Gram staining of extracellular material

During the course of studies on the role of proteins in the gram reaction[1,2] we found that the peripheries of several strains of organisms could be made markedly gram-positive as a result of the formation of salt-like complexes with added protein[3]. If these cells are subsequently mechanically ruptured the gram-positive peripheries retain the stain, in contrast to normal gram-positive organisms which are known to lose their staining ability when their structural integrity is destroyed[4]. As a result of this finding we were led to examine the staining properties of a variety of substances and of complexes formed between these substances and several proteins.

Reports have been made of positive gram staining of extracellular material, including fibrin[5], nucleoproteins[6], lipids[7], etc. These results have encountered some criticism on the grounds of failure to provide comparisons with known gram-positive cells and lack of presentation of details of staining techniques[8].

The materials we have examined were heat-fixed and stained according to the method of Hucker under conditions exactly analogous to those we use for staining of organisms. Furthermore we have been able to study the effect of enzymic and chemical hydrolysis on some substance, thus obtaining more convincing evidence of the staining characteristics of the native materials.

A typical example illustrating the technique used and results obtained is that of hyaluronic acid, which we found stained intensely gram-positive. To a neutral aqueous solution of highly polymerized acid (*ca.* 1–2 mg/ml) was added several μl of partially purified bacterial hyaluronidase. Immediately following this, and subsequently at intervals of 10–15 minutes, smears were made and heat-fixed on the same slide. After about 2 hours the preparations were stained and it was found that the intensity of staining decreased with degree of hydrolysis until the acid was practically gram-negative. Staining of the non-hydrolyzed acid, omitting either the crystal violet *or* the iodine, results in a gram-negative stain, thus demonstrating that we are dealing with a gram reaction and not a staining artifact. This latter point is important because starch, for example, stains intensely as a result of its affinity for iodine but this cannot be construed as evidence that it is gram-positive.

Several polysaccharides were found to be intensely gram-positive, including heparin, alginic acid, dextran, and sulfated dextran. In the case of dextran, the intensity of staining was found to decrease with increasing acid degradation. The mucopolysaccharide substrate of lysozyme, prepared from the cell walls of *Micrococcus lysodeicticus*, was found to be moderately gram-positive, while the partially insoluble complex formed by the addition of an excess of lysozyme stained more intensely, the intensity then decreasing with time as the polysaccharide was depolymerized. Glycogen was rather difficult to heat-fix on a slide, but appeared to be rather gram-negative.

Of several proteins examined, results varied from egg and serum albumins, which are negative, to protamine, a portion of which forms on the slide small positive granules, the remainder being only moderately positive. Gelatin is negative, but, in confirmation of the findings of MITCHELL AND MOYLE[9], the gel resulting from the treatment of gelatin with formaldehyde is intensely positive, although less so if not freshly made up. Surprisingly enough, glutamic acid retains the stain to some extent although we would not class it as clearly gram-positive as do GIANNI *et al.*[10].

Highly polymerized deoxyribonucleic acid was gram-negative as were its complexes with albumin and lysozyme. Apurinic acid[11], however, exhibited granules that stained intensely and fibrous material that was moderately positive. In the presence of magnesium ions, which are known to hydrolyse apurinic acid[11], the staining intensity decreased with time.

Of various samples of ribonucleic acid examined, two commercial preparations were intensely positive, even after hydrolysis with RNase to the point where all the RNA was acid-soluble, but after this point they began to lose the stain. However, the insoluble complex formed by the addition of lysozyme to this hydrolyzed RNA retained the stain much longer. One purified sample of RNA was gram-negative, while another purified, relatively highly polymerized sample was moderately positive and became less so when subjected to hydrolysis by RNase.

Lecithin was found to be intensely positive. We shall deal with the question of metaphosphates in connection with a current study on phosphate-starved microorganisms.

It is clear from the above that gram staining of extracellular material is by no means as unusual as has been assumed. A necessary corollary of this is that the extraction from bacterial cells of some component which itself stains gram-positive cannot be considered as proof that the gram-positivity of the original cells is due to this component (see [3,9,12]). An analogous conclusion follows implicitly from the fact observed as far back as 1920 that mechanically ruptured gram-positive cells no longer stain positively[4]. Similarly the demonstration in gram-positive cells of a larger proportion of some substance (such as the glycerophosphate complex of MITCHELL AND MOYLE[9]) does not necessarily mean that this component is responsible for gram staining, but only that it may be one of the manifestations of the differences in physiological and physicochemical characteristics between gram-positive and gram-negative organisms.

The results of enzymic and chemical hydrolysis of some of the substances examined points to the fact that their molecular structure plays some role in the Gram reaction. On the other hand, the fact that such a simple substance as glutamic acid retains the stain, even though only moderately, suggests that the gross structure of the heat-fixed material may be involved; the results obtained with protamine and apurinic acid support such a conception, although this is not the only possible explanation. Furthermore, the fact that dextran stains positively indicates that an ionic mechanism is not an essential feature of the gram reaction.

In any event it is likely that some of the above substances should prove useful in further investigations on the mechanism of gram staining, particularly if the observations can be made quantitative.

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