STUDIES OF THE BACTERIAL CELL WALL

VII. MONOSACCHARIDE CONSTITUENTS OF THE WALLS OF GRAM-NEGATIVE BACTERIA

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SUMMARY

The DISCHE reaction and paper chromatography have been used in detecting and determining the distribution of heptoses and other monosaccharide constituents in walls of Gram-negative bacteria and in wall-fractions obtained after removal of capsular polysaccharide, and after extraction with diethylene glycol, trichloroacetic acid and treatment with lysozyme and 1 % acetic acid. Heptoses were found in the lysozyme-insoluble fractions of walls containing these sugars. Diethylene glycol and trichloroacetic acid gave polysaccharides in soluble and insoluble fractions whereas treatment of walls with 1 % acetic acid releases most of the polysaccharide. It is concluded that the "surface" antigenic polysaccharides are part of the lipopolysaccharide-protein portion of the wall.

Unknown compounds which do not appear to be 3,6-dideoxy sugars have been detected in the walls of *Alcaligenes faecalis* and *Rhodospirillum rubrum*.

INTRODUCTION

The chemical constitution of the walls of Gram-negative bacteria is more complex than that of walls of Gram-positive organisms[1,2]. Mucocomplex substances (mucopeptides and mucopolysaccharides) together with the teichoic acids[3] may account for the entire cell-wall structure of many Gram-positive organisms, whereas the mucopeptide moiety of the walls of Gram-negative bacteria may constitute only a small percentage (10–20 %) of the weight of the cell wall[4,5]. Thus, the major groups of substances in the walls of Gram-negative bacteria include proteins, lipids, polysaccharides and mucocomplex components.

The monosaccharide constituents of the "surface antigens" of Smooth strains of Gram-negative bacteria[6] have been detected in isolated cell walls[1]. Dideoxy sugars[7,8] and heptoses[9,10] have been isolated from Smooth and Rough antigenic, bacterial polysaccharides respectively. WEIDEL[11] isolated a heptose from the wall of *Escherichia coli* B. Although the characteristic sugars of the antigenic polysaccharides have been detected in the wall preparations, there has been no direct experimental evidence establishing the polysaccharides as integral parts of the cell-wall structure, with the

Abbreviations: DEG, diethylene glycol; TCA, trichloracetic acid.

exception of the lipo-polysaccharide studied by WEIDEL *et al.*[4,11,12]. In the absence of any experimental information as to the anatomical status of the Smooth antigenic polysaccharide complexes, WILKINSON[13] and SALTON[14] have referred to these as "micro-capsular" constituents. The studies presented in this paper represent an attempt to obtain some information about the anatomical origin of the monosaccharide constituents in isolated cell-wall preparations obtained from Gram-negative bacteria.

MATERIALS AND METHODS

Organisms and isolation of cell walls

Cell walls were isolated from the following organisms: *Alcaligenes faecalis* (NCTC 8764); *Bacterium cadaveris* (NCTC 6578); *Chlorobium thiosulphatophilum*; *Chromobacterium kilense* (NCTC 4619); *Chromobacterium prodigiosum* (NCTC 2847); *Escherichia alkalescens* (NCTC 4338); *Escherichia dispar* (4169); *Klebsiella aerogenes* (NCTC 8172, and strains A3, A3(Sl), A3(O) kindly provided by Dr. J. F. WILKINSON), *Klebsiella cloacae* (NCTC 5920), Organism LC 1 (ELSDEN *et al.*[15]), *Photobacterium albensis* and *Photobacterium fischeri* kindly provided by Dr. R. SPENCER; *Proteus vulgaris*; Pseudomonas sp. kindly provided by Dr. A. D. BROWN; *Rhodospirillum rubrum*; *Salmonella gallinarum*; *Spirillum serpens*. Washed suspensions of cells harvested from suitable media were subjected to the wall isolation procedures previously described[16]. All of the cell-wall fractions were digested with trypsin prior to final washing and freeze-drying.

DISCHE *reaction for detection of certain monosaccharides*

Cell-wall preparations were examined by the DISCHE reaction[17] under conditions used for the detection of heptoses. 1–2 mg of dried wall or cell-wall fractions were placed in test tubes and 1 ml water added prior to the addition of the H_2SO_4 with cooling in an ice bath. After the addition of the H_2SO_4, the tubes were transferred to a water-bath at room temperature and then placed in a vigorously boiling water-bath and heated for 3 min. The tubes were cooled to room temperature and 0.1 ml 3 % w/v cysteine–HCl (or 0.1 ml H_2O for control tubes) was added and the contents mixed and the spectra were determined after standing for 24 h at room temperature.

Detection of dideoxy sugars

Compounds separating on paper chromatograms in the vicinity of dideoxy sugars were eluted from papers and subjected to the reaction for 3,6-dideoxy-aldohexoses described by FROMME, LÜDERITZ, STIERLIN AND WESTPHAL[18].

Paper chromatography of monosaccharide constituents

Cell-wall preparations were hydrolysed with 0.5 *N* H_2SO_4 for 8–16 h, or with 2 *N* H_2SO_4 for 2 h at 100° in sealed ampoules. Hydrolysates were neutralized with $Ba(OH)_2$ and after removal of the $BaSO_4$, the preparations were either dried and dissolved in pyridine for placing on the chromatogram paper or they were de-ionized with a mixed resin bed [Amberlite resins IR-120 (H) and IR-4B (OH)] concentrated and dissolved in distilled water to give solutions containing about 5 % sugars. Separation of monosaccharides was performed on Whatman paper No. 1, with *n*-butanol–pyridine–water (6:4:3, v/v) or *n*-butanol–acetic acid–water (6:1:2, v/v) as

the solvent systems. Paper chromatograms were sprayed with aniline phthalate[19] or with the $AgNO_3$ reagent described by TREVELYAN *et al.*[20]. Specific detection of glucose was performed with the "Glucostat" reagent (Worthington Biochemical Corporation) as described by SALTON[21].

RESULTS

Distribution of monosaccharides in wall fractions from capsulated, slime-producing and non-encapsulated strains of Kl. aerogenes

To determine whether the monosaccharides of the capsular and slime polysaccharides are detectable in the isolated cell-wall fractions, three strains of *Kl. aerogenes* previously investigated by WILKINSON, DUGUID AND EDMUNDS[22] were selected. The *Kl. aerogenes* A3 strain possesses a capsular polysaccharide which may be extracted with hot water[23]. The absorption spectra of DISCHE reaction products of wall fractions of the A3 strain before and after removal of the capsular polysaccharide are compared in Fig. 1 with that of the extracted polysaccharide material. The monosaccharide constituents detectable in the cell-wall fractions of the capsulated (A3),

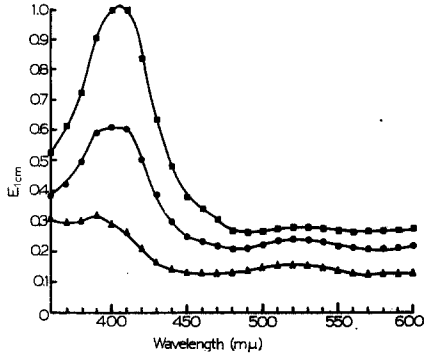

Fig. 1. ●—●, spectra of products of DISCHE reaction on *Kl. aerogenes* A3 untreated walls; ▲—▲, walls extracted with hot water; ■—■, hot-water extractable capsular polysaccharide. $E_{1\,cm}$ for 0.45 mg each fraction.

slime-producing (A3 Sl) and the non-encapsulated A3(O) strains of *Kl. aerogenes* are summarized in Table I, together with the results obtained with a serologically related strain[22], *Kl. cloacae*, and an additional strain of *Kl. aerogenes* (NCTC 8172).

Detection of heptoses by the DISCHE *reaction*

Cell walls have been subjected to the DISCHE reaction using the conditions favourable for the detection of heptoses[17]. From these preliminary tests with the DISCHE reaction, heptoses have been suspected in the cell walls of a number of organisms used in these studies. The heptoses give an absorption peak in the range 505–510 mμ (see ref. 17) and a typical example of results obtained with bacterial walls is presented in Fig. 2, showing the absorption spectra of *Proteus vulgaris* walls subjected to the DISCHE reaction. It will be seen from Fig. 2 (curve 2) that the walls treated with the H_2SO_4 alone (no cysteine added) also give appreciable absorption but without any marked peaks.

The separation of heptoses from other monosaccharides on paper chromatograms

TABLE I

MONOSACCHARIDE CONSTITUENTS OF "WALLS" OF ENCAPSULATED, SLIME-PRODUCING AND NON-ENCAPSULATED STRAINS OF *Klebsiella spp.*

Organism	*Strain*	*Preparation*	*Monosaccharides detected*
Kl. aerogenes	A3 capsulated	Cell walls	Galactose, glucose, fucose, uronic acid
		Wall extracted with hot water	Galactose, glucose
		Extracted capsular polysaccharide	Galactose, glucose, fucose, uronic acid
Kl. aerogenes	A3(Sl)	Cell walls	Galactose
Kl. aerogenes	A3(O)	Cell walls	Galactose
Kl. aerogenes	NCTC 8172	Cell walls	Galactose, glucose, mannose, rhamnose, uronic acid?, trace fucose?
Kl. cloacae	NCTC 5920	Cell walls	Glucose, rhamnose

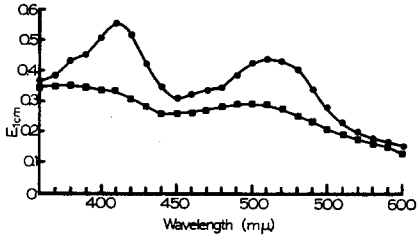

Fig. 2. Spectra of DISCHE reaction products of walls of *Proteus vulgaris*; ●—●, cysteine-H_2SO_4; ■—■, H_2SO_4 alone. $E_{1\ cm}$ for 0.2 mg wall.

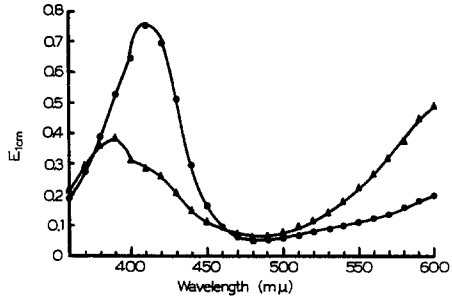

Fig. 3. ▲—▲, DISCHE reaction spectra given by 100 μg D-galactose; ●—●, 100 μg D-glucose.

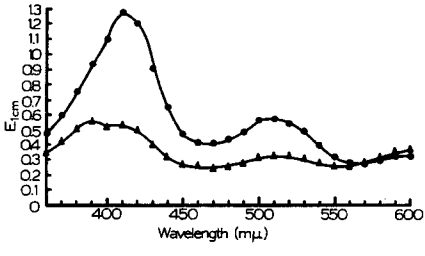

Fig. 4. ▲—▲, Spectra of DISCHE reaction products performed on *Chromobacterium kilense* cell-wall "galactose"; ●—●, "glucose" separated by paper chromatography of hydrolysed wall.

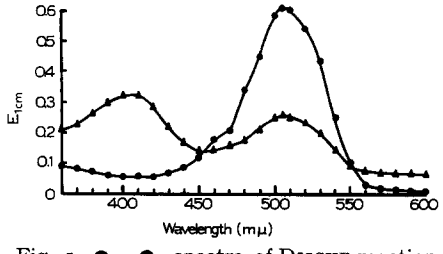

Fig. 5. ●—●, spectra of DISCHE reactions on 50 μg D-guloheptose; ▲—▲, *Spirillum serpens* cell-wall "glucose-heptose" fraction separated by paper chromatography of hydrolysed wall.

has been investigated by DAVIES[24]. With many solvents it is very difficult to obtain a clear separation of heptoses from glucose and galactose, two sugars frequently encountered in bacterial polysaccharides. Hydrolysates of the walls of several of the bacteria suspected of possessing heptoses have been submitted to the "double run" method used by DAVIES[24] for the identification of heptoses on paper chromatograms.

A satisfactory resolution of the heptoses of *Spirillum serpens* and *Proteus vulgaris* was not obtained and attempts to identify them by this means were abandoned until larger quantities of wall material would be available.

Confirmation of the presence of heptoses in the walls of several bacteria has been obtained by elution of the monosaccharides from paper chromatograms and subjecting them to the DISCHE reaction. As shown in Fig. 3, the conditions employed for the DISCHE test for heptoses also gives rise to coloured compounds for glucose and galactose. After hydrolysis and separation of the sugars in the wall of *Chromobacterium kilense*, the compounds corresponding to galactose and glucose on paper chromatograms were eluted and then subjected to the DISCHE reaction in the usual way. The absorption spectra for "glucose" and "galactose" presented in Fig. 4, both show the presence of material giving a peak between 500–510 mμ. No such absorption was detectable in a paper blank nor in the "mannose" separated on the same paper chromatograms. That the heptoses overlap the glucose and galactose was suspected from the pinkish colour of the chromatograms sprayed with aniline phthalate (aldohexoses give a brown colour[19], authentic heptoses gave a pink colour). Similar results were obtained with walls of *Chromobacterium prodigiosum* and *Photobacterium albensis*.

The presence of both glucose and a heptose in the wall of *Spirillum serpens* was confirmed by performing a DISCHE reaction on the "glucose" eluted from paper chromatograms, and comparing it with the spectrum obtained with D-glycero-D-gulo-heptose (Fig. 5). The "glucose" also showed an absorption maximum between 400–410 mμ and the presence of glucose was confirmed with the glucose oxidase spray as described by SALTON[21].

Although blank areas from paper chromatograms showed no absorption between 500–510 mμ when reacted with the DISCHE reagent, further confirmation that the above observations were not due to absorption by non-specific compounds (separating with glucose on paper chromatography of hydrolysed wall) was sought. The cell wall of *R. rubrum* reacted with cysteine–H_2SO_4 showed no absorption peak around 500–510 mμ. The "glucose" separated on paper chromatograms of hydrolysed *R. rubrum* wall was subjected to the DISCHE reaction and the result is illustrated in the spectrum shown in Fig. 6. It is evident that a small, non-specific absorption may be obtained, but it is insufficient to account for the peaks obtained with walls in which heptoses were suspected.

Cell-wall monosaccharides and their distribution after treating walls with various reagents

Cell walls of various Gram-negative bacteria have been examined by paper chromatography, combined with the DISCHE reaction of certain sugars separated on paper chromatograms as described in the preceding section. The results are presented in Table II. As shown in Table II the walls of *Alcaligenes faecalis* and *Rhodospirillum rubrum* contained substances reacting with aniline phthalate and migrating on paper chromatograms in the vicinity of the dideoxy sugars. Neither the compound from *A. faecalis* wall, nor that from *R. rubrum* wall gave the reaction for 3,6-dideoxy-aldohexoses, so they do not appear to belong to this class of sugars. The compound from *R. rubrum* also failed to give a positive direct WEBB AND LEVY test[25].

The diagnostic value of the DISCHE reaction for methyl pentoses (rhamnose and fucose) and heptoses has been used in combination with paper chromatography to follow the distribution of the monosaccharide constituents after subjecting the walls

TABLE II

MONOSACCHARIDE CONSTITUENTS OF WALLS OF GRAM-NEGATIVE BACTERIA

Walls from	*Monosaccharides detected*
Alcaligenes faecalis	Glucose, arabinose, fucose, rhamnose, unknown*
Bacterium cadaveris	Galactose, glucose, rhamnose
Chlorobium thiosulphatophilum	Galactose, glucose, mannose, rhamnose
Chromobacterium kilense	Galactose, glucose, heptose, mannose
Chromobacterium prodigiosum	Glucose, heptose, mannose, traces pentose (?) and rhamnose
Escherichia alkalescens	Galactose, glucose, rhamnose
Escherichia dispar	Galactose, glucose, rhamnose
Organism LC 1	Galactose, glucose, rhamnose
Photobacterium albensis	Glucose, heptose
Photobacterium fischeri	Galactose, glucose
Proteus vulgaris	Glucose, heptose
Pseudomonas sp.	Glucose, fucose, rhamnose
Rhodospirillum rubrum	Glucose, fucose, rhamnose, unknown*
Salmonella gallinarum	Galactose, glucose, mannose, rhamnose, tyvelose
Spirillum serpens	Glucose, heptose, rhamnose

* Both compounds migrating near dideoxy sugars, but give negative reactions for 2 and 3,6 deoxy sugars.

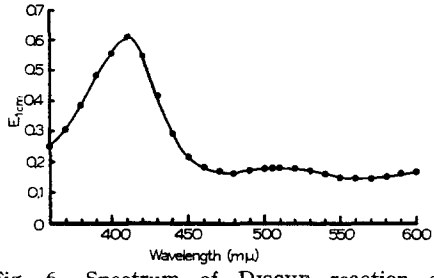

Fig. 6. Spectrum of DISCHE reaction on *R. rubrum* cell-wall "glucose" separated by paper chromatography of hydrolysed wall.

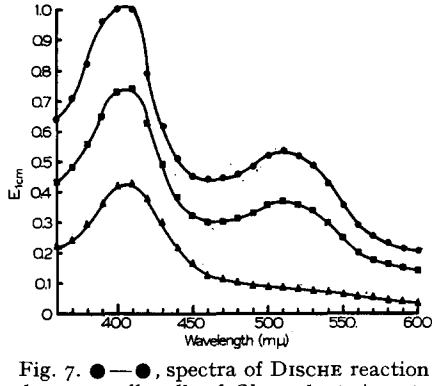

Fig. 7. ●—●, spectra of DISCHE reaction products on cell walls of *Chromobacterium prodigiosum* unextracted; ■—■, extracted with DEG for 7 days; ▲—▲, lysozyme soluble fraction.

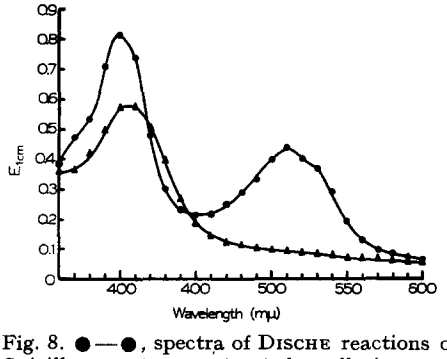

Fig. 8. ●—●, spectra of DISCHE reactions on *Spirillum serpens* untreated wall (1.5 μg); ▲—▲, lysozyme-soluble fraction (1.4 μg).

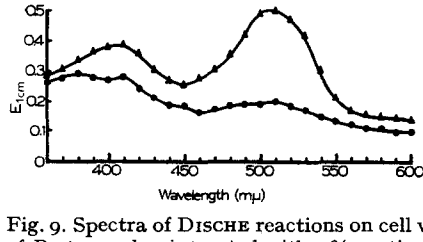

Fig. 9. Spectra of DISCHE reactions on cell walls of *Proteus vulgaris* treated with 1 % acetic acid, 4 h, 100°. ▲—▲, acetic acid "soluble" fraction; ●—●, insoluble residue.

to various extraction procedures. DEG, TCA and 1 % acetic acid have been used for the extraction of bacterial polysaccharides and lipopolysaccharide complexes[6,26]. Cell walls of *S. gallinarum*, *Kl. aerogenes* A3 (O), *Chromobacterium prodigiosum* and *R. rubrum* were extracted with DEG for 1 week at room temperature. Soluble and insoluble fractions were separated and in all instances DISCHE reactions and paper chromatography showed the presence of all the monosaccharide constituents in both fractions. Extraction of walls of *S. gallinarum* with 10 % TCA (3 days at 20°) and with 90 % w/w phenol for 24 h at 37° also gave soluble products and insoluble residues containing all of the monosaccharides present in the parent wall material.

Although extraction with DEG failed to remove the polysaccharides quantitatively from the wall, leaving the characteristic sugars in the insoluble residues as well, treatment of walls with lysozyme (using conditions outlined previously[27]) did give a clearer fractionation of the walls of several organisms. The DISCHE reactions on the unextracted, DEG-extracted walls of *Chromobacterium prodigiosum* are compared with the lysozyme soluble fraction in Fig. 7. With *Spirillum serpens* the spectrum of the DISCHE reactions products again showed a marked difference between the original walls and the lysozyme-soluble fraction as shown in Fig. 8. However, treatment of the walls of *S. gallinarum* with lysozyme did not give a complete fractionation of the monosaccharide constituents of the walls; all sugars were again detectable in both the lysozyme-soluble and lysozyme-insoluble fractions.

DAVIES[6] has used 1 % acetic acid treatment for 4 h at 100° to release the polysaccharide haptens of various Gram-negative bacteria. Employing these conditions with several organisms, a clearer fractionation was obtained, as indicated by the results of the DISCHE reaction on soluble and insoluble fractions of *Proteus vulgaris* walls (Fig. 9). Treatment of *S. gallinarum* walls with 1 % v/v acetic acid gave insoluble residues which no longer showed the sharp peak due to rhamnose reaction with cysteine–H_2SO_4.

DISCUSSION

The retention of at least some of the capsular material on the cell surface during mechanical disintegration and isolation of the walls is illustrated by the results obtained with *Kl. aerogenes* A3. All of the monosaccharide constituents of the capsular polysaccharide were detectable in the "wall" fraction, but after extraction with hot water (to remove the capsular material) virtually all of the fucose and uronic acid were removed. Thus, it has been possible to differentiate between true wall monosaccharide constituents and those derived from a capsular polysaccharide. The slime-producing mutant of *Kl. aerogenes* showed none of the sugar components of the slime polysaccharide in the wall fraction. This would be in agreement with the results of WILKINSON *et al.*[22], who have shown that the polysaccharide can be readily washed off the cells. It is of interest to note that the monosaccharides of the wall of the slime-producing mutant (derived from A3) are identical to those of the mutant A3(O), which produces neither slime, nor capsule.

Attempts to obtain a clear-cut fractionation of the monosaccharide constituents of the walls by extraction with DEG have not been very successful. BROWN[28] has also shown that extraction of walls with TCA removes material of fairly similar composition to what is left in the insoluble fractions of the walls. Lysozyme treatment on the

other hand has conclusively established the presence of heptoses in the lipo-polysaccharide-protein fraction of the wall and not in the mucopeptide part which is released into solution by lysozyme action[5]. 1 % acetic acid treatment for 4 h at 100° has also given a fairly selective release of the sugar components.

The results of these investigations are in accord with the view that the polysaccharides characteristic of the Smooth and Rough antigenic constituents of the bacterial cell are an integral part of the lipid-polysaccharide-protein fraction of the bacterial wall and some of the polysaccharide may even be linked to the mucopeptide material released by lysozyme (as with *S. gallinarum*). Thus, the description of the "surface" antigenic polysaccharide complexes as "micro-capsular"[13,14] constituents of the bacterial cell is misleading and should be discontinued. Whether the polysaccharide complexes are localized in a particular layer of the multilayered wall of a Gram-negative organism is not known. Preparation of ultra-thin sections of walls subjected to the more selective of the extraction procedures used in this study should greatly clarify an understanding of the structure of the walls of the Gram-negative bacteria.

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