The Relationship Between the Nature of the Cell Wall and the Gram Stain

By M. R. J. SALTON*

Department of Bacteriology, University of Manchester

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SUMMARY

None of the classes of major chemical constituents of the walls of bacteria and yeasts is exclusive to organisms which give a Gram-positive reaction. Walls of Gram-positive bacteria are richer in mucopeptide than are those of Gram-negative bacteria. The latter are characterized by high lipid contents. Yeast walls are rich in polysaccharide complexes. There appears to be a broad correlation between the Gram reaction and the decreased leakage of ^{32}P compounds from labelled cells on exposure to increasing ethanol concentrations in the range 50–100 % (v/v) ethanol. The data on the release of ^{32}P compounds are in accord with the cell-wall ‘permeability’ mechanism for the Gram reaction and imply quantitative rather than all or none differences in Gram behaviour. Mechanical rupture of the cell wall or digestion of the wall with lysozyme rendered organisms which had been previously Gram stained in suspension by the technique of Wensinck & Boevé (1957), susceptible to decolorization with 96 % (v/v) ethanol in water.

INTRODUCTION

Although the Gram reaction is probably the most widely used staining procedure in bacteriology, there has been no wholly acceptable explanation of the mechanism of its reaction despite the great proliferation of theories advanced since the description of the technique by Christian Gram in 1884. Many of the earlier views of the mechanism of the Gram reaction were discussed in some detail by Bartholomew & Mittwer (1952). As pointed out by Salton (1961*a*) it is now virtually impossible to propose any new theory to explain the Gram reaction, for the most likely possibilities have been covered at some time or other and all of the major classes of cellular constituents have been implicated. The theories propounded from the studies of the mechanism of the Gram reaction fall into two major groups: (i) those which involve the presence of a particular substance or class of substances which confer Gram positivity; (ii) those which invoke a difference in the ‘permeability’ of the cells to the dyes.

Much effort in the past has been concentrated on searching for specific bacterial substances which may account for the Gram-positive reaction. Thus a positive response to the Gram stain has been claimed to be due to lipoprotein (Stearn & Stearn, 1924, 1930), lipids (Eisenberg, 1910; Schumacher, 1928), nucleoproteins and nucleic acids (Deussen, 1921; Dubos & MacLeod, 1938; Henry, Stacey & Teece, 1945; Henry & Stacey, 1946; Webb, 1948); carbohydrate (Webb, 1948) and

* Present address: Department of Microbiology, University of New South Wales, Kensington, N.S.W., Australia.

glycerophosphate substances (Schumacher, 1928; Mitchell & Moyle, 1950, 1951, 1954). Fischer & Larose (1952) suggested a similarity in chemical structure between highly degraded wool and the cytoplasmic membrane of Gram-positive bacteria. Other chemical constituents believed to be related to the Gram reaction in some unknown way include the polyamines found in greatest amounts in the Gram-negative group of bacteria (Herbst, Weaver & Keister, 1958). Evidence that the Gram reaction was related to Mg-ribonucleate in Gram-positive bacteria was presented by Henry *et al.* (1945) but an explanation of the stain reaction based on this proved less satisfactory when Mitchell & Moyle (1954) made a thorough investigation of the ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) contents of various bacteria. Further doubts that the Gram reaction was due to a Mg-ribonucleate + basic protein complex (Stacey, 1949) also came from the studies of Shugar & Baranowska (1957), reinforcing the earlier conclusion of Lamanna & Mallette (1950) that much of the nucleic acid can be extracted from yeast cells without loss of a Gram-positive reaction.

As an alternative to the mechanism of the Gram reaction based on a nucleic acid + basic protein complex as proposed by Stacey (1949), Mitchell & Moyle (1950, 1951, 1954) suggested a correlation between Gram positivity and the presence of phosphate esters ('XSP'). These phosphate esters were subsequently identified as mixed glycerol and ribitol phosphate polymers (Mitchell & Moyle, 1958), substances now well known as the teichoic acids of bacterial walls (Armstrong, Baddiley, Buchanan, Carss & Greenberg, 1958). The polyglycerophosphate of *Staphylococcus aureus* was given the name 'positic acid' by Mitchell & Moyle (1958). However, Jones, Rizvi & Stacey (1958) showed that most organisms (both Gram-positive and Gram-negative) contained 'XSP'. Thus it now seems unlikely that 'positic acid' could be directly responsible for Gram positivity.

The opponents of the idea that a specific cellular substance is responsible for the Gram reaction have sought other explanations, and 'permeability' factors have long been suggested as the basis of the differential staining observed. Thus, Burke & Barnes (1929) concluded that the Gram reaction was due to differences in cell-wall permeability. Further support of the 'permeability' theories have come from the studies of Kaplan & Kaplan (1933) and more recently from Bartholomew, Cromwell & Finkelstein (1959). The studies of Wensinck & Boevé (1957) on the pattern of extractibility with ethanol, of the crystal violet + iodine (CVI) complex from bacteria, provide convincing evidence that some 'permeability' effect is involved.

That the cell wall is involved in some manner seemed inescapable, especially from the loss of Gram positivity following mechanical crushing (Benians, 1920), lysozyme treatment of heated cells (Webb, 1948) or protoplast formation (Gerhardt, Vennes & Britt, 1956). Although Chelton & Jones (1959) have shown that disintegrated yeast cells can, under certain conditions, give a positive Gram reaction, it has been widely established that loss of Gram reaction occurs concomitantly with loss of cellular integrity (Bartholomew & Mittwer, 1952). Since many of the theories of the mechanism of the Gram reaction were proposed before the more recent studies of the chemistry of bacterial cell walls, it seemed worth while to re-examine the problem in terms of present knowledge of the surface structure of bacteria. In this paper, details of the composition of bacterial cell walls from the present investiga-

tion and from earlier studies (Salton, 1953*a*; Salton, 1958; Salton & Pavlik, 1960) have been considered in relation to the Gram reaction and a possible method for studying the mechanism of the Gram differentiation is presented.

METHODS

Organisms and growth conditions. Unless otherwise specified, the following organisms used were laboratory strains: *Alcaligenes faecalis* (NCTC 8764); *Bacillus brevis* (NCTC 7577); *B. cereus* P2; *B. megaterium* KM; *Candida pulcherrima* (NCYC 373); *Chromobacterium prodigiosum*; *Clostridium perfringens* (C. *welchii*); *C. sporogenes*; *Corynebacterium hoffmanni*; *Corynebacterium xerosis*; *Escherichia coli* B; *E. dispar* (NCTC 4169); *Klebsiella aerogenes* (*Aerobacter aerogenes*) A 30; *Lactobacillus arabinosus* 17/5; *Leuconostoc mesenteroides* P60; *Micrococcus lysodeikticus* (NCTC 2665); *Neisseria catarrhalis* (kindly provided by Dr S. E. Hartsell); *Proteus vulgaris*; *Pseudomonas fluorescens*; *Pseudomonas* sp. (kindly provided by Dr J. M. Shewan); *Saccharomyces cerevisiae* (NCYC 366); *Salmonella gallinarum*; *Spirillum serpens*; *Staphylococcus aureus* Duncan; *Streptococcus faecalis* (NCTC 6782); *Vibrio metchnikovii* (NCTC 8443).

The organisms were grown on the following media: Clostridia on Robertson's cooked meat medium at 37°; *Lactobacillus arabinosus* and *Leuconostoc mesenteroides* on 2 % Bacto Casitone + 0.1 % Marmite + 1 % glucose + 2 % agar at 30°; *Spirillum serpens* on 2 % Bacto Casitone + 0.1 % Marmite + 1 % glucose broth at 25°–28°; yeasts on malt agar at 25°; all other organisms were cultivated on nutrient agar or nutrient broth at 30° or 37°. The yeasts and *Spirillum serpens* were harvested after incubation for 2 days and all other organisms after 24 hr. Cultures of the spore-forming *Bacillus* species were harvested after incubation for 12–18 hr. The harvested organisms were generally washed three times with distilled water on the centrifuge. Organisms which showed any tendency to lyse under these conditions were harvested and washed in physiological saline.

For studying the leakage of ³²P compounds from organisms, these were grown under exactly the same conditions as specified above, except that the media were supplemented with ³²P as orthophosphate (Radiochemical Centre, Amersham, Buckinghamshire, England) in sterile saline solutions; 0.1 ml. sterile ³²P solution containing about 10⁷ counts/min. was added to 20 ml. portions of agar or liquid medium. The ³²P-labelled organisms were harvested and washed as described above.

Gram reaction. Smears were stained by the method modified by Hucker (1921). All smears were blotted dry before decolorizing with 96 % (v/v) ethanol in water. The Gram method applied to bacterial suspensions by Wensinck & Boevé (1957) was used as described by these authors with the modification of dissolving 0.9 g. Gurr's crystal violet in 100 ml. 0.067 M-phosphate buffer (pH 7).

Leakage of ³²P compounds from micro-organisms. The effect of ethanol concentration on the leakage of ³²P compounds from labelled organisms was studied in the following way. Washed suspensions of organisms were pipetted into 1 cm. × 7 cm. test tubes and 1 ml. water or saline (when bacteria suspended in this fluid) added to each tube and the organisms packed by centrifugation. The volume of washed suspension added was adjusted so that each tube contained about 3–5 mg. dry weight organism. After centrifugation, the supernatant liquid was carefully sucked

off with a pump so that the packed organisms were undisturbed, leaving a minimum of residual liquid in the tube (usually less than 0.05 ml.). Packed organisms were suspended in 1 ml. water or aqueous ethanol solution (concentrations of ethanol ranging from 25–100 %, v/v) and allowed to stand at room temperature (about 20°) for specified periods. Organisms were deposited by centrifugation for 10 min. at room temperature and the supernatant liquids further clarified when necessary by centrifugation. The leakage of ^{32}P compounds was determined with a Geiger counter by measuring the radioactivity of 0.2 ml. samples of supernatant fluid dried on planchettes as described by Roberts, Abelson, Cowie, Bolton & Britten (1955).

The time course of leakage in 96 and 100 % (v/v) ethanol was determined by preparing a series of test tubes containing ^{32}P -labelled organisms as described above, adding the ethanol and filtering off the organisms on Oxoid bacteriological membrane filters. Filtration was rapid (10 sec. for about 1 ml. cell suspension) thus permitting samples to be taken at times much shorter than would have been possible by using centrifugation for removal of the extracted organisms. The ^{32}P contents of the filtrates were determined in the usual way.

Isolation and composition of cell walls. Cell walls were isolated as described by Salton & Horne (1951). Analytical procedures used were those given in earlier studies (Salton, 1953*a*; Salton & Pavlik, 1960). 'Total lipid' was determined by extraction with ether after preliminary hydrolysis with 6 N-HCl for 2 hr. at 100° (Salton, 1953*a*). Amino sugar contents were estimated by the Randle & Morgan (1955) method after previous hydrolysis of cell walls for 2 hr. with 2 N-HCl at 100°.

RESULTS

Chemical composition of walls of Gram-positive and Gram-negative organisms

Extensive investigations of the chemistry of bacterial cell walls (Salton, 1961*b*) and yeast walls (Falcone & Nickerson, 1956; Kessler & Nickerson, 1959; Northcote & Horne, 1952) have been made during the past 10 years; analyses have been

Table 1. *Major classes of cell-wall constituents and the Gram reaction of certain micro-organisms*

Gram-positive organisms	Major chemical components of cell walls
Yeasts	
<i>Saccharomyces cerevisiae</i>	Polysaccharide, protein, lipid
<i>Candida</i> spp.	Polysaccharide, protein
Bacteria	
<i>Micrococcus lysodeikticus</i>	Mucopeptide
<i>Staphylococcus aureus</i>	Mucopeptide, teichoic acid
Streptococci	Mucopeptide, teichoic acid, polysaccharide
<i>Corynebacterium</i>	Mucopeptide, polysaccharide
Gram-negative organisms	
<i>Escherichia coli</i>	Protein, polysaccharide, lipid, mucopeptide
<i>Aerobacter aerogenes</i>	
<i>Pseudomonas</i> spp.	
<i>Spirillum serpens</i>	

performed on a large enough variety of Gram-positive and Gram-negative species to give a comprehensive picture of the major chemical constituents of walls from both groups. The principal types of chemical constituents found in walls of Gram-

positive and Gram-negative organisms are summarized in Table 1. One of the most conspicuous differences between the walls of Gram-positive and Gram-negative bacteria observed in the earlier studies (Salton, 1953*a*) was the higher lipid contents of walls of the latter group. The amino sugar contents of the walls of Gram-positive bacteria were generally higher than those of Gram-negative organisms, such a difference probably being a reflexion of the total amount of mucopeptide (peptidopolysaccharide or glycopeptide) component in the wall. Thus, as suggested by Salton (1958) a 'spectrum' of lipid and amino sugar contents exists and quantitative results for the walls of a variety of Gram-negative bacteria are compared with the range observed for Gram-positive organisms (Salton & Pavlik, 1960) in Table 2.

Table 2. *Amino sugar and lipid contents of cell walls of Gram-negative and Gram-positive micro-organisms*

	Amino sugar (%)	Total lipid (%)
Gram-negative		
<i>Vibrio metchnikovii</i> *	1.8	11.2
<i>Aerobacter aerogenes</i>	2	14.6
<i>Chromobacterium prodigiosum</i>	2	12.8
<i>Escherichia coli</i> *	3	20
<i>Salmonella gallinarum</i>	3.5	22
<i>Proteus morganii</i>	4.1	—
<i>P. vulgaris</i>	5.1	17.6
<i>Escherichia dispar</i>	5.0	12.6
<i>Spirillum serpens</i> *	6.8	—
<i>Alcaligenes faecalis</i>	7.6	—
<i>Neisseria catarrhalis</i>	8.1	12.2
<i>Bacillus brevis</i>	14.5	2.7
Gram-positive		
<i>Candida pulcherrima</i>	1.2	5.4
<i>Saccharomyces cerevisiae</i> *	1.2	8.5-10
<i>Staphylococcus aureus</i>	17	—
<i>Bacillus megaterium</i>	18	0
<i>Micrococcus lysodeikticus</i> *	16-22	0
<i>Bacillus cereus</i> *	31-32	0

* Data from earlier studies: Northcote & Horne (1952); Salton (1953*a*, 1958); Salton & Pavlik (1960); Salton & Shafa (1958).

In considering the chemical nature of the cell wall in relation to the Gram reaction (Table 1), it became apparent that Gram positivity could not be correlated with the presence of one particular type of substance in the cell wall. Thus, the teichoic acids (Armstrong *et al.* 1958) are present in only certain Gram-positive bacteria, and similarly, polysaccharide components are found in some and not in others (Salton, 1961*b*). All of the Gram-positive bacteria contain mucopeptides (glycopeptides) and all of the yeast walls are rich in polysaccharides and polysaccharide complexes (Northcote & Horne, 1952; Kessler & Nickerson, 1959). Although the walls of Gram-negative bacteria were rich in lipids, the lipid content of yeast walls may be as high as 10 % and as low as 1 % (Kessler & Nickerson, 1959). These considerations of the chemistry of cell walls led to the conclusion that the Gram reaction is not due to the presence of any specific substance in the wall and that several types of polymeric substances may serve equally well as wall structures for Gram-positive organisms.

Influence of ethanol concentration on the leakage of ^{32}P compounds across the walls of Gram-positive and Gram-negative bacteria

In their analysis of the Gram reaction Wensinck & Boevé (1957) showed that the amounts of crystal violet and iodine taken up was similar for Gram-positive and Gram-negative organisms. The main divergence in behaviour was found in the extractability of the CVI complex from the stained organisms washed in aqueous ethanol solutions ranging from about 90–100 %, v/v. Thus under these conditions the CVI in Gram-positive organisms remained largely unextractable despite the fact that the CVI complex formed by mixing solutions of the Gram reagents was completely solubilized in such concentrations of aqueous ethanol. If the CVI is not rendered alcohol-insoluble by being bound to specific chemical constituents present in only Gram-positive organisms then other explanations of the results of Wensinck & Boevé (1957) would have to be sought. As an alternative to the possession of a specific 'Gram-positive' chemical entity it seemed conceivable that the Gram differentiation brought about by decolorizing with 96 % ethanol may result from a dehydration of the wall structures and consequent decrease in pore size, thus impeding the passage of small molecules across the wall and rendering the CVI complex inaccessible to extraction. Any information on the passage of small molecules across the walls (release from inside as well as passage from the external medium into the cell) when organisms are suspended in ethanol concentrations used in Gram differentiation may lead to a greater understanding of the mechanism of the Gram reaction.

To test the possibility that the passage of small molecules across the outer envelope is impeded when Gram-positive organisms are exposed to ethanol concentrations which bring about the Gram differentiation, the release of ^{32}P compounds from organisms suspended in aqueous ethanol solutions (25–100 %, v/v) was investigated. The influence of ethanol concentration on the leakage of ^{32}P compounds from a variety of Gram-positive and Gram-negative bacteria was studied; the results for two Gram-positive organisms (*Saccharomyces cerevisiae* and *Streptococcus faecalis*) and two Gram-negative bacteria (*Escherichia coli* and *Proteus vulgaris*) are illustrated in Figs. 1 and 2, respectively.

It was conceivable that the decreased leakage of ^{32}P compounds from Gram-positive organisms might have been due simply to a smaller fraction of the cells having had their plasma membranes damaged by the treatment. That this explanation of the type of results given in Figs. 1 and 2 was unlikely was shown by the results of the following experiments. ^{32}P -labelled *Staphylococcus aureus* was treated with ethanol solutions, the organisms deposited by centrifugation and the supernatant fluids removed. The organisms were then suspended in distilled water and the release of residual ^{32}P compounds determined after 10 min. at room temperature. *Escherichia coli* was treated in the same manner. The results for *S. aureus* and *E. coli* are presented in Figs. 3 and 4, respectively; they show that the larger fraction of ^{32}P material 'trapped' inside *S. aureus* treated with ethanol concentrations between 80–100 % readily leaked out when the organisms were subsequently suspended in water.

To test the possibility that the differences in the patterns of ^{32}P leakage in ethanol solutions were not due to a slower release from Gram-positive bacteria, the

time course of leakage from Gram-positive and Gram-negative bacteria was studied. Organisms of *Escherichia coli* and *Staphylococcus aureus* were dispersed in 100 % ethanol and filtered as described in the Methods, at intervals of time up to 20 min. The time course for the release of ^{32}P compounds from *S. aureus* and *E. coli* are shown in Fig. 5. Although a smaller fraction of the total ^{32}P content leaked out from *S. aureus* in 100 % ethanol, the fraction was released at about the same speed as that observed for *E. coli*.

Fig. 1

Fig. 2

Fig. 1. Leakage of ^{32}P compounds from *Streptococcus faecalis* and *Saccharomyces cerevisiae*. Organisms suspended in ethanol solutions for 10 min. at room temperature.

Fig. 2. Leakage of ^{32}P compounds from *Escherichia coli* and *Proteus vulgaris*. Organisms suspended in ethanol solutions for 10 min. at room temperature.

Fig. 3

Fig. 4

Fig. 3. Leakage of ^{32}P compounds from *Staphylococcus aureus* suspended in ethanol solutions for 10 min. at room temperature (curve 1) and the subsequent release of ^{32}P compounds into distilled water when deposits of cocci from curve 1 samples were suspended in distilled water for 10 min. at room temperature (curve 2).

Fig. 4. Leakage of ^{32}P compounds from *Escherichia coli* suspended in ethanol solutions for 10 min. at room temperature (curve 1) and the subsequent release of ^{32}P compounds into distilled water when deposits of organisms from curve 1 samples were suspended in distilled water for 10 min. at room temperature (curve 2).

Experiments were performed with a collection of Gram-positive and Gram-negative organisms to determine the release of ^{32}P compounds in 100 % ethanol. The leakage in 100 % ethanol was determined in relation to the maximum leakage occurring at lower ethanol concentrations (usually at 50–75 %, v/v, ethanol in

water) and has been expressed as a percentage of the maximum release in the results summarized in Table 3.

These differences between the Gram-positive and Gram-negative organisms only establish that the passage of certain small molecules (inorganic phosphate, nucleotides, etc.; Salton, 1951) across the wall or wall-membrane of Gram-positive bacteria is impeded when they are suspended in high concentrations of ethanol, whereas many of the Gram-negative bacteria were affected to a lesser degree. This finding in itself gives not more than a clue about a likely mechanism of the Gram reaction and suggests that the crystal violet + iodine complex is 'trapped' inside the organisms when the 'permeability' of the outer wall is decreased on treatment with concentrations of ethanol exceeding 90 %.

Fig. 5. The time course for the release of ³²P compounds from *Escherichia coli* and *Staphylococcus aureus* suspended in 100 % ethanol at room temperature.

Unfortunately the role of mordanting with iodine in the Gram reaction could not be investigated in relation to the leakage of ³²P at different ethanol concentrations, as pretreatment of the bacteria with the iodine solution resulted in an immediate loss of intracellular ³²P compounds from Gram-positive and from Gram-negative bacteria. It seems likely that a more successful approach to the problem of the role of mordanting with iodine might be to study the penetration of small molecules into organisms suspended in ethanol before and after treatment with iodine solutions.

Effects of mechanical rupture and wall removal on extractability of crystal violet + iodine complex from suspensions of Gram-stained organisms

If, as the above results and the iodine permeability effects reported by Kaplan & Kaplan (1938) and Bartholomew *et al.* (1959) suggest, the pore size of the wall of Gram-positive bacteria is decreased by mordanting with iodine and dehydration

with 95 % ethanol, thereby trapping the crystal violet+iodine (CVI) complex within its boundary, then mechanical rupture or enzymic removal of the wall after Gram staining should then render the CVI complex accessible to extraction.

Table 3. *The relationship between the Gram reaction and the leakage of ^{32}P compounds from labelled bacteria exposed to about 100 % ethanol*

Gram reaction	Organism	^{32}P released (%) relative to maximum*
—	<i>Pseudomonas</i> sp.	96
—	<i>Proteus vulgaris</i>	90
—	<i>Escherichia coli</i>	84
—	<i>Alcaligenes faecalis</i>	78
—	<i>Vibrio metchnikovii</i>	75
—	<i>Salmonella gallinarum</i>	75
—	<i>Aerobacter aerogenes</i>	75
—	<i>Chromobacterium prodigiosum</i>	69
—	<i>Neisseria catarrhalis</i>	65
—	<i>Pseudomonas fluorescens</i>	60
—	<i>Spirillum serpens</i>	56
—	<i>Escherichia dispar</i>	54
—	<i>Bacillus brevis</i>	54
+	<i>Leuconostoc mesenteroides</i>	43
+	<i>Clostridium perfringens</i>	36
+	<i>Micrococcus lysodeikticus</i>	35
+	<i>Candida pulcherrima</i>	34
+	<i>Streptococcus faecalis</i>	33
+	<i>Clostridium sporogenes</i>	30
+	<i>Lactobacillus arabinosus</i>	30
+	<i>Staphylococcus aureus</i>	26
+	<i>Bacillus megaterium</i>	22
+	<i>Corynebacterium hoffmanni</i>	17
+	<i>C. xerosis</i>	13
+	<i>Bacillus cereus</i>	11
+	<i>Saccharomyces cerevisiae</i>	10

* Maximum ^{32}P released from organism usually occurred in 50–75 % (v/v) ethanol in water solution.

Heated washed suspensions of *Bacillus megaterium*, *Micrococcus lysodeikticus* and *Saccharomyces cerevisiae* were Gram stained as described by Wensinck & Boevé (1957). The Gram-positive organisms were washed several times with 96 % ethanol (no counter stain used), the supernatant solutions decanted off and the packed organisms drained before dispersing in distilled water. These suspensions were mixed with Ballotini beads and shaken in the Mickle apparatus under the conditions normally used for cell-wall isolation (Salton & Horne, 1951). The beads were separated by allowing them to settle and after several washes with distilled water the fluid containing the disintegrated organisms was centrifuged. The deposits from disintegrated organisms and untreated Gram-positive organisms were suspended in 96 % ethanol and centrifuged again. On extraction with 96 % ethanol the control organisms not submitted to mechanical disintegration still remained strongly Gram-positive whereas the material from the ruptured organisms was decolorized.

Salton (1953*b*) showed that the walls of heated *Bacillus megaterium* and *Micrococcus lysodeikticus* were removed on incubation with egg-white lysozyme, leaving

the coagulated protoplasmic bodies. Similar experiments were performed with suspensions of organisms Gram stained by the method of Wensinck & Boevé (1957). After decolorizing by washing several times with 96 % ethanol on the centrifuge, the packed organisms were drained and suspended in 0.067 M-phosphate buffer (pH 7) and incubated with 50 μ g. lysozyme/ml. for 1 hr. at 37°. Control preparations without lysozyme were incubated under the same conditions. Lysozyme-treated and untreated organisms were then deposited by centrifugation, the supernatant fluids decanted, the deposits washed with distilled water and the washing fluid drained off. The deposits were then dispersed in 96 % ethanol and the organisms sedimented by centrifugation. Under these conditions only residual amounts of crystal violet remained in the deposits from the lysozyme-treated preparations; the organisms not exposed to lysozyme action were still strongly Gram-positive.

DISCUSSION

Burke & Barnes (1929) suggested that the Gram reaction could be explained by the impermeability of the walls of Gram-positive bacteria to the crystal violet + iodine complex. Both before and since the proposal of this mechanism, attempts have been made to relate the Gram reaction to specific chemical compounds in Gram-positive bacteria. Convincing correlations between the presence of certain cellular constituents and a positive Gram reaction have ultimately broken down and as pointed out by Shugar & Baranowska (1957) the demonstration of certain substances in Gram-positive organisms '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 physico-chemical characteristics between Gram-positive and Gram-negative organisms'. Indeed, a wide variety of simple and polymeric substances can behave as Gram-positive material (Shugar & Baranowska, 1957).

Strong support for the 'permeability' mechanism of the Gram reaction came from the earlier studies of Kaplan & Kaplan (1933) and more recently from the work of Bartholomew *et al.* (1959). A clearer interpretation of the mechanism of the Gram reaction has only been possible as a result of the studies of Wensinck & Boevé (1957) who were the first to provide convincing quantitative information about the sequence of steps in the Gram reaction and the points of departure in behaviour between the Gram-positive and Gram-negative organisms on decolorization with ethanol. The similarity between their patterns of extractability of crystal violet + iodine (CVI) complex and the release of ^{32}P compounds at different ethanol concentrations shown in the present investigations (Figs. 1, 2), is striking. Thus, the ethanol concentration used as the differentiating step in the Gram stain decreases the accessibility of the CVI complex and the same concentrations have been shown to impede the passage of intracellular ^{32}P compounds across the walls of Gram-positive organisms. These results together with the observations on disrupted Gram-stained organisms involving either simple mechanical damage or enzymic removal of wall, strongly support the view that a Gram-positive reaction is due to the CVI complex being 'trapped' within the organisms owing to dehydration and diminution in pore size of the walls of Gram-positive organisms. It also appears likely that mordanting with iodine could have a twofold effect of forming a CVI complex as well as an

association between iodine atoms and OH groups of polysaccharide (composed of sugars and/or amino sugars) components of the walls. This might further decrease the pore size on dehydration of the structure in ethanol, in a similar manner to the decrease in the internal diameter of the starch helix which accompanies the association of the large iodine atom with this polysaccharide (Greenwood, 1956).

If the Gram reaction is thus due to the physico-chemical state of the wall during differentiation with 96 % ethanol, then many of the conflicting results of earlier investigation can be readily understood. Any breach in the mechanical integrity of the wall either before or after Gram staining would render the CVI complex more accessible to extraction. Gerhardt *et al.* (1956) indeed attempted such a conclusive experiment with *Bacillus megaterium* but their results were not entirely satisfactory and not as clear cut as the data obtained from washed Gram-stained suspensions. The loss of Gram positivity on disintegration, autolysis and digestion of heated organisms with lysozyme (Webb, 1948) and even the effects of ageing, are all readily understandable if an intact rigid wall is needed as a barrier for the retention of the CVI complex. The presence of cell-wall degrading enzymes in bacteria has been well established (Mitchell & Moyle, 1957; Salton, 1956) and could account for the conversion of bacteria to the Gram-negative state.

It is now generally agreed that the Gram reaction is not an 'all or none' phenomenon, for gradations between Gram-positive and Gram-negative extremes have long been recognized (Neide, 1904; Churchman & Siegel, 1928; Shugar & Baranowska, 1958). Some of these quantitative differences may well be accounted for by the presence of more mucopeptide component in the walls of organisms within the Gram-negative group. The amino sugar content of the walls is probably a good index of the amount of cell-wall mucopeptide and it is of interest to note that several of the organisms intermediate between the extremes of ^{32}P leakage in ethanol (Table 3) also contain appreciable quantities of amino sugar in the walls (Table 2). Thus the total amount of mucopeptide and polysaccharide complex in the walls of bacteria and yeasts may have an important bearing on the quantitative aspects of the Gram reaction. The physicochemical state of the wall mucopeptides and polysaccharides may also be of considerable importance in determining the response in the Gram reaction. It appears likely that the mucopeptide polymers in the walls of Gram-positive bacteria form thick continuous sheets, whereas in a Gram-negative organism such as *Escherichia coli* the mucopeptide component responsible for the rigidity of the wall is present as a very thin layer (Weidel, Frank & Martin, 1960). Gram-variable properties may thus be explicable on the basis of mucopeptide layers of thickness intermediate between those found for the two extremes of Gram-positive and Gram-negative bacteria. More information about the physico-chemical structure of microbial cell walls is obviously needed before a clearer picture will emerge of the effects of the differentiation with ethanol which leads to a Gram-positive or Gram-negative reaction; and the role of iodine and the possible groups in the wall with which it may associate must be further investigated.

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