of the Gram stain, the mordant is added after crystal violet has interacted with the cells. The iodide forms a precipitate with the basic crystal violet, whereas the iodine probably forms a charge transfer complex with the dye (Biswas et al. 1970). Various anions including picrate, phenolate, dichromate or permanganate, also form insoluble complexes with crystal violet, but the most reliable and convenient mordant is an iodine:iodide mixture (Mittwer et al. 1950).

The decolorizing agent can be absolute ethanol, a mixture of ethanol with acetone, ethyl ether, isopropyl alcohol and others (Conn 1928). Methanol can be used also, but it must be used with caution as it may remove too much crystal violet from Gram-positive cells.

New Stains for the Gram Character

It is now known that many carbohydrate-binding proteins, called lectins, can form complexes with cell walls of microorganisms (Doyle 1994). One of the lectins, wheat germ agglutinin (WGA), can bind both N-acetylglucosamine and N-acetylmuramic acid. With this in mind, Sizemore et al. (1990) surveyed numerous bacteria and observed that WGA aggregated only Gram-positive cells. These investigators concluded the amino sugar residues were readily available for interaction with WGA in the Gram-positive bacteria, but not in the Gram-negative cells. This is a logical conclusion, because the amino sugar-rich peptidoglycan of Gram-positive cells is usually exposed at the cell surface, whereas the peptidoglycan of the Gram-negative cells is covered by an outer membrane. Their survey did not cover all common Gram-negative bacteria, such as *Neisseria gonorrhoeae* or *Yersinia* and *Brucella* species. These Gram-negative bacteria rapidly aggregate in the presence of WGA (see Doyle 1994 for review). For this reason, it appears unlikely that WGA, or other lectins, will be of practical value in differentiation of Gram-positive and Gram-negative cells.

Noda and Toei (1992) observed that the anionic dye, tetrabromophenolphthalein (TBP), stained most bacteria. When the stained bacteria were mixed with the cationic octyltrimethylammonium (OTA), only Gram-negative cells retained the stain. Presumably, some kind of salt complex is retained by Gram-negative bacteria. In contrast to the standard Gram-staining procedure, the TBP-positive cells (retained stain) are Gram-negative, whereas the TBP-negative cells are Gram-positive. The TBP-OTA reagent appears to be a promising stain. The mechanism and specificity of this stain in animal cells, animal cells infected with intracellular bacteria, and yeasts would be of further interest.

Table 1. Chronology of the Gram Stain[a]

Observation(s)	Authors
Friedlander used Gram's unpublished method for studying pneumococci in lung	Friedlander (1883)
Gram first reported details of a stain for bacteria	Gram (1884)
First use of Gram stain in clinical microbiology laboratory	Roux (1886)
The Gram-positive staining depends on structure and integrity of cell membrane	Benians (1920)
Hucker's modification, now popularly used	Hucker (1921)
pH extremes yield poor Gram staining	Stearn and Stearn (1924)
Yeasts are Gram-positive	Henrici (1914)
Gram-positive bacteria in tissues can be readily identified by Gram stain	Lillie (1928)
Extruded protoplasm cannot be made Gram-positive. Cell wall is a factor in Gram staining	Burke and Barnes (1929)
Cell wall not a factor in the Gram reaction	Burke and Barnes (1928, 1929) Stearn and Stearn (1930)
Retention of dye-iodine by Gram-positive cells based on permeability of cell surface	Kaplan and Kaplan (1933)
Gram staining detected in thin sections	Krajian (1943)
Mg-ribonucleate contributes to crystal violet binding	Henry and Stacey (1943, 1946) Henry et al. (1945)
Cell walls are responsible for retention of dye	Lamanna and Mallette (1950)
Growth of bacteria in presence of penicillin decreases stainability. Mg-ribonucleate is not a factor in Gram staining	Mitchell and Moyle (1950)
1:2 Molar ratio of dye:iodine in Gram-positive and Gram-negative bacteria	Wensinck and Boevé (1957)
Uptake and retention of (^{131}I) greater in Gram-positive than Gram-negative bacteria	Shugar and Baranowska (1958)
Gram variability observed in broken suspensions of clostridia and yeast	Chelton and Jones (1959)
Gram-positive cells bind more iodine than Gram-negative bacteria	Bartholomew et al. (1959)
Treatment of microorganisms with 50–100% (v/v) ethanol results in greater loss of (^{32}P) labeled compounds from Gram-negative than from Gram-positive species	Salton (1963)
Efforts to quantitate Gram stain in microorganisms	Scherrer (1963) Shugar and Baranowska (1958) Smyth and Gershenfeld (1960)
Treatment with chloroform-methanol renders Gram-negative *Escherichia coli* Gram-positive	Basu et al. (1968a, b)
Crystal violet forms insoluble, electron-opaque complex with trichloro (η^2-ethylene)-platinum(II), permitting an electron microscopic investigation of the Gram reaction	Davis et al. (1983)
Ethanol removed electron opaque complex of crystal violet-trichloro (η^2-ethylene) platinum(II) from *Escherichia coli*, but not from the Gram-positive *Bacillus subtilis*	Beveridge and Davies (1983)
Gram-positive cells stressed at division sites tend to stain Gram-negative. S-layered bacteria were more difficult to decolorize than cells without S-layer	Beveridge (1990)
Wheat germ agglutinin aggregates only Gram-positive bacteria	Sizemore et al. (1990)
Methanospirillum hungatei, a peptidoglycan-deficient bacterium, exhibited Gram-positive staining at ends of filaments	Beveridge et al. (1991)
Tetrabromophenolphthalein stained Gram-negative, but not Gram-positive bacteria	Noda and Toei (1992)

[a] The authors have counted about 250 papers dealing with some aspect of the Gram stain. The papers cited above were arbitrarily considered by the authors to have had the greatest impact on the development of Gram stain. No effort is made in the text to discuss each of the papers in this chronology.

Mechanism of the Gram Stain

It has been recognized for years that retention of the Gram stain is a function of cell surface characteristics (Béguet 1929a,b, Burke and Barnes 1928, 1929) (Table 1). Retention of the stain is independent of cell surface isoelectric point (all bacteria except one are negatively charged) and of cell surface composition. Gram-positive fungi (Henrici 1914) have surface chemistries readily distinguishable from Gram-positive bacteria. Some members of the genus *Bacillus* (such as *B. anthracts)* possess no highly negative teichoic acids in their cell walls, but

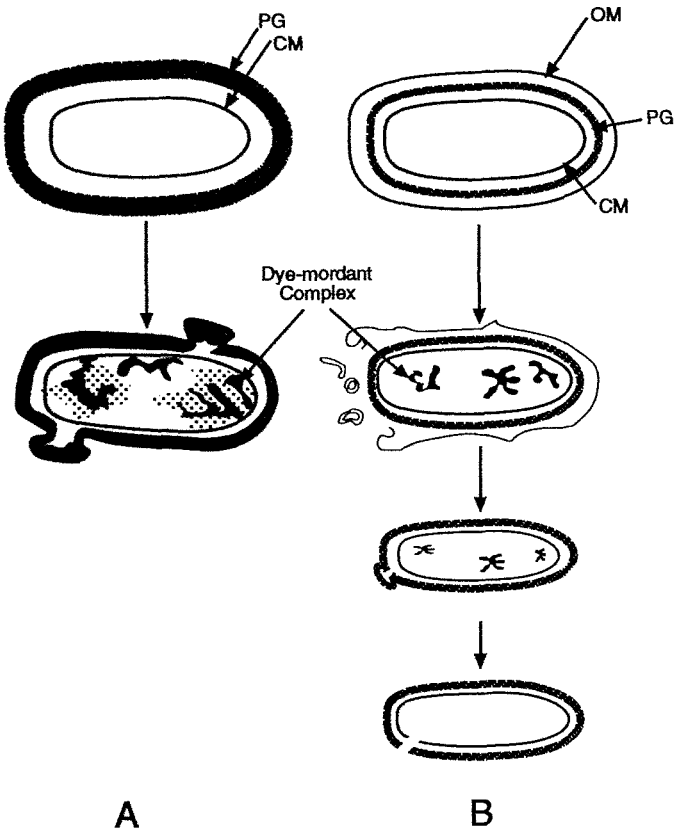

Fig. 2. Mechanism of the Gram stain. A) Gram-positive cells. When mixed with $I^-(-I_2)$ (or other counterion or mordant) an insoluble complex is formed. When alcohol or other nonaqueous solvent is added, uncomplexed dye is washed away. Only minor damage to the cell surface can be detected by electron microscopy. An insoluble dye, trichloro (η^2-ethylene)–platinum (II) complex, can easily be detected by electron microscopy. B) Sequence of effects of Gram staining on a Gram-negative cell. The chemistry of the stain is the same for both Gram-positive and Gram-negative cells. For the Gram-negative cell, decolorization results in loss of outer membrane integrity. The peptidoglycan layer is not thick enough to retard loss of the insoluble dye-mordant complex. The staining-decolorization procedures may damage the surfaces of both cell types, but the outer membrane and PG layers of the Gram-negative cell is more severely damaged. Basic features of the model for A and B above derived from Davies et al. (1983) and Beveridge and Davies (1983). PG, peptidoglycan; CM, cytoplasmic; OM, outer membrane.

they do contain uronic acid polymers called teichuronic acids. Other members of the genus *Bacillus* produce only wall teichoic acids and are free of teichuronic acids. Both kinds of bacilli are Gram-positive. Similarly, animal cells (Gram-negative) have surface chemistries far different from most Gram-negative bacteria. If not chemistry, the effects of Gram staining must be governed by physical properties, such as the porosity of various cell walls.

Work in the lab of Terry Beveridge (University of Guelph) beginning in the early 1980's and continuing to the present has produced a good understanding of how the Gram stain works (Beveridge et al. 1991, Beveridge and Davies 1983, Beveridge 1990, Davies et al. 1983). These investigators synthesized an electron-opaque mordant, trichloro (η^2–ethylene) platinum (II) (TEP) and studied its disposition following each Gram staining step. TEP served as a replacement for the $I:I_2$ mixture employed in the standard Gram staining protocol. The TEP formed an insoluble complex with crystal violet in aqueous solution analogous to the complex formed between crystal violet and $I:I_2$. Following each staining step, *B. subtilis* and *E. coli* were examined in the electron microscope for regions of high electron opacity. In some experiments, sections were made to study the site of deposition of the TEP. It was observed that in the Gram-negative *E. coli*, the outer membrane had been damaged, thereby permitting efflux of

Table 2. Cell Constituents Reported to Bind Crystal Violet or to Contribute to Gram Staining

Carbohydrates	Webb (1948)
Glycerol (or ribitol) phosphates	Mitchell and Moyle (1958) Benians (1920)
Glycerophosphates	Schumacher (1928) Mitchell and Moyle (1950, 1954)
Lipids	Eisenberg (1910) Schumacher (1928) Shugar and Baranowska (1957)
Lipoproteins	Stearn and Stearn (1924, 1930)
Nucleic Acids	Deussen (1921) Dubos and Macleod (1938) Henry et al. (1945) Henry and Stacey (1946) Webb (1948)
Peptidoglycans	Beveridge and Davies (1983)
Polyamines	Herbst et al. (1958)
Polysaccharides	Shugar and Baranowska (1957)
S-Layers	Beveridge (1990)

the dye:TEP complex. Damage to the cell surface of *B. subtilis* was also noted, but not to the degree that the dye:TEP complex could be washed away.

Figure 2 provides a diagrammatic summary of the effects of Gram staining and proposes a model to account for loss of dye or retention of dye. According to this model, any microorganism with a cell wall able to retard efflux of the dye:mordant complex should be Gram-positive. It is known that the peptidoglycan layer of Gram-positive bacteria is 10–15 times thicker than that of peptidoglycan-containing Gram-negative bacteria. Similarly, fungi have relatively thick cell walls and are Gram-positive. This mechanism requires that, regardless of the cell constituent to which crystal violet may bind (Table 2), the ultimate retention of Gram's stain is a function of physical properties of the cell wall unrelated to its chemical composition. The mechanism further implies that decolorization causes significant damage to the cell surfaces of Gram-negative bacteria, but only limited damage to the surfaces of Gram-positive organisms. This suggests that following Gram staining, Gram-negative bacteria are more permeable or "leaky" than prior to staining. Salton (1963) showed increased loss of ^{32}P-labeled compounds in Gram-negative bacteria, compared to unstained controls.

The model depicted in Fig. 2 also encompasses the phenomenon of "Gram variability." In cases of Gram variability, parts of a cell may appear Gram-positive, whereas other parts are Gram-negative. Beveridge (1990), using the chloroplatinate mordant and electron microscopy, observed that some bacteria known to be Gram-positive could not retain the dye-mordant complex at thin division sites. Another form of Gram variability may be related to unevenness in wall thicknesses, such as when autolysins (Doyle and Koch 1987) degrade certain sites during cell aging.

Many have attempted to correlate retention of the Gram stain with particular cell constituents. At one time it was thought that a Mg: ribonucleate complex was critical for Gram staining of bacteria (Henry and Stacey 1943, 1946, Henry et al. 1945). Herbst et al. (1958) suggested that polyamines were important in the Gram stain, whereas Mitchell and Moyle (1958) claimed that glycerol (or ribitol) phosphates (now known as monomers of teichoic acids) were critical for Gram retention. There is no doubt that crystal violet can complex with numerous cell constituents by ionic interactions, hydrophobic effects, or charge transfer complexes. It is no surprise that so many cell constituents have been reported to complex with crystal violet (Table 2).

The Gram stain has spawned a large volume of literature (Table 1). Numerous studies related to choice of stain, mordant, decolorizing agent, or counterstain are available. Factors such as temperature, concentration of crystal violet, mordant, decolorizing agent and counterstain have been published. Other factors, including the influence of enzymes, cell cycle and role of bacteriophages have been studied in detail. Attempts to ascribe Gram stainability to a particu-

lar cell component have been published, but the results are not convincing.

Electron microscopy, coupled with the synthesis of new electron opaque mordants, led to the current view of the mechanism of the Gram stain (Fig. 2) that only thick-walled organisms can be Gram-positive because the wall acts as a permeability barrier restricting diffusion of the crystal violet:mordant complex. A complicating factor in interpreting Gram stains and Gram-reactivity is illustrated by *Methanospirillum hungatei*, an archaeobacterium. Although it has a relatively thick cell wall (Beveridge et al. 1991), *M. hungatei* is Gram-negative because the dye is unable to enter the cell. In contrast, other archaeobacteria, possessing walls similar to those of the *Bacillaceae*, retain the Gram stain (Beveridge 1993). The latter observations show that Gram staining is not absolutely dependent on wall thickness.

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