STUDIES OF THE BACTERIAL CELL WALL

IV. THE COMPOSITION OF THE CELL WALLS OF SOME GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA

by

M. R. J. SALTON*

Department of Colloid Science, University of Cambridge (England)

INTRODUCTION

The chemical constitution of bacterial cell walls has for long remained uncertain and much of our knowledge of their nature has been largely based upon indirect evidence. In reviewing the literature on the structure and chemical composition of the bacterial cell wall, KNAYSI[1] draws attention to the "very limited and primitive data available" and concludes "that the composition of the cell wall is highly complex and that it may vary considerably from strain to strain and to a certain extent in the same strain."

The recent development of suitable techniques for the preparation of bacterial cell walls in a high state of purity has led to a direct approach to the study of their constitution. These studies have been implemented by the use of the electron microscope which has enabled a more rigorous check on the morphological homogeneity of cell wall preparations (DAWSON[2], SALTON AND HORNE[4] and WEIDEL[6]).

That the composition of the bacterial cell wall varies from one organism to another has become abundantly clear from the recent reports on the chemical constitution of these structures. WEIDEL[5,6] has shown that the cell "membrane" of E. *coli* B is a lipoprotein complex of unusual properties. The mucopolysaccharide nature of the cell wall of *Streptococcus faecalis* has been demonstrated by SALTON[7,8]. The "envelope" of *Staphylococcus aureus* was reported to be a glycerophospho-protein complex by MITCHELL AND MOYLE[9]. The cell wall material of *Corynebacterium diphtheriae* investigated by HOLDSWORTH[10] has been shown to be a carbohydrate-protein complex, but unfortunately no evidence as to the morphological homogeneity of the preparation has been given.

This paper presents the results of an investigation of the composition of the cell walls of some Gram-positive and Gram-negative bacteria.

EXPERIMENTAL

Materials and Methods

Organisms

The following organisms were used; *Streptococcus pyogenes* (precipitin Type 4, Lab. No. 21522/51 —strain isolated and kindly given by Dr J. BOISSARD): *Micrococcus lysodeikticus* Fleming (N.C.T.C. No. 2665): *Sarcina lutea* (laboratory strain kindly given by Dr E. F. GALE): *Bacillus subtilis* Marburg

* Beit Memorial Research Fellow.

References p. 523.

(N.C.T.C. No. 3610), together with *Escherichia coli* H and *Salmonella pullorum* used in earlier investigations (SALTON AND HORNE[3,4]).

Growth conditions and harvesting

Strep. pyogenes was grown on blood-agar plates (trypsin digest of beef heart muscle base, 5% horse blood-agar) for approximately 24 h at 37° C. All other organisms were grown on a medium consisting of 3% tryptic digest of casein, 0.1% Marmite, 1% glucose and 2% agar, and harvested, unless otherwise specified, after incubation for 16 h at 37° C. *Strep. pyogenes* was harvested from the surface of the blood-agar plates by means of a wire loop, the cells removed from the loop by emulsifying in distilled water. Cells of the other organisms were washed from the agar surface with distilled water. Suspensions of all organisms were centrifuged and the harvested cells washed twice with distilled water on the centrifuge. The cells were finally suspended in distilled water to give suspensions containing approximately 10–20 mg dry weight bacteria/ml.

Preparation of cell walls

The procedure used in the preparation of cell walls was essentially the same as that described by SALTON AND HORNE[4] and later employed in the preparation of sufficient quantities of *Strep. faecalis* cell wall for chemical studies[7,8]. This involved mechanical disintegration of the cells with "ballotini" glass beads in the Mickle disintegrator. The introduction of a slight modification, *viz.* washing cell walls with 1 *M* NaCl before washing with distilled water, enabled a more rapid and efficient removal of contaminating cytoplasmic debris from the cell wall preparations of *B. subtilis* *E. coli* and *Salmonella pullorum*. Preparations washed with NaCl in this way were then thoroughly washed with distilled water on the high-speed centrifuge until supernatant washings no longer gave a test for chloride ion with silver nitrate.

Small amounts of cell wall material of *E. coli* and *Salmonella pullorum* were prepared by the heat-treatment rupture of cell walls at 100° C as described in an earlier communication[4].

In all instances cell wall preparations were examined for purity in the electron microscope and if necessary subjected to further washings on the high-speed centrifuge before finally centrifuging at 3 000 r.p.m. and freeze-drying the supernatant cell-wall suspensions.

Total nitrogen. Digestion of samples was carried out by the Kjeldahl method and the nitrogen was estimated colorimetrically by the procedure described by JOHNSON[11]. Estimations were performed on triplicate samples.

Total phosphorus was estimated colorimetrically by the method of FISKE AND SUBBAROW[12].

Reducing power of hydrolysates (expressed as glucose) was determined by the Hagedorn and Jensen method as modified by HANES[13].

Hexosamine was estimated by the colorimetric method of ELSON AND MORGAN[14], using glucosamine-HCl as a standard.

Chromatographic methods. Identification of the amino acids in the cell wall hydrolysates was carried out by running two-dimensional paper chromatograms (Whatman filter-paper No. 1) according to the procedure of CONSDEN, GORDON AND MARTIN[15]. Chromatograms were irrigated for 16 h, with phenol-ammonia in one direction and with *n*-butanol—acetic acid—water (40%; 10%; 50% v/v) at right angles. The amino acids were detected by spraying with ninhydrin. The sugars in the cell wall hydrolysates were identified by paper chromatography, using the method described by PARTRIDGE[16]. Hydrolysates were placed on Whatman filter-paper No. 1, run for 22 h with ethyl acetate-pyridine—water (5;2;5 v/v) as the solvent[18], or for 44 h when *n*-butanol—acetic acid—water (4;1;5 v/v) was used as the solvent mixture. Duplicate chromatograms were sprayed with silver nitrate—ammonia[16] and with aniline hydrogen phthalate[17].

GENERAL PROPERTIES

Preparations of cell walls from *Strep. pyogenes, M. lysodeikticus, Sarcina lutea, B. subtilis, E. coli* and *Salmonella pullorum* were subjected to a number of qualitative tests, for the detection of protein, carbohydrate and nucleic acid. All of the preparations gave strong positive Biuret reactions. With the exception of *E. coli* which gave only a very weak positive reaction, the Molisch tests of the cell walls were positive. For the detection of nucleic acid or purine-pyrimidine substances, cell wall suspensions were examined for their ultraviolet absorption in the Beckman spectrophotometer. Ultraviolet absorbtion spectra of the cell wall preparations over wave-lengths of 220 to 400 mμ were similar to those reported for *Strep. faecalis* and *E. coli* in an earlier study[4]. None of the cell wall preparations of the series of organisms studied in the present investigation showed

References p. 523.

absorption maxima corresponding to that of nucleic acid or purine–pyrimidine compounds. As shown in a subsequent section of this paper, the absence of ribose is in accord with these observations.

Liberation of reducing substances

SALTON[8] showed that hydrolysis of the cell wall of *Strep. faecalis* with 2*N* HCl resulted in a rapid liberation of reducing substances and hexosamine. To ascertain whether the cell walls of other bacteria behaved similarly when hydrolysed with acid, preparations of *M. lysodeikticus*, *Sarcina lutea* and *B. subtilis* were investigated.

The rate of hydrolysis with 2 *N* HCl was followed by sealing 25 mg samples of the cell wall preparations in glass ampoules with 5 ml acid and heating for appropriate lengths of time in a boiling water bath. The sealed ampoules were cooled in ice-cold water, insoluble material removed by filtration and the hydrolysates immediately neutralized in the cold with sodium hydroxide solution. Estimations of liberated reducing substances and hexosamine were then performed on aliquots of the neutralized hydrolysates.

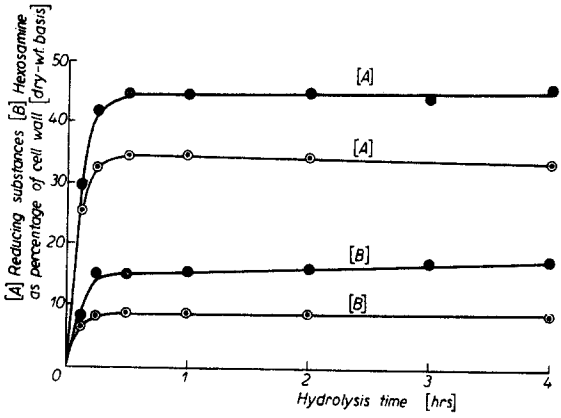

Fig. 1. Rates of hydrolysis of *M. lysodeikticus* (●——●) and *B. subtilis* (◉——◉) cell wall preparations by 2 *N* HCl at 100° C, as determined by liberation of (A) reducing substances (expressed as glucose) and (B) hexosamine.

The results obtained with the cell wall preparations of *M. lysodeikticus* and *B. subtilis* are shown in Fig. 1. Similar results showing a rapid liberation of reducing substances and hexosamine on hydrolysis were also obtained with the cell wall preparation of *Sarcina lutea*.

Extraction of cell wall lipid

Owing to the relatively small quantities of some of the cell wall preparations, it has not been possible to make an extensive study of the extraction or nature of the cell wall lipid material.

Cell wall preparations of *E. coli* and *B. subtilis* have been subjected to the following extraction procedures, using approximately 50 mg samples throughout.

1. Extraction with four successive 10 ml lots of boiling anhydrous, freshly-distilled ether, each extraction being carried out for 30 min. Ether was evaporated and each extract was weighed separately. Extraction of both cell wall preparations was virtually complete with the third successive 10 ml lot of ether. Extracts were redissolved in ether, combined and transferred to a clean weighing bottle, ether evaporated and the material dried *in vacuo* over P_2O_5 and the total ether-extractable lipid determined by weighing. 2. REICHERT[19] has shown that lipid-protein complexes can be broken down by refluxing with 95% methanol. Cell wall preparations were heated under reflux with 10 ml 95% methanol for 1 h. The methanol was evaporated and the material dried *in vacuo* over P_2O_5. Lipid was then removed by repeated extraction with boiling anhydrous ether. The whole procedure of refluxing with methanol and extraction with ether was repeated and the combined extracts evaporated and the material dried *in vacuo* over P_2O_5 and weighed. 3. Preparations were hydrolysed with strong acid in order to determine whether the cell walls contained additional, "firmly bound" lipid which may not be available for extraction unless other cell wall components have been degraded or destroyed. Samples were hydrolysed with 5 ml of 6 *N* HCl in sealed glass ampoules, heating in a boiling water bath for 2, 4 and 8 hours. The contents of the ampoules were transferred quantitatively to small separating funnels, the ampoules thoroughly washed out with ether, ether washings added

References p. 523.

to the contents of the separating funnel and the whole was then thoroughly extracted with ether. Ether extracts were evaporated *in vacuo* over P_2O_5 and NaOH pellets. The lipid material was finally dissolved in a small quantity of anhydrous ether and transferred to a clean weighing bottle, the ether evaporated and the material dried *in vacuo* over P_2O_5 and NaOH pellets, and weighed.

The results are shown in Table I.

TABLE I

SHOWING THE EXTRACTION OF LIPID MATERIAL FROM CELL WALL PREPARATIONS

Organism	*Lipid as percentage dry weight*				
	Ether extraction	*Refluxed with 95% methanol, ether extracted*	*6N HCl hydrolysis (100°) followed by ether extraction. Hydrolysis time in h*		
			2	*4*	*8*
E. coli (from 16 h culture)	8.2 (36)*	8.6 (38)*	22.5	22.7	22.5
E. coli (from 9 h culture)	7.6 (36)*	—	20.8	—	—
B. subtilis	0.3 (11)*	1.6 (59)*	2.5	2.7	2.7

* Figures in brackets show extracted lipid expressed as percentage of maximum lipid content.

Properties of the cell wall preparations

The N and P contents of the cell walls and the amounts of reducing substances and hexosamine liberated by acid hydrolysis were determined. Estimations of the "total lipid" contents were carried out by hydrolysing the preparations for 2 h at 100°C with 6*N* HCl and extracting with ether according to the procedure outlined in the previous section. All determinations have been expressed in terms of dry weight of the cell wall, the latter having been estimated by drying samples to constant weight in an air-oven at 105°C. The data are summarized in Table II.

TABLE II

SOME PROPERTIES OF THE CELL WALLS OF CERTAIN GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA

Organism	*Mean determinations expressed as percentage dry weight cell wall*				
	Total N	*Total P*	*Reducing substances**	*Hexosamine**	*Total lipid*
Gram-positive					
Strep. pyogenes	10.6	0.62	33.1	11.8	—
M. lysodeikticus	8.7	0.09	44.9	16.1	1.2
Sarcina lutea	7.6	0.22	46.5	16.3	1.1
B. subtilis	5.1	5.35	34.0	8.5	2.6
Gram-negative					
E. coli 1.	10.1	1.52	16.0	3.0	20.8
E. coli 2.	10.0	1.52	—	—	22.6
Salmonella pullorum A.	6.4	0.88	46.0	4.8	19.0
Salmonella pullorum B.	5.5	—	—	—	—

* Determined after 2 *N* HCl hydrolysis for 2 h at 100° C.
E. coli 1. preparation from 9 h culture of bacteria.
E. coli 2. preparation from 16 h culture of bacteria.
Salmonella pullorum A. cell walls prepared by mechanical disintegration.
B. cell walls prepared by heat-treatment rupture.

References p. 523.

IDENTIFICATION OF PRODUCTS OF ACID HYDROLYSIS

Amino acids

The amino acids of the cell wall preparations were identified by filter-paper chromatography.

Samples of 10–15 mg of the cell wall preparations were hydrolysed with 5 ml of 6 *N* HCl for 24 h in a boiling water bath, hydrolyses being carried out in sealed ampoules. Hydrolysates were evaporated to dryness *in vacuo* over conc. H_2SO_4 and NaOH pellets, redissolved in water and dried *in vacuo*. This was repeated several times before filtering off humin particles washing with hot water and finally evaporating *in vacuo*. The residues were then dissolved in 0.2 ml distilled water and the solutions examined by filter-paper chromatography.

For the detection of cystine and methionine, the hydrolysates were treated on the paper with H_2O_2 according to the procedure described by DENT[20]. Under these conditions cystine is converted to cysteic acid and methionine to methionine sulphone. To confirm the presence of methionine sulphoxide the hydrolysates were treated on the paper with H_2O_2 and ammonium molybdate (DENT[21]). Hydrolysates which gave chromatogram spots corresponding to methionine sulphoxide when treated in this way gave spots in the position of methionine sulphone.

A spot appeared on chromatograms of cell wall hydrolysates of *B. subtilis*, *E. coli* and *Salmonella pullorum* in the vicinity of the region where cystine is normally observed. On treatment with H_2O_2 however, the spot persisted and it therefore appeared unlikely that it could be due to cystine. This spot occupied a position similar to that of the unknown amino acid observed by WORK[22,23] in hydrolysates of *C. diphtheriae*. WORK[24] isolated this amino acid and its identity was established as diaminopimelic acid. The cell wall material examined by HOLDSWORTH[10] was also found to contain diaminopimelic acid. To determine whether diaminopimelic acid was present in the cell wall hydrolysates of *B. subtilis*, *E. coli* and *Salmonella pullorum*, the following procedure was adopted.

1. The basic and acidic amino acids were removed from the hydrolysates by means of filter-paper electrophoresis. The technique and equipment employed was essentially the same as that described by DURRUM[25]. Strips of 57 cm lengths of Whatman filter-paper No. 1 were saturated with *M*/20 ammonium acetate buffer at pH 5.9 and the hydrolysate applied as a narrow band at the centre of the strip. The ends of strips were placed in electrode vessels containing buffer and an electrical potential applied by connecting the electrodes to 220 volts D.C. Electrophoresis was carried out for 12 h and the positions to which the amino acids had migrated were determined by spraying a narrow strip with ninhydrin. 2. The central, unsprayed portion of the strip was cut out and the neutral amino acids eluted with water. The eluate was dried *in vacuo*, the dry residue dissolved in water, placed on filter-paper chromatogram and treated with H_2O_2 to convert any cystine to cysteic acid. Controls of diaminopimelic acid isolated from *C. diphtheriae* (kindly provided by Dr E. WORK), cystine and cystine treated with H_2O_2 were run in parallel with the neutral amino-acid fractions of the cell wall hydrolysates. Chromatograms were then irrigated with phenol-ammonia and developed with ninhydrin.

Well-defined spots corresponding to diaminopimelic acid were obtained in preparations of *B. subtilis*, *E. coli* (both mechanical disintegration and heat-treatment methods) and *Salmonella pullorum* (mechanical disintegration method only). No spots corresponding to cysteic acid were observed, thus confirming the absence of cystine in the neutral amino acid fraction of the hydrolysates. The addition of diaminopimelic acid to the cell-wall neutral amino acid fractions did not give rise to the appearance of any new spots on the chromatograms.

That the amino acid constitution of the cell wall of *Strep. pyogenes* may be complicated by the retention of the type-specific M-protein antigen appeared conceivable in

References p. 523.

view of the observations reported by ZITTLE AND MUDD[26]. The retention of the M-protein by the cell wall was readily demonstrated by performing precipitin tests on $N/5$ HCl extracts of the walls. Extracts were neutralized with unbuffered $N/5$ NaOH and using a typing serum known to be moderately good, the extracts gave specific precipitates with the homologous anti-serum and no cross reactions observed. LANCEFIELD[27] reported that the M-protein can be easily removed from intact cells of group A Streptococci by exposure to trypsin, the viability of the cells remaining unaffected. It was of interest therefore, to compare the amino acids of the cell wall preparation before and after removal of the M-protein with trypsin. Removal of the M-protein was effected by dispersing 12 mg of *Strep. pyogenes* cell wall in a 5 ml solution of trypsin (final concentration 1 %) in $M/15$ PO_4 buffer at pH 8 and incubating for 2 h at 37°C according to the procedure described by LANCEFIELD[27]. The insoluble cell wall fraction was deposited by centrifugation in a high-speed centrifuge, washed three times with PO_4 buffer (pH 8) and four times with distilled water on the high-speed centrifuge. The cell wall deposit was hydrolysed with $6N$ HCl and the amino acids identified as previously described.

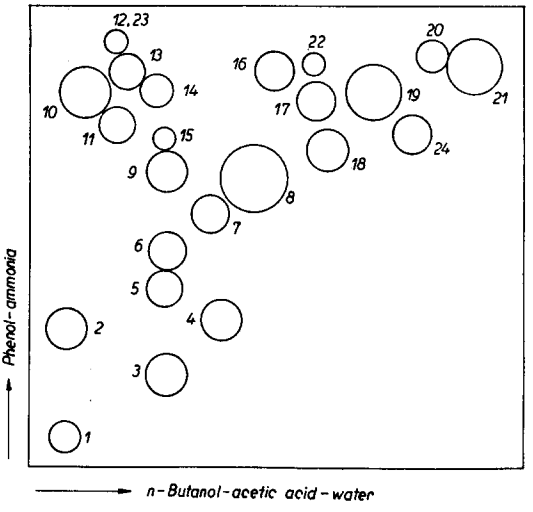

Fig. 2. Two-dimensional chromatogram "map of spots" showing positions of amino acids, amino-sugar and the unknown ninhydrin-reacting substances of cell wall hydrolysates. 1. cysteic acid, 2. diaminopimelic acid, 3. aspartic acid, 4. glutamic acid, 5. serine, 6 glycine, 7. threonine, 8. alanine, 9. glucosamine, 10. lysine, 11. histidine, 12. methylhistidine, 13. arginine, 14. methionine sulphoxide, 15. methionine sulphone, 16. proline, 17. γ-aminobutyric acid, 18. tyrosine, 19. valine and methionine, 20. phenylalanine, 21. leucine and *iso*leucine, 22. unknown "Spot 11", 23. unknown. 24. unknown.

Chromatograms of cell wall hydrolysates have been compared with those obtained from synthetic mixtures of amino acids and glucosamine and the complete data on the identification of the amino acids together with the detection of an amino-sugar (hexosamine) are summarized in Table III. Three unknown ninhydrin-reacting substances which do not correspond to any of the amino acids commonly found in proteins, have been encountered. The locations of these substances together with those of known amino acids are illustrated in the two-dimensional chromatogram "map of spots" shown in Fig. 2. "Spot 11" originally observed in the cell wall of *Strep. faecalis*[8] has now been detected in cell wall preparations of other bacteria. An unknown substance (spot 23, Table III, Fig. 2) appeared in the position usually occupied by methylhistidine, but unlike this amino acid it gave a faintly purple spot instead of the characteristic green of methylhistidine. The third unidentified substance (spot 24, Table III, Fig. 2) gave a weak brown spot on reaction with ninhydrin.

Sugars

For the identification of the sugars, cell wall preparations were hydrolysed with $2N$ H_2SO_4 for 2 h at 100°C, in sealed glass ampoules.

References p. 523.

TABLE III

AMINO ACIDS AND AMINO-SUGAR DETECTED IN HYDROLYSATES (6 *N* HCl, 24 h at 100° C) OF BACTERIAL CELL WALLS

Amino acid	*Gram-positive*						*Gram-negative*			
	Strep. pyogenes	*Strep. pyogenes (trypsin treated)* *	*Strep. faecalis* **	*M. lysodeikticus*	*Sarcina lutea*	*B. subtilis*	*E. coli (A)*	*E. coli (B)*	*Salmonella pullorum (A)*	*Salmonella pullorum (B)*
Alanine	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Aspartic acid	++	++	+	—	+	+	++	++	++	++
Glutamic acid	+++	++	++	++	+++	+++	+++	+++	++	++
Serine	++	+	+	—	—	+	++	++	++	++
Glycine	++	+	+	++	++	+	++	++	++	++
Threonine	+	+	+	—	—	+	++	++	+	+
Lysine	++	++	++	+	++	+	++	+	+	+
Valine	++	+	+	—	—	+	++	++	++	++
Leucine/*I*soleucine	+++	+	++	—	—	+	+++	+++	++	++
Proline	+	—	—	—	—	—	+	+	+	+
Arginine	+	—	—	—	—	—	+	+	+	+
Cysteic acid	+	—	—	—	—	—	+	+	—	—
Cystine	—	—	—	—	—	—	—	—	—	—
Methionine sulphoxide	+	—	—	—	—	—	+	+	+	+
Methionine		—	—	—	—	—	+		+	
Tyrosine	+	—	—	—	—	—	+	+	+	+
Phenylalanine	+	—	—	—	—	—	+	+	+	+
Diaminopimelic acid	—	—	—	—	—	+++	+	+	+	—
"Spot 11"	+	+	+	—	—	—	+	+	+	+
Unknown (Spot 23)	—	—	—	—	—	—	+	+	+	+
Unknown (Spot 24)	+	—	—	—	—	—	+	+	+	+
Amino-sugar										
Hexosamine	++	++	++	++	++	++	+	+	+	+

* M-protein removed by treatment with trypsin as described in text. A. cell walls prepared by mechanical disintegration. B. cell walls prepared by heat-treatment rupture.

** Data taken from SALTON[7,8].

The weights of preparations hydrolysed were adjusted (according to the reducing values shown in Table II) so that the final concentration of the solution for application to the chromatogram was approximately 5% (w/v expressed as glucose). Hydrolysates were neutralized with barium hydroxide solution to pH 4.5, the barium sulphate removed by centrifugation washed several times and the hydrolysates and washings evaporated to dryness *in vacuo*. Residues were dissolved in sufficient distilled water to give 5% solutions of sugars. The hydrolysates were then examined by paper chromatography running in parallel with known sugars (singly or in mixtures) and the sugar spots located by spraying duplicate chromatograms with aniline hydrogen phthalate and silver nitrate–ammonia. Two solvent systems were employed, ethyl acetate–pyridine–water and *n*-butanol–acetic acid–water and the sugars detected in the cell wall hydrolysates were identical in both instances.

The results of the identification of the sugars are presented in Table IV.

TABLE IV

IDENTIFICATION OF SUGARS IN 2 *N* SULPHURIC ACID HYDROLYSATES OF CELL WALL PREPARATIONS

Organism	*Galactose*	*Glucose*	*Mannose*	*Rhamnose*	*Ribose*
Gram-positive					
Strep. pyogenes	—	—	—	++	—
*Strep. faecalis**	+	+	—	++	—
M. lysodeikticus	—	++	—	—	—
Sarcina lutea	—	++	—	—	—
B. subtilis	—	++	—	—	—
Gram-negative					
E. coli	+	+	—	—	—
Salmonella pullorum A.	++	+	+	+	—
Salmonella pullorum B.	++	+	+	+	—

Salmonella pullorum A. cell walls prepared by mechanical disintegration.
B. cell walls prepared by heat-treatment rupture.
* Data taken from SALTON[7,8].

The sugars detected by spraying chromatograms with aniline hydrogen phthalate were identical with those shown on spraying with silver nitrate–ammonia. In addition to the sugars shown in Table IV, weak spots corresponding to the position of the amino sugar, glucosamine, were obtained on the chromatograms sprayed with aniline hydrogen phthalate, the hexosamine spots being considerably stronger however, on the duplicate sprayed with silver nitrate–ammonia.

GROUP-SPECIFIC "C" SUBSTANCE OF *Strep. pyogenes* CELL WALL

The identification of rhamnose and hexosamine as the two reducing substances of the cell wall of *Strep. pyogenes* was of particular interest in view of their presence in the isolated group A Streptococcus polysaccharide recently investigated by SCHMIDT[28]. It appeared likely, therefore, that the group-specific polysaccharide may be a component of the rigid wall of *Strep. pyogenes*. Extraction of cell wall preparations by standard procedures (LANCEFIELD's acid method, FULLER's formamide method and MAXTED's enzyme method) yielded good extracts which gave rapid specific precipitation with rabbit group A anti-sera in ring precipitin tests, thus confirming the presence of group-specific substance in *Strep. pyogenes* cell wall. Anti-sera to Streptococci belonging to groups B, C and G were included in the test but no cross reactions were observed. An

References p. 523.

enzyme preparation from *Streptomyces albus*, which MAXTED[29] had previously shown to be capable of releasing the "C" substance from β-haemolytic Streptococci, effected a rapid lysis of turbid cell wall suspensions of *Strep. pyogenes* on incubation at 55°C

DISCUSSION

It is now apparent that bacterial cells are capable of producing an amazing diversity of complex substances which perform at least one function of providing the cell with a mechanically rigid wall. The cell wall of *E. coli* is essentially of lipo-protein nature, probably containing a small carbohydrate moiety or residue. The results of the investigation of the composition of the cell wall of this organism are in general agreement with those reported for *E. coli* B by WEIDEL[5,6], the major difference being the absence of carbohydrate in the cell walls studied by the latter worker. *Salmonella pullorum* cell wall possesses lipid, protein and carbohydrate components and contains rather more of the latter than does the cell wall of *E. coli*. The cell walls of *Strep. pyogenes*, *M. lysodeikticus* and *Sarcina lutea* are clearly of mucoid nature, possessing amino acid and polysaccharide components. That the cell walls of *M. lysodeikticus* and *Sarcina lutea* were found to be mucoid substances was not surprising, considering their susceptibility to lysozyme and the dissolution of the separated walls of *M. lysodeikticus* by this enzyme (SALTON[30]). Furthermore, owing to the sensitivity of *B. subtilis* to lysozyme, the presence of a mucoid component in the cell wall may have been expected. But unlike the cell walls of *M. lysodeikticus* and *Sarcina lutea* the mucoid component does not appear to be the sole structural unit of the cell wall of *B. subtilis*. The high P content of the cell wall of *B. subtilis*, together with the low lipid content, suggests that part of the wall of this organism may be a glycerophospho-protein complex of the type reported for *Staph. aureus* by MITCHELL AND MOYLE[9].

Several interesting differences between the properties of the Gram-positive and the Gram-negative cell walls are apparent. The lipid contents of the cell walls of the Gram-negative bacteria are substantially higher than those of the Gram-positive organisms studied. That such a difference may have been expected has been inferred from the greater resistance of Gram-negative bacteria to various chemical disinfectants (DUBOS[31]). Although some 36 % of the total lipid content of the cell wall of *E. coli* (see Table I) can be extracted with ether, there is some evidence to suggest that "loosely bound" lipid does occur in the surface of the intact cell of certain Gram-negative bacteria (CHAPLIN[32]). It is conceivable, therefore, that this lipid is indeed present in the cell wall of the intact cell and that it is not merely adventitious material nor an artefact of the method of preparing the cell walls.

With the exception of *Strep. pyogenes* cell wall which has retained the type-specific M-protein, the cell walls of the Gram-positive organisms have a limited complement of amino acids. However, on removal of the M-protein from the *Strep. pyogenes* wall, its amino acid constitution conforms to the general features observed for the other Gram-positive preparations and indeed shows a striking similarity to the amino acid constitution of the cell wall of *Strep. faecalis*. The cell walls of the two Gram-negative bacteria investigated, possess a much more complete range of amino acids, including aromatic, certain sulphur-containing amino acids, arginine and proline. The existence of such a marked difference in the amino acid constitution of the cell wall of the Gram-positive and the Gram-negative organism is of considerable interest and may be yet another reflection

References p. 523.

of the different biochemical properties of the two groups of bacteria (*e.g.* the ability of the Gram-negative organisms to synthesize many of their amino acids on the one hand and on the other, the requirement of a wide variety of amino acids for growth and the capacity to accumulate certain amino acids in the free state within the cell, shown by the Gram-positive bacteria—GALE[33], TAYLOR[34]).

It would have been of interest to compare more fully the composition of the cell walls prepared by mechanical disintegration with that of the walls obtained by heat-treatment rupture of *E. coli* and *Salmonella pullorum*. At least the similarity in appearance of the cell walls of *E. coli* as seen in the electron microscope is parallelled by the similarity in the range of amino acids detected. Although the same sugars were present in the cell wall material of *Salmonella pullorum* prepared by the two methods, the lower N content and the absence of diaminopimelic acid together with the fragmented appearance of the heat-ruptured walls[3,4] suggest that this method has degraded the cell wall.

The retention of the M-protein by the cell walls of *Strep. pyogenes* and its removal by trypsin illustrate the presence of molecular species which are not involved in the structural rigidity of the cell wall. Electron microscopic examination however, reveals no morphological differentiation of the whole cell wall structure into layers. KNAYSI[1] has already pointed out the possibility of the presence of thin slime layers surrounding the cell wall. It is, therefore, conceivable that such components are present in the cell walls of other bacteria. Although the rigid cell wall structures examined in these studies are single morphological entities, possessing a homogeneous appearance in the electron microscope, it is evident that the constituiton of some is characterized by molecular and antigenic heterogeneity. Studies of the composition of cell walls provide valuable information concerning the class of substance or substances occurring in the walls, but much more work remains to be done before these data can be extended to give a clear understanding of the sub-microscopic structure of the complex bacterial cell wall.

ACKNOWLEDGEMENTS

I wish to thank Dr J. H. SCHULMAN O.B.E and Dr E.F. GALE for their constant interest and help in these investigations, and Dr V. E. COSSLETT for making available the electron microscope for examinations of cell wall preparations. My thanks are also due to the Superintendent of the Low Temperature Research Station for kindly providing facilities for paper chromatography, to Dr J. BOISSARD for the strain of *Strep. pyogenes* and for performing the immunological tests, to Dr E. WORK for a generous gift of diaminopimelic acid and to Dr S. M. PARTRIDGE for helpful discussions and for reading the manuscript.

SUMMARY

Some general properties of the cell walls of four Gram-positive and two Gram-negative bacteria have been studied. The rate of hydrolysis of cell walls of several bacteria, by 2 *N* HCl at 100° C, has been followed by estimation of liberated reducing substances and hexosamine. Several extraction procedures have been used in a study of the lipid content of the cell walls of *E. coli* and *B. subtilis*.

References p. 523.

Cell wall preparations have been analysed for their N and P contents, reducing substances and hexosamine liberated on acid hydrolysis and total lipid contents. The lipid content of the cell walls of the two Gram-negative bacteria, *E. coli* and *Salmonella pullorum* is substantially higher than that of any of the Gram-positive cell wall preparations.

The products of acid hydrolysis of cell walls have been examined by paper chromatography. The amino acids of the cell walls have been identified. The amino acids of *Strep. pyogenes* wall have been examined before and after removal of the type-specific M-protein with trypsin. Cell walls of some Gram-positive species show very limited amino-acid constitutions, being devoid of aromatic and sulphur-containing amino acids. A much more complete range of amino acids including aromatic, certain sulphur-containing amino acids, arginine and proline, was found in the cell walls of the two Gram-negative species. The recently isolated amino acid, diaminopimelic acid, has been identified in the walls of certain bacteria. Three unknown ninhydrin-reacting substances have appeared on chromatograms of cell wall hydrolysates. The sugar components of the cell walls have been identified.

The presence of group specific substance in the cell wall of *Strep. pyogenes* has been demonstrated.

RÉSUMÉ

Nous avons étudié certaines propriétés générales des parois cellulaires de quatre bactéries Gram-positives et deux Gram-négatives. Nous avons suivi la vitesse d'hydrolyse des parois cellulaires de plusieurs bactéries par HCl 2 *N* à 100° en évaluant les substances réductrices et l'hexosamine libérées. Lors de l'étude de la teneur en lipoïdes des parois cellulaires d'*E. coli* et de *B. subtilis*, nous avons fait emploi de plusieurs procédés d'extraction. Nous avons analysé, dans des préparations de paroi cellulaire, les teneurs en N et P, les substances réductrices et l'hexosamine libérées par hydrolyse acide et la teneur totale en lipoïdes. La teneur en lipoïdes des parois cellulaires des deux bactéries Gram-négatives. *E. coli* et *Salmonella pullorum* est considérablement plus élevée que celle de n'importe laquelle des préparations de parois cellulaires Gram-positives.

Nous avons étudié les produits de l'hydrolyse acide des parois cellulaires par chromatographie sur papier. Les acides aminés des parois cellulaires ont été identifiés. Nous avons examiné les acides aminés de *Strep. pyogenes* avant et après destruction de la protéine M "type-spécifique". Les parois cellulaires de certaines espèces Gram-positives ont une composition d'acides aminés très restreinte ne contenant ni acides aminés aromatiques ni sulphurés. Une gamme beaucoup plus complète d'acides aminés, comprenant des acides aminés aromatiques et certains sulphurés de l'arginine et de la proline, se trouve dans les parois cellulaires des deux espèces Gram-négatives. Nous avons trouvé l'acide diamino-pimélique, acide aminé isolé récemment, dans les parois de certaines bactéries. Sur les chromatogrammes d'hydrolysats de parois cellulaires, trois substances inconnues, réagissant avec la ninhydrine, ont été observées. Nous avons identifié les sucres contenus dans les parois cellulaires.

Nous avons démontré la présence de matière "groupe-spécifique" dans la paroi cellulaire de *Strep. pyogenes*.

ZUSAMMENFASSUNG

Es wurden einige allgemeine Eigenschaften der Zellwände von vier Gram-positiven und zwei Gram-negativen Bakterien untersucht. Die Geschwindigkeit der Hydrolyse der Zellwände von mehreren Bakterien mit 2 *N* HCl bei 100° wurde durch Bestimmung der freigemachten reduzierenden Substanzen und Hexosamine verfolgt. Bei einer Untersuchung des Lipoidgehaltes der Zellwände von *E. coli* und *B. subtilis* wurden mehrere Extraktionsverfahren benutzt. Zellwandpräparate wurden auf ihren Stickstoff- und Phosphorgehalt, auf durch Säurehydrolyse freigemachte reduzierende Substanzen und Hexosamine und auf den Gesamtlipoidgehalt untersucht. Der Lipoidgehalt der Zellwände der zwei Gram-negativen Bakterien, *E. coli* und *Salmonella pullorum*, ist wesentlich höher als der irgend eines anderen der Gram-positiven Zellpräparate. Die Produkte der Säurehydrolyse der Zellwände wurde durch Papierchromatographie untersucht. Die Aminosäuren der Zellwände wurden identifiziert. Die Aminosäuren der Zellwand von *Strep. pyogenes* wurden vor und nach der Entfernung des typenspezifischen M-Proteins mit Trypsin untersucht. Die Zellwände einiger Gram-positiver Arten zeigten eine sehr beschränkte Zusammensetzung von Aminosäuren, ohne jegliche aromatische und schwefelenthaltende Aminosäuren. Eine viel vollständigere Reihe Aminosäuren, einschliesslich aromatischer, bestimmter schwefelenthaltender Aminosäuren, Arginin und Prolin, wurden in den Zellwänden der zwei Gram-negativen Arten gefunden. Die kürzlich isolierte Diaminopimelinsäure wurde in den Zellwänden bestimmter Bakterien identifiziert. Drei unbekannte mit Ninhydrii- reagierende Substanzen wurden auf dem Chromatogram der Zellwandhydrolysate beobachtet. De Zuckerkomponenten der Zellwände wurden identifiziert. Es wurde die Anwesenheit von gruppen spezifischer Substanz in den Zellwänden von *Strep. pyogenes* gezeigt.

References p. 523.

REFERENCES

1 G. KNAYSI, *Elements of Bacterial Cytology*, 2nd Ed., Ithaca, N.Y., 1951.
2 I. M. DAWSON, *The Nature of the Bacterial Surface*, p. 119, Oxford, 1949.
3 M. R. J. SALTON AND R. W. HORNE, *Biochim. Biophys. Acta*, 7 (1951) 19.
4 M. R. J. SALTON AND R. W. HORNE, *Biochim. Biophys. Acta*, 7 (1951) 177.
5 W. WEIDEL, *Viruses* 1950, California Institute of Technology, p. 119, 1950.
6 W. WEIDEL, *Z. Naturforschung*, 6b (1951) 251.
7 M. R. J. SALTON, Ph. D. Thesis, University of Cambridge, 1951.
8 M. R. J. SALTON, *Biochim. Biophys. Acta*, 8 (1952) 510.
9 P. MITCHELL AND J. MOYLE, *J. Gen. Microbiol.*, 5 (1951) 981.
10 E. S. HOLDSWORTH, *Biochim. Biophys. Acta*, 8 (1952) 110.
11 M. J. JOHNSON, *J. Biol. Chem.*, 137 (1941) 575.
12 C. H. FISKE AND Y. SUBBAROW, *J. Biol. Chem.*, 66 (1925) 375.
13 C. S. HANES, *Biochem. J.*, 23 (1929) 99.
14 L. A. ELSON AND W. T. J. MORGAN, *Biochem. J.*, 27 (1933) 1824.
15 R. CONSDEN, A. H. GORDON AND A. J. B. MARTIN, *Biochem. J.*, 38 (1944) 224.
16 S. M. PARTRIDGE, *Biochem. J.*, 42 (1948) 238.
17 S. M. PARTRIDGE, *Nature*, 164 (1949) 443.
18 M. A. JERMYN AND F. A. ISHERWOOD, *Biochem. J.*, 44 (1949) 402.
19 R. REICHERT, *Helv. Chim. Acta*, 27 (1944) 961.
20 C. E. DENT, *Biochem. J.*, 41 (1947) 240.
21 C. E. DENT, *Biochem. J.*, 43 (1948) 169.
22 E. WORK, *Biochim. Biophys. Acta*, 3 (1949) 400.
23 E. WORK, *Biochim. Biophys. Acta*, 5 (1950) 204.
24 E. WORK, *Biochem. J.*, 49 (1951) 17.
25 E. L. DURRUM, *J. Am. Chem. Soc.*, 72 (1950) 2943.
26 C. A. ZITTLE AND S. MUDD, *Ann. N.Y. Acad. Sci.*, 43 (1942) 47.
27 R. C. LANCEFIELD, *J. Exptl. Med.*, 78 (1943) 465.
28 W. C. SCHMIDT, *J. Exptl. Med.*, 95 (1952) 105.
29 W. R. MAXTED, *Lancet*, 2 (1948) 255.
30 M. R. J. SALTON, *Nature*, in the Press.
31 R. J. DUBOS, *The Bacterial Cell*, Harvard University press, 1945.
32 C. E. CHAPLIN, *J. Bact.*, 63 (1952) 453.
33 E. F. GALE, *J. Gen. Microbiol.*, 1 (1947) 53.
34 E. S. TAYLOR, *J. Gen. Microbiol.*, 1 (1947) 86.

Received July 22nd, 1952