administered. Ligation of the vasa efferentia and depletion of epididymal spermatozoa decreases the amount of [^{32}P]phosphate incorporated into both the lecithin and glycerylphosphorylcholine.

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Cell Walls of *Propionibacterium* Species: Fractionation and Composition

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The mucopeptide components of cell walls of different species of bacteria are characterized by their amino acids and hexosamines (see reviews by: Work, 1957, 1961; Salton, 1960; Sharon, 1963; Perkins, 1963). D-Glutamic acid, D- and L-alanine, muramic acid, glucosamine and either lysine or diaminopimelic acid are always present; glycine, D- and L-aspartic acid and galactosamine may also occur. In addition, there is often a polysaccharide which is intimately connected with the mucopeptide and which may contain up to four major sugar components. These constituents provide a basis for considerable variation of wall structure between different species. Among Gram-positive bacteria many varieties of mucopeptides occur, but in Gram-negative organisms there is little or no species differentiation with regard to the mucopeptide components (Mandelstam, 1962). Mucopeptides containing lysine are confined almost entirely to the Gram-positive cocci and lactobacilli, while those containing *meso*-diaminopimelic acid are widely distributed, being present in all Gram-negative bacteria and many Gram-positive forms. LL-Diaminopimelic acid is mainly found in species of *Propionibacterium*, *Streptomyces*, *Actinomyces*

and *Micromonospora* (Hoare & Work, 1955; Antia, Hoare & Work, 1957; Cummins & Harris, 1958; Cummins, 1962).

The object of the present investigation is the analysis of cell walls that contain LL-diaminopimelic acid, as this type of wall has not yet been studied in detail. Walls from two species of *Propionibacterium* were prepared by a modification of conventional techniques; since their compositions were very similar, walls from only one species, *P. peterssonii*, were further examined after fractionation by density-gradient centrifuging, a technique applied to cell-wall preparations by Roberson & Schwab (1960) and Yoshida, Hedén, Cedergren & Edebo (1961). The effects of hot formamide extraction on the composition and lysozyme sensitivity of walls are described. A preliminary report has already been presented (Allsop & Work, 1962).

METHODS

Organisms. Propionibacterium peterssonii, NCTC 5962, and *P. rubrum*, NCIB 8901, were grown in tomato-juice broth (Briggs, 1953) or casein–yeast extract–lactate–glucose medium (Marshall & Kelsey, 1960) at 30° for

3–5 days in screw-capped bottles (1 l. capacity) completely filled with medium.

Preparation of unfractionated cell walls. The cells (5–9 g. dry wt.) from 5 l. of medium were harvested on the continuous-flow head of the Servall centrifuge SS-1, washed twice in 0·9% (w/v) NaCl and resuspended in water (120 ml.). Cell disintegration was carried out on the International Centrifuge model PR-2 in the shaker head described by Shockman, Kolb & Toennies (1957). Each of the four metal capsules contained 30 g. of Ballotini beads, 30 ml. of cell suspension and 1 drop of octanol. The centrifuge was run at 1000 rev./min. for 30 min. at −10°. The beads were removed by filtration through a sintered-glass funnel and washed with distilled water; the filtrate and washings were centrifuged at about 1000g for 7 min. to sediment unbroken cells. If a large pad was obtained, the cells were resuspended and disintegrated again. The supernatant liquids were centrifuged at 2° at 22 000g for 40 min.; the pad was resuspended in 0·1 M-phosphate buffer, pH 8·0 (200 ml.), containing 0·3% of trypsin and incubated at 37° until the extinction had fallen to a constant level (4–5 hr.). In this and all subsequent steps, chloroform-saturated reagents were used to prevent bacterial contamination. The low- and high-speed centrifugings were repeated and the walls (material sedimented at 22 000g) were washed by resuspending and centrifuging (22 000g, 15 min.) twice in M-NaCl, twice in 0·1 M-phosphate buffer, pH 7·0, three times in M-NaCl, and three times in water.

Density-gradient centrifuging of walls. The washed walls were resuspended in 48 ml. of M-NaCl and 12 ml. was layered on top of each of four linear gradients of sucrose (0–40%, w/v, in M-NaCl) (Yoshida *et al.* 1961). The gradients were made in 250 ml. glass centrifuge bottles by the method of Ribi & Hoyer (1960). The bottles were centrifuged for 1 hr. in a swing-out head at 2500 rev./min. (1400g), after which the individual bands from each gradient were collected by pipette, pooled and centrifuged and the pads resuspended in M-NaCl. The larger fractions were resedimented on fresh gradients until they were free from other fractions. The debris from the bottom of the tubes was resuspended and resedimented. Finally each fraction was dialysed against running tap water until free from sucrose, and freeze-dried.

Treatment with lysozyme. Suspensions containing 0·5–1·0 mg. of walls/ml. were treated at 37° or room temperature with lysozyme at final concentrations up to 200 μg./ml. in 0·1 M-phosphate buffer, pH 6·8. Digestion was followed by observing the fall in extinction of the suspensions. The degree of digestion was determined more exactly after overnight incubation of 50 mg. or more of walls, by centrifuging the digest in a weighed tube, and washing, drying and weighing the insoluble residue.

Analytical techniques. Standard suspensions were made by shaking known weights of freeze-dried walls in water in the Mickle shaker for 10 min. For amino acid and hexosamine analysis, hydrolysis was carried out in sealed tubes with 4 N-HCl for 8 hr. at 105°. Hydrochloric acid was removed *in vacuo* over NaOH by repeated addition and removal of water, and the sample was resuspended in water (1 ml. equivalent to 5 mg. of walls).

Chromatography. Two-dimensional chromatograms on Whatman no. 4 paper were used to separate amino acids and amino sugars; the solvents were aq. phenol (NH₃ atmosphere) and butan-1-ol–acetic acid–water (67:10:23,

by vol.). The isomer of diaminopimelic acid was identified on Whatman no. 1 paper, with the solvent methanol–water–10 N-HCl–pyridine (32:7:1:4, by vol.) (Rhuland, Work, Denman & Hoare, 1955; Hoare & Work, 1957). Amino acid and hexosamine spots were developed by dipping the paper in ninhydrin in acetone (1%, w/v) and heating at 105° for 5 min. Hexosamine spots were also developed by Ehrlich's reagent (Partridge, 1948). For sugar analysis, hydrolysis by 2 N-H₂SO₄ at 105° for 2 hr. was followed by neutralization with Ba(OH)₂ and the liquid was concentrated *in vacuo* at room temperature. Sugars were separated in butan-1-ol–acetic acid–water (4:1:1, by vol.) or in two dimensions with aq. phenol and butan-1-ol–pyridine–water (6:4:3, by vol.) (Primosigh, Pelzer, Maass & Weidel, 1961). Spots were developed by dipping in aniline phthalate in acetone (3·5%, w/v) and heating at 105° for 10 min.

DNP-amino acids were separated on paper by using the top layer prepared from isopentanol–phenol–water (1:1:1, by wt.) in an ammoniacal atmosphere (Biserte & Osteux, 1951). Thin-layer chromatograms developed with chloroform–methanol–acetic acid (95:5:1, by vol.) were also used (Brenner, Niederwieser & Pataki, 1961).

Examination for teichoic acids was carried out on products from hydrolysis in 2 N-HCl for 3 hr. at 100°. Solvents were either propan-1-ol–NH₃ solution (sp.gr. 0·88)–water (6:3:1, by vol.) or butan-1-ol–ethanol–water–NH₃ solution (sp.gr. 0·88) (40:10:49:1, by vol.). The chromatograms were sprayed with sodium metaperiodate–Schiff reagent (Baddiley, Buchanan, Handschumacher & Prescott, 1956). A similar hydrolysate prepared from teichoic acid of *Staphylococcus aureus* H (Baddiley, Buchanan, Raj-Bhandary & Sanderson, 1962) was used as a marker.

Amino acid analysis. Amino acids, muramic acid and NH₃ were determined by ion-exchange chromatography (Moore, Spackman & Stein, 1958); an amount of hydrolysate equivalent to 3 mg. of original walls was used for each column. Ammonia was also estimated in microdiffusion cells (Conway, 1957).

Alanine isomers. The isomers of alanine were determined by D-amino acid oxidase by a method suggested by J. L. Strominger (personal communication). Hydrolysed walls (150–500 μg.) were mixed with 1 μl. of FAD (900 μg./ml.), 1 μl. of catalase (Boehringer suspension) and 50 μl. of D-amino acid oxidase (2 mg./ml. buffer, L. Light and Co. Ltd.) and the volume was made up to 0·5 ml. with cold, oxygenated 0·05 M-sodium pyrophosphate buffer, pH 8·3. The mixture was incubated for 2 hr. at 37°, then treated with 15% (w/v) trichloroacetic acid (10 μl.); 30 mM-quinolylhydrazine (10 μl.) was added to form a complex with the pyruvic acid produced from D-alanine. The samples were incubated for a further 30 min. at 37° and then diluted with 0·01 N-HCl to 5 ml., and the extinction at 305 mμ was measured against a reagent blank. There was no interference by other mucopeptide constituents and there was 100% recovery of 7 μg. of D-alanine added to 150 μg. of hydrolysed walls.

Glutamic acid isomers. L-Glutamic acid was determined by the method of Sowerby & Ottaway (1961) by use of L-glutamate dehydrogenase. Hydrolysed cell walls (1–2 mg.) were mixed with 11·2 mM-3-p-iodophenyl-2-p-nitrophenyl-5-phenyltetrazolium chloride (0·3 ml.), 1·75 mM-phenazine methosulphate (0·03 ml.), 60 mM-KCN (0·3 ml.) and 0·5 ml. of enzyme mixture (made fresh daily) consisting of

0·02 ml. of glutamate dehydrogenase (Boehringer suspension 20 mg./ml.), 2 mg. of NAD, 30 mM-EDTA (0·1 ml.) and 0·88 ml. of 0·09M-tris buffer, pH 7·6. Tris buffer (pH 7·6) was added to a final volume of 3 ml. and final concentration 0·06M, and the reaction mixture was incubated at 37° for 30 min. The formazan, formed by the reaction between 3-p-iodophenyl-2-p-nitrophenyl-5-phenyl-tetrazolium chloride and reduced NAD, was dissolved by adding 5 ml. of acetone to the mixture, and the extinction at 460 mμ of the solution was read against a reagent blank. D-Glutamic acid was estimated by subtraction. The least amount of L-glutamic acid measured by this method was 10 μg.

Hexosamine estimation. Glucosamine and galactosamine in 0·4 ml. of hydrolysed cell walls were separated on Zeo-Karb 225, 8% cross-linked, with 0·03N-HCl as eluting fluid, by the method of Gardell (1953), and estimated in the eluate by his modification of the Elson–Morgan reaction.

Nitrogen estimation. Nitrogen was determined by the micro-Kjeldahl method.

Carbohydrate estimation. Total carbohydrate was determined by the colorimetric method of Dubois, Gilles, Hamilton, Rebers & Smith (1956). Walls (400 μg.) in 2 ml. of water were treated with 0·05 ml. of aq. phenol (80%, w/v) and 5 ml. of conc. H_2SO_4. After standing for 5 min. the tubes were cooled in tap water and the extinction at 490 mμ was measured against a reagent blank. A glucose solution was used as a standard and the results were expressed as glucose equivalent.

Phosphorus estimation. Phosphorus was determined by the method of Long (1943), by using 2·5 mg. of walls digested in 60% (w/v) perchloric acid.

Amino-end group analysis. DNP derivatives of the walls were prepared and hydrolysed by the method of Ingram & Salton (1957). The water-soluble mono-DNP-diaminopimelic acid was identified by paper chromatography in butan-1-ol-acetic acid–water (4:1:1) with a marker prepared from a hydrolysate of DNP-treated walls of *Bacillus megaterium*; for this purpose, the ether-insoluble aqueous phase was adjusted to N-HCl and put on a column of talc (50 g.) and Hyflo Super-Cel (20 g.): after washing with N-HCl (100 ml.), mono-DNP-diaminopimelic acid was eluted with 400 ml. of ethanol–N-HCl (4:1, v/v) and the eluate was concentrated *in vacuo*. Ether-soluble DNP-amino acids were estimated by elution with 1% (w/v) NaHCO₃ solution from paper chromatograms developed in isopentanol–phenol–water: the extinction at 360 mμ was compared with that of known amounts of DNP-amino acids treated in the same way.

Formamide treatment. Walls (150 mg.) were treated with 25 ml. of formamide at 170° for 20 min. (Krause & McCarty, 1962). After cooling, the insoluble residue was centrifuged and washed once with ethanol. The supernatant was treated with 2·5 vol. of acid-ethanol (5% of 2N-HCl, v/v) and any small precipitate obtained was mixed with the insoluble material. The solids were dialysed against running tap water. The acid-ethanol solution was treated with 5 vol. of acetone to precipitate the 'polysaccharide' (see Results).

Optical measurements. Spectrophotometric measurements were made in Unicam spectrophotometers SP. 500 and SP. 600 in 1 cm. light-path cells. Extinctions of suspensions were measured in the EEL portable colorimeter, model A, filter no OGR 1.

RESULTS

Composition of unfractionated walls of P. peterssonii *and* P. rubrum. The freeze-dried unfractionated cell walls of both species were brown, those of *P. rubrum* being darker. The yield of walls was 16–20% of the bacterial dry weight. Chromatography of hydrolysed walls from both organisms showed that the main constituents were glucose, galactose, muramic acid, glucosamine, galactosamine, glutamic acid, glycine, alanine and LL-diaminopimelic acid. The nitrogenous compounds are referred to below as mucopeptide constituents. The walls from the two species were very similar in composition (Table 1). The molar ratios of glutamic acid, alanine, diaminopimelic acid and glycine were approximately 1:2:1:1 respectively. No L-glutamic acid was detected: as the limit of detection was 5% of the total glutamic acid present in the sample, it was presumed that at least 95% was D-glutamic acid. About one-third of the total alanine was D-alanine. In addition to the mucopeptide constituents there were minor amounts of other amino acids. About 10% of the total glycine was found to have free amino groups; no detectable ether-soluble DNP derivatives were produced from other amino acids. Only a trace of the mono-DNP derivative of diaminopimelic acid was found, indicating that the greater part of both its amino

Table 1. *Analysis of unfractionated walls*

Molar ratios are expressed in relation to glutamic acid. –, Not detected.

	P. peterssonii		P. rubrum	
	%	Molar ratio	%	Molar ratio
D-Glutamic acid	4·79	1·00	5·20	1·00
Alanine (total)	5·06	1·70	5·59	1·77
D-Alanine	1·97	0·66	1·86	0·58
LL-Diaminopimelic acid	5·72	0·92	7·09	1·06
Glycine	2·63	1·07	3·30	1·25
Muramic acid*	4·13	0·51	3·80	0·43
Glucosamine*	4·06	0·70	4·46	0·70
Galactosamine*	2·79	0·48	3·24	0·51
Ammonia	1·83	3·31	1·18	1·96
Aspartic acid	0·22	0·05	0·37	0·08
Threonine	–	–	0·20	0·05
Serine	0·22	0·06	0·29	0·08
Proline	–	–	–	–
Valine	0·21	0·06	0·68	0·16
Isoleucine	0·96	0·02	0·31	0·07
Leucine	0·29	0·07	0·51	0·11
Tyrosine	–	–	–	–
Phenylalanine	–	–	–	–
Lysine	0·61	0·13	0·41	0·08
Histidine	0·64	0·13	0·99	0·18
Arginine	–	–	–	–
Nitrogen	7·5	.	4·9	.
Phosphorus	0·14	.	0·23	.

* Uncorrected for destruction during hydrolysis.

groups was bound. The phosphorus content was less than 0·3 % and was not changed by extraction with cold trichloroacetic acid.

Fractionation of walls of Propionibacterium peterssonii *by density-gradient centrifuging.* When walls of *P. peterssonii* were fractionated by density-gradient centrifuging, two main bands (2 and 3) were obtained at positions corresponding to sucrose concentrations of 11–14 and 33 % respectively. The freeze-dried material from these bands was white. A third major fraction ('debris') sedimented to the bottom of the gradient; after freeze-drying, it was pale cream in colour. The proportion of starting material found in each of these three fractions varied from one preparation to another; in three successive preparations the respective percentages of the wall weight occurring in the various fractions were as follows: fraction 2: 50, 16 and 77; fraction 3: 20, 52 and 8; debris: 25, 30 and 8. The materials from bands 2 and 3 were very different in composition (Table 2). The less dense material (fraction 2) contained 23 % of carbohydrate, and besides the mucopeptide constituents (hexosamines and four amino acids) it had only very minor amounts of other amino acids. Fraction 3 was apparently a mixture of protein, polysaccharide and mucopeptide, as it contained significant amounts of all protein amino acids; the content of aspartic acid was particularly high, being almost equal to that of glutamic acid. Both fractions resembled unfractionated walls, in having 10 % of their glycine with free amino groups, and a small amount of diaminopimelic acid with one amino group free. The debris was examined only by paper chromatography; it resembled fraction 3 in containing protein as well as mucopeptide, but here aspartic acid was no more prominent than other protein amino acids.

Trypsin, chymotrypsin or pepsin had no effect on the extinction of suspensions of any fractions and trypsin did not remove protein amino acids from fraction 3. Both papain and the proteolytic enzyme from *Streptomyces griseus* (Pronase, Kaken Chemical Co. Ltd., Tokyo, Japan) reduced the extinction of fraction 3 and debris, and also removed most but not all of their protein.

Electron microphotographs of negatively stained preparations (kindly carried out by Mr R. W. Horne) from fraction 2 showed large clumps of reasonably clean cell walls. Only debris of various shapes and sizes could be seen in preparations of fraction 3; possibly excessive clumping of the walls had prevented their being spread in the phosphotungstic acid.

Digestion of walls by lytic enzymes. Overnight incubation of suspensions of unfractionated walls of either species at 37° with lysozyme (200 μg./ml.) produced no change in extinction. The walls were not rendered susceptible to lysozyme by the following treatments: 18 hr. at 2°, or 15 min. at 90°, with trichloroacetic acid (10 %, w/v); 1 hr. at 37° with 0·01 N-sodium hydroxide; 24 hr. at 25° with anhydrous formic acid; 1 hr. at 25° with 90 % phenol.

Treatment of walls from both species with formamide at 170° for 20 min., followed by washing and dialysis, rendered the walls susceptible to lysozyme. Preliminary dialysis was necessary, otherwise the results were not repeatable, possibly owing to inhibition of lysozyme by residual formamide. Suspensions of treated walls showed 70 % decreases in extinction after 2 hr. at 18° with lysozyme (133 μg./ml.); only 5 % by weight of material remained insoluble after incubation overnight at 37°. Heating with formamide at 150° also induced lysozyme susceptibility, but the degree of digestion by the enzyme was less; heating at 100° or overnight at 37° was not effective. Walls previously treated with trichloroacetic acid, formic acid or sodium hydroxide, and subsequently extracted by formamide, were digested by lysozyme. Fractions 2 and 3 from density-gradient centrifuging were both insensitive to lysozyme, but after hot formamide treatment only 2 and 8 % (by weight) respectively remained insoluble after lysozyme digestion. Treatment of fraction 3 with papain or Pronase did not induce lysozyme sensitivity.

J. M. Ghuysen (personal communication) found that untreated unfractionated walls of *P. peterssonii* were digested by the enzyme from *Streptomyces* now known as *N*-acetylhexosaminidase (Dierickx & Ghuysen, 1962), previously called F_1 enzyme (Ghuysen & Salton, 1960). Incubation of walls for 24 hr. with 5 μg. of enzyme/ml. produced a 75 % reduction in extinction; no further digestion resulted when F_2B enzyme containing amidase was also present.

Composition of formamide-extracted walls of P. peterssonii. The insoluble residues remaining after formamide extraction of walls represented only 30–40 % of the original weight. This range was found for *P. rubrum* as well as *P. peterssonii*, also for crude as well as fractionated walls. The residues were always dark brown in colour, even if the starting preparations were white. Analytical results (Table 2) showed that formamide had removed virtually all the non-mucopeptide amino acids and most of the carbohydrate and phosphorus from both fractions 2 and 3. The amino acids of both residues were limited to glutamic acid, alanine, diaminopimelic acid and glycine, in molar proportions of 1:1·8:1:1 respectively. D-Alanine (1·1 moles relative to glutamic acid) made up 60 % of the total alanine. The end-amino groups in both fractions after formamide treat-

33-2

Table 2. *Analyses of fractions from walls of* Propionibacterium peterssonii

Washed trypsin-treated walls were layered on sucrose gradients (0–40 % in M-NaCl) and centrifuged for 1 hr. at 2500 rev./min. Fraction 2 is a band which separated at 11–14 % of sucrose, fraction 3 at 33 % of sucrose. After several gradients, walls were dialysed free of sucrose and freeze-dried. Formamide treatment was carried out for 20 min. at 170°; the insoluble residue was washed, dialysed and dried. Molar ratios are expressed in relation to glutamic acid. +, Present but not estimated; –, not detected.

| | Fraction 2 | | | | Fraction 3 | | | |
| | Untreated | | Formamide-treated | | Untreated | | Formamide-treated | |
	%	Molar ratio	%	Molar ratio	%	Molar ratio	%	Molar ratio
D-Glutamic acid	6·25	1·00	11·12	1·00	7·05	1·00	7·03	1·00
Alanine (total)	6·89	1·82	12·68	1·87	6·41	1·50	7·33	1·72
D-Alanine	.	.	7·46	1·07	.	.	4·46	1·04
LL-Diaminopimelic acid	7·21	0·89	13·35	0·93	3·30	0·36	7·45	0·82
Glycine	4·08	1·28	5·86	1·03	4·04	1·12	3·76	1·05
Muramic acid*	3·64	0·34	10·11	0·53	1·31	0·11	4·52	0·38
Glucosamine*	} 6·59	} 0·87	5·31	0·40	2·97	0·35	3·03	0·36
Galactosamine*			5·22	0·39	3·14	0·37	2·85	0·33
Ammonia	1·34	1·86	2·24	1·74	2·53	3·10	1·80	2·22
Aspartic acid	0·68	0·12	–	–	4·25	0·67	–	–
Threonine	0·38	0·07	–	–	2·54	0·44	–	–
Serine	0·51	0·11	–	–	2·23	0·44	–	–
Proline	–	–	–	–	1·89	0·34	–	–
Valine	0·56	0·11	0·32	0·04	2·34	0·42	0·46	0·08
Isoleucine	–	–	–	–	1·48	0·24	0·67	0·11
Leucine	0·69	0·12	–	–	3·71	0·59	0·72	0·11
Tyrosine	–	–	–	–	1·47	0·17	–	–
Phenylalanine	–	–	–	–	1·47	0·19	–	–
Lysine	1·20	0·19	–	–	+	+	–	.
Histidine	1·71	0·26	–	–	+	+	–	–
Arginine	–	–	–	–	+	+	–	–
Nitrogen	8·5	.	8·1	.	12·2	.	7·3	.
Carbohydrate†	22·92	.	2·62	.	13·14	.	1·75	.
Phosphorus	0·51	.	0·19	.	0·50	.	0·17	.
Weight recovered‡	57·9	.	58·2	.	55·1	.	33·7	.
Nitrogen recovered	66·5	.	101·2	.	66·9	.	81·0	.

* Uncorrected for destruction during hydrolysis. † Expressed as glucose. ‡ Allowing for water of hydrolysis.

ment were identical with those of the original materials. In either fraction, equal amounts of glucosamine and galactosamine were present.

The residues after formamide treatment of fraction 2 were richer than the original material in all the mucopeptide constituents, particularly in muramic acid. With fraction 3, only diaminopimelic acid and muramic acid showed marked rises in concentration after formamide treatment. The formamide residues of fractions 2 and 3 were not identical in composition, although the molar ratios of the amino acids were similar; fraction 2 contained higher concentrations of all mucopeptide constituents. Although all the nitrogen of fraction 2 residue was found in the mucopeptide constituents, only 58 % of the weight was accounted for, after allowing for N-acetylation of hexosamine residues and muramic acid. In fraction 3 residue, only 64 % of the nitrogen and 32 % of the weight were accounted for. Electron microphotographs of negatively stained preparations from both residues resembled those of homogeneous cell-wall preparations.

Material extracted from walls by various reagents. Formamide extracts of walls produced, on treatment with acetone, a white powder, referred to below as 'polysaccharide'. It represented about 50 % of the weight of the extracted material when the extraction was carried out at 170°; at 150° there was much less 'polysaccharide', and none was obtained from extracts prepared at 100° or 37°. 'Polysaccharide' from unfractionated walls of both species of *P. peterssonii* contained all amino acids, including diaminopimelic acid, but the mucopeptide constituents (except muramic acid) were predominant. Paper chromatography showed the presence of glucose, galactose and some unidentified substances giving positive reactions for reducing sugars and hexosamines. Fractionated walls (fractions 2 and 3) of *P. peterssonii* produced 'polysaccharides' that resembled those from unfractionated walls in their sugar patterns. Both contained mucopeptide constituents, but the protein amino acids in the 'polysaccharide' from fraction 3 were much more predominant than in the 'polysaccharide' from fraction 2. These 'polysac-

charides' contained 1% or more of phosphorus; when hydrolysates were examined by paper chromatography for teichoic acid it was evident that they contained anhydroribitol and some unidentified polyols. The material remaining in solution after precipitation with acetone was recovered after removal of solvents by distillation and washing with ethanol and water. The black insoluble residues contained all protein amino acids, but no diaminopimelic acid.

When the formic acid extract (10 ml.) from 77 mg. of fraction 2 was diluted by adding ice and dialysed against cold distilled water, a white precipitate (2·5 mg.) was produced inside the dialysis sac. It contained mucopeptide constituents and gave a strong positive reaction for polysaccharides with the phenol–sulphuric acid reagent. It was not examined for teichoic acids. Hot trichloroacetic acid extracted similar mixtures of constituents and also small amounts of phosphorus (0·3–0·5%) and teichoic acid-like substances.

The possible presence of nucleic acids in fraction 3 was investigated spectroscopically on the hot-trichloroacetic acid extract. There was a small amount, representing only about 5% of the starting material. Fraction 3 (54 mg.) was also examined for lipids by extraction with either chloroform or ethanol–ether (3:1, v/v); insignificant amounts were removed by either solvent. Aqueous phenol (90%, w/v) at room temperature did not extract anything that could subsequently be precipitated with ether.

DISCUSSION

The analytical results on the unfractionated walls of the two species of *Propionibacterium* examined show that they are remarkably similar in composition. In addition to the usual mucopeptide components (glutamic acid, alanine, diaminopimelic acid, muramic acid and glucosamine) both species contained glycine, galactosamine, glucose and galactose. These constituents, with mannose as well, were found in *P. peterssonii* by Cummins & Harris (1958) during the course of a wide qualitative survey of cell walls of Actinomycetes and related groups. Walls of *P. rubrum* have not been previously examined.

The separation of walls of *P. peterssonii* into three main fractions by density-gradient centrifuging shows that this type of material, prepared by extensive washing and differential centrifuging of trypsin-treated walls, does not consist of a single species of particle. Full analyses were carried out only on the two fractions that had separated into apparently homogeneous bands in the sucrose gradients, as there was no criterion of homogeneity applicable to the third 'debris' fraction which sedimented to the bottom of the gradient. The less

dense fraction 2 was relatively free from protein contaminants, whereas the heavier fraction 3 and the debris contained considerable amounts of all the normal amino acids of proteins. With few exceptions, minor amounts of protein amino acids have been found in all cell-wall preparations reported in the literature. Nothing is known about the significance or nature of the proteins contributing these amino acids except that they are resistant to various animal proteolytic enzymes, as in *P. peterssonii*. No explanation can be put forward for the observed differences in composition and density of the fractions, nor for their varying proportions in different batches. Since the phosphorus content of the protein-rich fraction 3 was no higher than that of fraction 2 and there was no lipid present, it is unlikely that the protein originated from contamination by the cytoplasmic membrane, which, being a lipoprotein, would have contributed both phosphorus and lipid as well as protein.

Previous reports of fractionation of walls by density-gradient centrifuging (Roberson & Schwab, 1960; Yoshida *et al.* 1961) have not included full detailed analyses. Yoshida *et al.* (1961) examined five species of Gram-positive organisms: three gave only one band, and the others yielded two discrete bands; the less dense band consisted of cell walls and the heavier one was described as 'intact cells which were Gram-negative, fuchsin-positive and optically dense'; the material sedimenting to the bottom of the tube was said to be non-disintegrated cells. These preparations had not been treated with trypsin, so are not directly comparable with the walls from *P. peterssonii*, where no intact cells were observed in any fraction.

The cause of insensitivity to lysozyme of walls of various species of bacteria is not generally known. Treatment of resistant walls with either dilute alkali or trichloroacetic acid can, in some cases, render them lysozyme-sensitive, possibly as a result of removal of *O*-acetyl groups, polysaccharides or teichoic acids according to the treatment (Brumfitt, 1959; Ikawa & Snell, 1960; Ikawa, 1961; Mandelstam & Strominger, 1961; Salton & Pavlik, 1960). Hot formamide is known to remove group-specific polysaccharide from streptococcal walls and to render them lysozyme-sensitive (Krause & McCarty, 1961, 1962). Although 'polysaccharide' and mucopeptide and probably teichoic acid were extracted from *Propionibacterium* walls by formic acid, hot trichloroacetic acid and hot formamide, lysozyme sensitivity was produced only by formamide; dilute alkali was also without effect. It is therefore not yet possible to attribute lysozyme resistance in *Propionibacterium* to any one constituent of the walls. The susceptibility of untreated *Propionibacterium* walls to *N*-acetylhexos-

aminidase of *Streptomyces* cannot yet be explained. The type of bonds split by hot formamide are not known; since no new free amino groups appeared after treatment, it is evident that amide bonds are not attacked. The results of the analyses on fraction 2 before and after formamide treatment could be explained by supposing that intact mucopeptide units containing slightly more than the average content of glycine were removed at the same time as a polysaccharide containing hexosamines, but not much muramic acid. The same explanation could be applied to fraction 3 from which a considerable amount of protein and aspartic acid was also removed, resulting in marked rises in concentrations of the two specific mucopeptide constituents, diaminopimelic acid and muramic acid. Although the mucopeptide components accounted for all the nitrogen of formamide-extracted fraction 2 they made up only 58 % of the weight; the nature of the other components is not known. In fraction 3, after formamide treatment, mucopeptide contributed even less to the total weight. This failure to account for all components has been a common finding in cell-wall analyses.

Little can be deduced about the structure of the mucopeptide unit present in formamide-treated *P. peterssonii* walls. D-Glutamic acid, LL-diaminopimelic acid, glycine and D-alanine were present in almost equimolar ratios; there was slightly less than 1 molar proportion of L-alanine and 0·5 or less of muramic acid and each of the hexosamines. These proportions resemble those found in mucopeptides from Gram-negative cells, which do not, however, contain glycine (Mandelstam, 1962; Schocher, Bayley & Watson, 1962); mucopeptides from Gram-positive bacteria usually contain proportionately more alanine, glucosamine, muramic acid and glycine, although in some cases similar proportions occur to those reported here (see review by Perkins, 1963). Equal amounts of glucosamine and galactosamine were found in purified wall fractions of *P. peterssonii* both before and after formamide extraction. This was not the case with group C streptococcal walls, where most of the galactosamine but little glucosamine was extracted by formamide (Krause & McCarty, 1962). Nothing is known about the position of galactosamine in cell walls.

Examination of the literature shows that the basal amino acid patterns of mucopeptides vary according as they contain *meso*-diaminopimelic acid, LL-diaminopimelic acid or lysine (see review by Work, 1961). Walls containing lysine may have either glycine or aspartic acid present (Cummins & Harris, 1956; Cummins, 1962), possibly bound to some if not all of the ε-amino groups of lysine (Salton, 1961). In walls where diaminopimelic acid is present only as the *meso*-isomer, glycine and

aspartic acid are never found (Cummins & Harris, 1958; Cummins, 1962), but often the diaminopimelic acid has many free ω-amino groups (Salton, 1961; Ghuysen, Leyh-Bouille & Dierickx, 1962). LL-Diaminopimelic acid is often accompanied by glycine, but never by aspartic acid (Cummins, 1962; Cummins & Harris, 1958), and in *Propionibacterium* it has very few free ω-amino groups. The existence of so few end-amino groups in *Propionibacterium* walls (about 10 % of the total glycine) suggests that there is extensive cross-linking among the peptide chains. Perhaps glycine forms a link between the ω-amino group of diaminopimelic acid and a carboxyl group of an adjacent peptide chain. This type of cross-linkage has already been suggested for certain mucopeptides containing lysine, in some of which there is more than one glycine residue per mucopeptide unit (Salton, 1961; Ghuysen, 1961; Mandelstam & Strominger, 1961).

The identity of the carbon chains of *meso*- and LL-diaminopimelic acid and lysine, one of which always occurs in mucopeptides together with D-glutamic acid and D- and L-alanine, suggests that the same basal peptide chain may occur in all bacterial cell walls. If this were the case, it would follow that the orientations of the carbon side chains of lysine and of both the diaminopimelic acid isomers were similar, i.e. their α-L-carbon atom would lie in the main peptide chain; as a consequence, when *meso*-diaminopimelic acid is present, its D-carbon atom would be in the ω-position in the side chain. The amino acid sequence in a mucopeptide subunit has been proved only for *Escherichia coli*; it is L-alanine (bound through its amino group to muramic acid), D-glutamic acid, *meso*-diaminopimelic acid, D-alanine (Pelzer, 1962); circumstantial evidence has suggested a similar sequence (with lysine replacing diaminopimelic acid) in *Staphylococcus aureus* (Mandelstam & Strominger, 1961).

SUMMARY

1. Cell walls from *Propionibacterium peterssonii* and *P. rubrum* have been prepared and analysed. They were very similar in composition and contained D-glutamic acid, D- and L-alanine, LL-diaminopimelic acid, glycine, muramic aid, glucosamine, galactosamine, glucose and galactose.

2. The walls were not digested by lysozyme, but could be made susceptible by treatment with formamide at 170°. No other reagents had this effect.

3. Fractionation of walls of *P. peterssonii* by density-gradient centrifuging produced two homogeneous fractions. There was also some material which sedimented to the bottom of the gradient. In addition to mucopeptide components, the

lighter of the two fractions contained 23% of carbohydrate but very little protein, and the denser fraction contained 13% of carbohydrate and considerable amounts of protein.

4. Hot formamide treatment of walls removed most of the carbohydrate and protein and a small amount of a teichoic acid-like material. The residues resembled typical walls under the electron microscope. The approximate molar ratios of the remaining amino acids of both fractions were D-glutamic acid 1, D-alanine 1, L-alanine 1, LL-diaminopimelic acid 1, glycine 1; there was 0·5 mole or less of muramic acid and each of the two hexosamines.

5. In all types of wall fractions glycine was almost the only end-amino group.

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