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The Chemical Composition of the Membranes of Protoplasts and L-Forms of *Staphylococcus aureus*

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Membrane fractions were prepared from *Staphylococcus aureus* H and 100 after dissolution of the cell walls by a lytic enzyme from *Streptomyces griseus*. Membranes were also prepared from the L-forms derived from the same strains. The membranes were analysed for protein, lipid, carbohydrate and RNA contents, and the fatty acid composition of the lipids was determined. A branched-chain saturated C_{15} acid was the major component in all samples, and the correspondence between L-forms and parent bacteria was fairly close. The lipids were separated into non-polar-lipid, glycolipid and phospholipid fractions; the L-forms contained a little more neutral lipid and much more glycolipid than the parent bacteria. In all membranes the glycolipid, which accounted for all the carbohydrate present, was a diglucosyl diglyceride. The major phospholipids of the protoplast membranes were phosphatidylglycerol and some lipoamino acids (lysine and a little alanine). On the other hand, diphosphatidylglycerol was the chief phospholipid found in L-form membranes.

Protoplast membranes have been isolated from several Gram-positive bacteria since their initial isolation from lysed protoplasts of *Bacillus megaterium* (Weibull, 1953). Studies have been made on both the chemical nature and the enzymic activity of these membranes. In addition to membranes from those organisms that are susceptible to lysozyme, such as *Bacillus licheniformis*, *Bacillus megaterium* KM, *Micrococcus lysodeikticus* and *Sarcina lutea* (Weibull, 1957; Gilby, Few & McQuillen, 1958; Brown, 1961; Salton & Freer, 1965; Yudkin, 1966), membrane preparations have been obtained from other organisms resistant to the action of lysozyme. These have included *Staphylococcus aureus* (Mitchell & Moyle, 1957), *Streptococcus faecalis* (Shockman, Kolb, Bakay, Conover & Toennies, 1963; Ibbott & Abrams, 1964) and group A streptococci (Freimer, 1963). In general, protoplast membranes are largely composed of protein and lipid, together with variable amounts of carbohydrate and RNA.

The membranes of bacterial L-forms, usually prepared from lysates of whole organisms, have also been the subject of several investigations. Much of this work has been directed towards determining the presence or absence of specific cell-wall components such as diaminopimelic acid and muramic acid in L-forms derived from both Gram-positive (Sharp, 1963; Panos, Barkulis & Hayashi, 1959; Pratt, 1966) and Gram-negative (Morrison & Weibull, 1962; Kandler, Hund & Zehender, 1958) organisms.

Comparisons have also been made of the chemical (James, Hill & Maxted, 1965; Panos, Cohen & Fagan, 1966; Cohen & Panos, 1966) and enzymic (Panos & Cohen, 1966) compositions of the membranes of protoplasts and L-forms derived from the same strains of *Streptococcus pyogenes* and *Proteus mirabilis* (Nesbitt & Lennarz, 1965; Weibull, Bickel, Haskins, Milner & Ribi, 1967).

The present paper concerns the membranes of protoplasts and L-forms derived from two strains of *Staphylococcus aureus*. Membranes have been prepared from lysates of both these cell-wall-less forms of the bacterium and subjected to chemical analysis.

METHODS

Organisms. Staph. aureus H and its derived L-form were grown at 37° in Bacto Brain–Heart Infusion (Difco Laboratories, Detroit, Mich., U.S.A.), supplemented with 0·5% (v/v) Bacto PPLO serum fraction (Difco Laboratories) and 3% (w/v) NaCl.

Staph. aureus 100 (Fodor & Miltényi, 1964) and its derived L-forms were grown at 37° in medium of the following composition (g./l.): Bacto proteose peptone (Difco Laboratories), 5; Bacto casein hydrolysate (Difco Laboratories),

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10; NaCl, 35; $Na_2HPO_4,12H_2O$, 16·5; KH_2PO_4, 0·5; $MgSO_4,7H_2O$, 0·01; $MnSO_4,7H_2O$, 0·01; sucrose, 50. The pH was adjusted to 7·5 before the medium was autoclaved.

Stock cultures of the L-forms were maintained on these media at room temperature after overnight incubation at 37°. Such cultures were transferred weekly. The staphylococci were maintained on nutrient agar at 4° after overnight incubation at 37° and subcultured monthly.

Isolation of L-forms. L-forms of *Staph. aureus* H were isolated and stabilized as described by Chatterjee, Ward & Perkins (1967) on a modification of the above medium containing Ionagar no. 2 (0·75%, w/v) (Oxoid Ltd., London, E.C. 4) and benzylpenicillin (100 units/ml.). The L-forms of *Staph. aureus* 100 were received from Dr M. Fodor as stabilized cultures.

Preparation of membranes. Staphylococci and their derived L-forms were harvested during the late exponential phase of growth. The bacteria, after being washed twice with ice-cold water, were resuspended at a concentration of 25mg. dry wt./ml. in tris–HCl buffer, pH 7·8, *I* 0·1, containing sucrose (0·6M). Staphylolytic enzyme 2 from *Streptomyces griseus* (Ward & Perkins, 1968) was added to a final concentration of 250μg./ml. and the suspension was incubated at 37°. The production of 'protoplasts' was monitored by following the changes in extinction of samples diluted in buffer not containing sucrose, and also by phase-contrast microscopy. Formation of 'protoplasts' was judged to be complete after the extinction of diluted samples had become stabilized, and when phase-contrast microscopy revealed only single cells, not clumps as normally found in staphylococcal cultures. Maximum osmotic fragility usually occurred after approximately 2hr. incubation. The 'protoplasts' were harvested by centrifuging at 8000*g* for 10min. and the supernatant, containing most of the solubilized cell wall, was removed, dialysed exhaustively against water and freeze-dried. The 'protoplasts' were resuspended in an equal volume of ice-cold 10mM-tris–HCl buffer, pH 7·8, containing $MgCl_2$ (10mM), and stirred vigorously for 15min. Deoxyribonuclease [Seravac Laboratories (Pty.) Ltd., Maidenhead, Berks.] was added to a final concentration of 50μg./ml. The lysate was allowed to come to room temperature (15–20min.), after which it was centrifuged at 0° for 20min. at 28000*g*, and the supernatant containing the soluble 'cytoplasm' was removed. The pellet of membranes was washed again with the tris–HCl–$MgCl_2$ buffer and the supernatant was decanted and added to the cytoplasm fraction. The membrane pellet was washed a further five times with the buffer, washed twice with water and then resuspended in water, dialysed to remove final traces of the buffer salts and freeze-dried.

The L-forms were washed with ice-cold 10mM-tris–HCl buffer, pH 7·8, containing $MgCl_2$ (10mM) and NaCl (4·5%, w/v), resuspended in a small volume of this buffer and incubated with deoxyribonuclease (50μg./ml.) at 37° for 15min. The organisms were harvested by centrifuging at 15000*g* for 20min., washed once with the buffered NaCl–$MgCl_2$ and ruptured by being resuspended and stirred vigorously in buffer without added NaCl. Subsequent procedures were identical with those described above for the isolation of the same fractions from whole cells.

Analytical techniques. To facilitate handling of samples for analytical estimations, membranes were dispersed in water by ultrasonic disintegration. Quantities (20–30mg.) of the freeze-dried preparations were cooled in an ice bath and subjected to ultrasonic oscillations with a Soniprobe (Dawe Instruments Ltd., London, W. 3) with a $\frac{1}{2}$in. probe. Treatment was continued for five periods of 1min., alternating with 1min. cooling periods, and the resulting suspension was made to a known volume.

The protein content of the membranes was determined by the quantitative biuret reaction as described by Mahler, Wittenberger & Brand (1958) with crystalline bovine serum albumin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex) as standard. Carbohydrate was determined as glucose by the phenol–H_2SO_4 method of Dubois, Gilles, Hamilton, Rebers & Smith (1956). RNA was measured by the orcinol reaction (Ceriotti, 1955) and DNA by the method of Burton (1956), with yeast RNA and highly polymerized calf thymus DNA (British Drug Houses Ltd., Poole, Dorset) as standards. Total phosphorus was determined by the method of Allen (1940). Amino nitrogen of the lipid samples was estimated by the method of Lea & Rhodes (1954) and total nitrogen by the method of Jacobs (1962). Hexosamines were determined on samples of membranes hydrolysed at 105° in 4N-HCl for 4hr., by the method of Cessi & Piliego (1960), or automatically in a Technicon autoanalyser (Technicon Instruments Co. Ltd., Chertsey, Surrey). For the identification of monosaccharides, samples of defatted and intact membranes were hydrolysed in 2N-HCl for 2hr. at 105°, freed from excess of HCl *in vacuo* and chromatographed on Whatman no. 1 paper with butan-1-ol–pyridine–water (6:4:3, by vol.), (solvent A) or ethyl acetate–pyridine–water (10:4:3, by vol.), (solvent B). Spots were detected with alkaline $AgNO_3$ (Trevelyan, Procter & Harrison, 1950). Amino acid analysis of defatted membranes was carried out on material hydrolysed in 6N-HCl for 18hr. at 105°, in a Technicon autoanalyser.

Extraction and analysis of lipid. Lipids from the various membrane and 'cytoplasm' fractions were extracted with methanol and chloroform. Preliminary experiments showed that no more lipid could be removed by further extraction with acidified solvents, including conc. HCl–methanol (5:95, v/v) under reflux for 60min. Samples of freeze-dried membranes (20–40mg.) and 'cytoplasm' (up to 100mg.) were extracted with 5ml. of aq. 95% (v/v) methanol at 40° for 10min. After the solution had cooled to room temperature, chloroform (10ml.) was added and the suspension was allowed to stand for a further 10min. with occasional stirring. Insoluble material was sedimented by centrifuging and the extraction was repeated twice. The combined extracts were evaporated to dryness, dissolved in chloroform–methanol (2:1, v/v) and washed by the method of Folch, Lees & Sloane-Stanley (1957). Water was removed by the addition of anhydrous Na_2SO_4 and the extract was then dried *in vacuo* to constant weight. The composition of the total fatty acids of the lipids was determined by gas–liquid chromatography with a Pye Argon Chromatograph (W. G. Pye Ltd., Cambridge). Apiezon L and polyethylene glycol adipate (10%, w/w) on Celite (80–120 mesh) at 196° and 180° were used as stationary phases. Methyl esters of the fatty acids were prepared by the inter-esterification technique of Coppock, Daniels, Gresham & Howard (1962) at a temperature of 60°, as described by Knivett & Cullen (1965). The methyl esters were identified by comparison with commercially available standards (Applied Science Laboratories Inc., State College, Pa., U.S.A.). Where reference compounds were not available, identification was based on comparison with published

retention values and chromatography of fatty acid esters derived from *M. lysodeikticus*. This organism is known to contain more than 80% of its fatty acids as the *anteiso* C_{15} acid (Macfarlane, 1961*b*; Cho & Salton, 1966). The percentage composition of the fatty acids was estimated by the method of Carroll (1961).

Lipid components were fractionated on columns (1·5cm. × 15cm.) of silicic acid (100 mesh; Mallinckrodt Chemical Works, St Louis, Mo., U.S.A.) as described by Cohen & Panos (1966). Fractions were examined qualitatively by thin-layer chromatography on Kieselgel G (E. Merck A.-G., Darmstadt, Germany) with the solvent systems chloroform–methanol–acetic acid (65:25:8, by vol.) (Nichols, 1963) and chloroform–methanol–water (65:25:4, by vol.) (Wagner, Horhammer & Wolff, 1961), and the two-dimensional system of Nichols (1964): chloroform–methanol–7N-NH_3 (65:25:4, by vol.) and chloroform–methanol–acetic acid–water (170:25:25:4, by vol.). Components were detected by spraying with chromic acid (Mangold & Kammereck, 1962), Zinzadze reagent (Dittmer & Lester, 1964), periodate followed by benzidine, or ninhydrin (0·1%, w/v, in acetone).

Water-soluble components of the lipid fractions were obtained after deacylation with methanolic KOH (Maruo & Benson, 1959). The phosphate esters were separated both before and after acid hydrolysis (N-HCl for 10min. at 100°) by two-dimensional paper chromatography with phenol–water (100:38, w/v) and butan-1-ol–propionic acid–water (142:71:100, by vol.) (Benson & Strickland, 1960). Spots were detected by the molybdate reagent of Hanes & Isherwood (1949) followed by irradiation with u.v. light (Dawson, 1960). Identification was based on co-chromatography with authentic compounds and comparison with reported R_F values (Benson & Strickland, 1960).

Water-soluble fractions obtained from the glycolipids by deacylation (Maruo & Benson, 1959) were separated by paper chromatography in solvent A. Products from acid hydrolysis (3N-HCl for 90min. at 100°) of the non-reducing glycoside and the glycosylglycerol moiety were chromatographed in solvent B.

Ester groups were estimated by the method of Stern & Shapiro (1953), with tripalmitin (Mann Research Laboratories Inc., New York, N.Y., U.S.A.) as a standard, and glycerol as described by Burton (1957). Correction was made for the interference of glucose in this assay, the amount present being measured by glucose oxidase (Glucostat; Worthington Biochemical Corp., Freehold, N.J., U.S.A.) or the phenol–H_2SO_4 method (Dubois *et al.* 1956). Glycerol recoveries of about 95% were obtained when glucose–glycerol mixtures of known composition were assayed.

Amino acids, present in acid hydrolysates of lipoamino acids were separated by paper chromatography with saturated aq. phenol (in an NH_3 atmosphere) and butan-1-ol–acetic acid–water (67:10:23, by vol.) as solvents, and detected with ninhydrin (0·1%, w/v, in acetone).

RESULTS

Chemical composition of membranes isolated from protoplasts and L-forms. The isolated membranes were analysed for protein, total lipid, carbohydrate, RNA and phosphorus. In addition, certain of the preparations were analysed for the presence of residual DNA, the amounts found being 0·03–0·06% in three preparations of L-form membranes and 0·03 and 0·04% in two preparations of protoplast membranes. The results of analysis of three separate preparations of membrane from the organisms being investigated are given in Table 1. Membranes prepared from either protoplasts or L-forms were largely lipoprotein in nature, the lipoprotein complex accounting for some 90% of the total membrane.

The protoplast membranes isolated from *Staph. aureus* 100 and H were essentially similar in chemical composition. The absence of detectable amounts of muramic acid and glucosamine indicated that the protoplast membrane preparations were not contaminated with cell-wall material. However, comparison of the chemical composition of the L-form membranes with that of the protoplasts did reveal some differences. In the L-forms

Table 1. *Chemical analysis of staphylococcal protoplast and L-form membranes*

The results given are expressed as percentages of the dry weight of the membrane.

Organism	Batch	Protein	Lipid	Carbohydrate	Phosphorus	RNA
Staph. aureus 100 protoplast	1	66·0	25·0	0·89	1·06	2·74
	2	62·6	23·0	0·81	1·19	3·80
	3	67·0	21·8	0·80	1·20	2·03
L-form L_1	1	62·8	28·3	1·38	1·03	2·78
	2	63·5	25·0	1·45	1·04	2·04
	3	64·0	29·9	1·51	1·13	1·98
L-form L_{20}	1	61·0	27·5	1·57	0·99	2·26
	2	59·0	32·5	2·02	1·04	2·20
	3	56·5	36·0	1·38	0·96	3·10
Staph. aureus H protoplast	1	62·4	24·6	0·81	1·14	4·47
	2	67·1	23·4	0·97	1·08	4·78
	3	70·6	21·8	0·86	1·18	4·60
L-form HL	1	56·8	29·8	2·86	0·95	4·26
	2	59·1	29·7	2·42	0·82	3·04
	3	61·0	31·5	2·37	0·76	3·13

Table 2. *Distribution of lipid in the fractions prepared from staphylococcal protoplasts and L-forms*

The results given are the means of duplicate determinations on two samples.

Cell fraction	Cell wall		Membrane		Protoplasm	
Organism	% of cellular dry wt.	% of total lipid	% of cellular dry wt.	% of total lipid	% of cellular dry wt.	% of total lipid
Staph. aureus 100 protoplast	21·3	0	10·1	92·1	68·6	7·9
L-form L_1	—	—	11·2	93·6	88·8	6·4
L-form L_{20}	—	—	8·9	90·2	91·1	9·8
Staph. aureus H protoplast	20·4	0	9·3	88·7	70·3	11·3
L-form HL	—	—	10·6	94·2	90·4	5·8

Table 3. *Chemical analysis of the lipid extracted from staphylococcal protoplast and L-form membranes*

Except where otherwise stated, results are given as percentages of the dry weight of the total extracted lipid.

Organism	Batch	Total lipid (% of cellular dry wt.)	Carbohydrate	Phosphorus	Amino N	Total N
Staph. aureus 100 protoplast	1	25·0	2·86	2·79	0·11	0·123
	2	23·0	3·65	3·44	0·71	0·78
	3	21·8	2·54	2·89	—	—
L-form L_1	1	28·3	4·73	2·62	0·038	0·05
	2	25·0	4·61	2·93	—	—
	3	29·9	5·12	2·79	0·120	0·145
L-form L_{20}	1	27·5	5·34	2·16	—	—
	2	32·5	4·73	2·65	—	—
	3	36·0	4·87	3·02	0·131	0·129
Staph. aureus H protoplast	1	24·6	2·35	2·34	0·221	0·363
	2	23·4	3·24	2·68	0·317	0·370
	3	21·1	2·74	2·48	—	—
L-form HL	1	29·8	7·80	1·47	0·140	0·197
	2	29·7	8·65	1·71	0·134	0·163
	3	31·5	8·29	1·65	0·123	0·204

the amount of lipid and carbohydrate present was greater and the protein content lower than those found in the protoplast membrane. The RNA content of the membranes also showed some variation, that of *Staph. aureus* H and its derived L-form being considerably higher than that found in the other membrane preparations examined. This may represent a difference between the two staphylococcal strains, since all the membranes were subjected to the same washing procedures in preparation. Glucose and ribose were the only monosaccharides detected in acid hydrolysates of membranes from both protoplasts and L-forms.

Distribution and composition of the lipids. The organisms were separated into cytoplasm, membrane and cell-wall fractions. Table 2 gives the contribution of each of these fractions to the total cell weight and the distribution of the cell lipid between the fractions. The contribution of cytoplasm to the total dry weight was obtained by difference, since removal of contaminating buffer by dialysis resulted in a loss of low-molecular-weight components. The Table shows that about 90% of the cellular lipid was located in the membrane fraction. The remaining 10% was found in the cytoplasm, of both cocci and L-forms. No lipid was found in the cell walls of the cocci.

The lipid extracted from the various membrane preparations was analysed for carbohydrate, phosphorus, total nitrogen and amino nitrogen (Table 3). Total-phosphorus estimations on the lipid extracted from *Staph. aureus* 100 and its derived L-forms gave average values of 3·04% for the protoplast-membrane lipid and 2·61 and 2·78% for the L-form lipid, corresponding to 61% and 71–83% respectively of the phosphorus present in the intact membranes. Values obtained with *Staph. aureus* H and its L-form gave 51 and 57% of the

Table 4. *Fatty acid composition of the extracted membrane lipids*

Methyl esters of the fatty acids were prepared and analysed as described in the Methods section. The fatty acids are designated thus: the number before the colon is the number of carbon atoms, that after the colon is the number of double bonds, and br. represents a branched-chain fatty acid. The values given are the percentages of the total fatty acid methyl esters in each sample, and are the means of duplicate determinations.

Fatty acid	*Staph. aureus* 100	L-form L_1	L-form L_{20}	*Staph. aureus* H	L-form HL
15:0 (br.)	54·8	48·2	59·5	38·9	40·8
16:0	0·1	4·0	0·6	2·9	1·2
17:0 (br.)	12·1	10·9	17·2	22·5	19·2
18:0	10·4	15·5	4·4	14·2	18·0
19:0 (br.)	9·0	6·3	8·9	6·5	3·0
20:0	2·9	3·9	2·2	8·8	7·0

membrane phosphorus present in the lipid. The extracted lipids also contained 78–96% of the total carbohydrate found in the membranes. Total nitrogen was usually less than 0·5%, and was largely accounted for by amino nitrogen.

Fatty acid composition of the total membrane lipids. Examination of the fatty acids present in the extracted lipids gave the results in Table 4. In the Table the branched-chain fatty acids have been included in a single column. However, on the basis of relative retention values, these fatty acids were present mainly as the *anteiso* form, the major fatty acid in all lipids being *anteiso* C_{15}. Differences were evident in the amounts of the various fatty acids, L-form L_{20} containing relatively few saturated straight-chain acids but rather more branched-chain acids than L-form L_1 and *Staph. aureus* 100. *Staph. aureus* H and its derived L-form contained relatively less C_{15} branched-chain acids but more C_{17} branched-chain and saturated straight-chain acids. These differences may have been due to the different growth media used for the two groups of organisms, or the physiological age of the respective cultures, a factor known to affect the fatty acid composition of bacterial lipids (Marr & Ingraham, 1962; Knivett & Cullen, 1965, 1967). However, cells from both forms were obtained in the late exponential phase of growth.

Fractionation of the membrane lipids. Lipids extracted from the various membrane preparations were separated on columns of silicic acid by discontinuous elution with mixtures of acetone in chloroform and methanol in chloroform. The non-polar lipids eluted with chloroform alone accounted for 15–17·6% of the total lipid of the protoplast membrane and 21–26% of the L-form membrane total lipid. Comparative elution diagrams of the total lipid extracts of *Staph. aureus* 100 and of one of its derived L-forms (L_{20}) are given in Fig. 1. The main glycolipid from all sources investigated was eluted with acetone, and the phospholipids were eluted with increasing concentrations of methanol in chloroform. Considerable variation was obtained in the elution pattern of the phospholipids, this being due to differences in the quantity of individual phospholipids rather than the presence of other compounds, as judged by thin-layer chromatography of the peak fractions. The R_F values shown were obtained by thin-layer chromatography of the peak fractions with chloroform–methanol–water (65:25:4, by vol.) as the solvent. Virtually all the glucose and phosphorus found in the total extracted lipid was recovered in the individual fractions.

On the basis of the column-chromatographic separation of non-polar lipid, glycolipid and phospholipid, the composition of the membrane lipids was determined in terms of these components (Table 5). The non-polar-lipid content of the lipid extracted from the membranes of all the L-forms examined was significantly greater (40–60%) than the amount present in protoplast-membrane lipid. In both the L-forms derived from *Staph. aureus* 100 the amount of membrane glycolipid was about double that of protoplast-membrane glycolipid, and the phospholipid content was 14% (L_1) and 23% (L_{20}) lower. *Staph. aureus* H and its derived L-form showed even more striking differences. The glycolipid content of the membrane lipid from the L-forms was three times that of the protoplast-membrane lipid, and the phospholipid content was 40% lower.

Identification of the glycolipid. The glycolipids obtained by column chromatography from both protoplast- and L-form-membrane lipid behaved identically on thin-layer chromatography. They were ninhydrin- and Zinzadze-negative, indicating the absence of phosphate and free amino groups, but gave a positive result with periodate–benzidine. Acid hydrolysis yielded glycerol, glucose and fatty acids as the major components. These substances were identified by chromatographic procedures, in the case of glycerol and glucose, and by gas–liquid chromatography of methyl esters prepared from the fatty acids. In addition, glucose was characterized enzymically with glucose oxidase. The

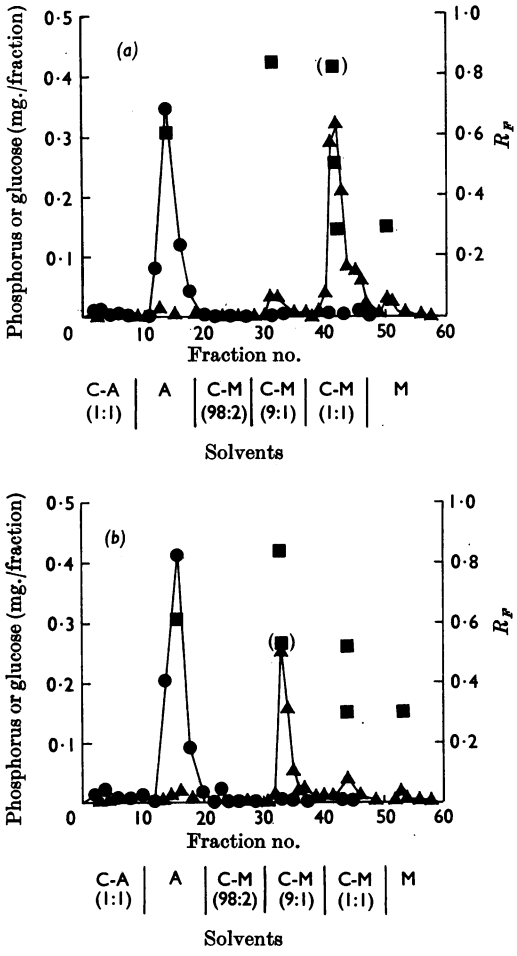

Fig. 1. Silicic acid column chromatography of the total lipids extracted from (*a*) *Staph. aureus* 100 protoplast membranes and (*b*) the membranes of its derived L-form, L_{20}. The ordinate gives the phosphorus (▲) or glucose (●) content of the fractions (5ml.) and the R_F (■) values of the components of peak fractions determined by thin-layer chromatography (solvent: chloroform–methanol–water, 65:25:4, by vol.). R_F values of trace components are indicated by symbols in parentheses. The amounts of lipid fractionated were 50·0mg. and 32·5mg. from protoplast and L-form membranes respectively. Solvents: C, chloroform; A, acetone; M, methanol. The compositions of all the solvent mixtures are v/v.

water-soluble products formed on deacylation of the glycolipids with dilute methanolic potassium hydroxide were separated by chromatography. The product obtained was non-reducing and had $R_{glucose}$ 0·44 in solvent A. This value was close to that reported by Brundish, Shaw & Baddiley (1965) for diglucosylglycerol obtained on deacylation of glycolipid extracted from whole cells of *Staph. aureus* H.

Glucose and glycerol were quantitatively estimated on the acid hydrolysates of the glycolipid and the non-reducing glycoside obtained by deacylation. A glucose/glycerol molar ratio of close to 2:1 was obtained with all the compounds examined, indicating a diglucosylglycerol structure. The ester/glucose molar ratio found was close to 1:1. Therefore the glycolipid present in both protoplast and L-form membranes could be characterized as diglucosyl diglyceride.

Identification of the phospholipids. On the basis of thin-layer chromatography in a number of solvent systems, including the two-dimensional system of Nichols (1964), the fraction eluted with chloroform–methanol (1:1, v/v) on silicic acid column chromatography of membrane lipid of L-form L_{20} was considered to be representative of this fraction, isolated in smaller or greater amount from all the membranes examined. Thin-layer chromatography with chloroform–methanol–water as the solvent revealed a major component (R_F 0·85) with only traces of a second component (R_F 0·5). Each spot was Zinzadze-positive, indicating the presence of phosphorus, and ninhydrin-negative. The major spot was periodate–benzidine-negative, but the minor spot was positive.

Examination of the water-soluble products, obtained on deacylation with methanolic potassium hydroxide, by the chromatographic system of Benson & Strickland (1960) revealed a major spot in the diglycerophosphorylglycerol (G-P-G-P-G) position as well as a minor spot in the glycerophosphorylglycerol (G-P-G) position. Further identification came from the determination of the phosphorus/glycerol molar ratio, which was found to be 2·0:2·86.

The major phospholipid component of the lipid extracted from the protoplast membranes was eluted from silicic acid columns with chloroform–

Table 5. *Composition of the extracted membrane lipids*

The extracted membrane lipids were fractionated by silicic acid column chromatography, as described in the Methods section. The results given are expressed as percentages of the dry weight of the extracted lipid.

Fraction	*Staph. aureus* 100	L-form L_1	L-form L_{20}	*Staph. aureus* H	L-form HL
Non-polar lipid	15·0	21·0	23·9	17·6	25·5
Glycolipid	8·1	15·4	16·6	10·3	32·5
Phospholipid	69·2	59·7	53·1	68·0	40·6

Table 6. *Amino acid composition of defatted membrane protein*

The results are given as moles/10^4g., and are the mean of the determinations on three separate batches.

Amino acid	Amino acid composition of defatted membrane protein				
	Staph. aureus 100	L-form L_1	L-form L_{20}	*Staph. aureus* H	L-form HL
CyS	0·7	0·5	0·7	3·3	3·7
Asp	8·5	8·7	8·2	8·2	8·8
Met	1·7	1·6	1·6	1·7	1·8
Thr	5·0	4·5	4·3	4·8	4·3
Ser	5·0	4·4	4·3	4·5	4·3
Glu	7·8	8·1	7·9	8·0	8·5
Pro	2·7	2·8	2·5	2·9	2·8
Gly	6·9	5·9	5·8	6·4	6·5
Ala	11·2	6·0	6·1	9·5	6·4
Val	5·2	5·0	4·8	5·4	4·3
Ile	4·4	4·3	4·3	4·2	4·2
Leu	6·4	6·0	5·8	6·2	6·0
Tyr	2·6	2·6	2·3	2·5	2·4
Phe	3·6	3·0	3·0	3·6	3·1
Lys	5·0	5·3	5·8	5·6	5·4
His	1·6	1·7	1·5	1·6	1·5
Arg	3·0	2·7	2·6	2·7	2·5

methanol (1:1, v/v) (Fig. 1) and revealed two major spots on thin-layer chromatography with chloroform–methanol–water as solvent. Each spot was Zinzadze-positive, indicating the presence of phosphorus. The faster spot (R_F 0·55) was periodate–benzidine-positive and ninhydrin-negative, but the opposite was true of the slower one (R_F 0·3). In addition, traces of a third spot having a similar R_F and staining characteristics to that of the G-P-G-P-G lipid described above were observed. The two major components were purified by rechromatography on silicic acid paper (Whatman SG81; H. Reeve Angel and Co., London, E.C. 4) with chloroform–methanol–water (65:25:4, by vol.) as solvent.

Deacylation of the faster, ninhydrin-negative, component and chromatography of the water-soluble products revealed only G-P-G. Acid hydrolysis yielded glycerophosphate and glycerol. Similar products, together with amino acids, were obtained on deacylation and acid hydrolysis of the slow component. The amino acids were identified by paper chromatography as lysine and a smaller amount of alanine. On the basis of R_F values obtained on thin-layer chromatography and their staining characteristics with specific reagents, it is probable that the smaller amounts of phospholipid eluted with chloroform–methanol (1:1, v/v) during silicic acid chromatography of L-form membrane lipids were of similar composition.

The amino acids present in the phosphoaminoacylglycerol components, eluted with chloroform–methanol (1:1, v/v) and methanol alone, from both protoplast and L-form lipids were investigated after acid hydrolysis. In all the membrane lipids, lysine was the predominant amino acid present. However, in acid hydrolysates of the membrane lipids of protoplasts, but not L-forms, alanine was also present.

Composition of the defatted membrane fractions. Preliminary chromatograms of acid hydrolysates of defatted membranes revealed the presence of most of the commonly occurring amino acids. Quantitative analysis of the individual amino acids showed that the amounts present in the defatted membranes were fairly constant from batch to batch. The amino acid composition of the membrane proteins is given in Table 6. These results show that the protoplasts and L-forms differ only slightly in the relative amounts of the amino acids. However, both protoplast-membrane preparations contained considerably more alanine (33–49% more) than the L-form membranes.

Acid hydrolysates of the defatted membrane preparations were examined for the presence of monosaccharides by paper chromatography. The only monosaccharide observed on the chromatograms was ribose. This result points to the glucose found in similarly treated intact membranes being present only as glycolipid. There was no evidence for the presence of a carbohydrate polymer.

DISCUSSION

The overall chemical composition of the staphylococcal protoplast and L-form membranes revealed that they were largely lipoprotein. The protein content ranged from 56% to 71%, the

protoplast membranes (62–71%) containing on average slightly more than those of the L-forms (56–64%). Some of this difference may have been due to contamination with lytic enzyme. Weibull, Zacharias & Beckman (1959) found that lysozyme, although bound primarily by the cell-wall fraction, was also present in the membrane ('ghost') and protoplasm fractions of *B. megaterium*. Similar results have been reported by Shockman *et al.* (1963) and Panos *et al.* (1966). However, the amount of lytic enzyme used here was only 1% of the weight of the cocci, so that gross contamination of membranes with enzyme seems unlikely.

The quantity of RNA found in the membranes was somewhat variable, though the range was very similar to that observed by Salton & Freer (1965) and Yudkin (1962). Since the preparation of the membranes involved the use of pancreatic deoxyribonuclease, which is known to contain small amounts of ribonuclease, it may be that some of the membrane RNA was destroyed. There seems little doubt that some RNA properly belongs to the membrane of *B. megaterium* (Yudkin & Davis, 1965), in which Yudkin (1962) found an RNA content varying from 3 to 14%. Some adherent ribosomal RNA may have been present in the staphylococcal membranes, since they were not washed in magnesium-free medium (Schlessinger, 1963). However, Yudkin & Davis (1965) found that such washing removed very little RNA from the membranes of *B. megaterium*.

The membrane composition of staphylococcal protoplasts was examined by Mitchell & Moyle (1951), who found 41% of protein and 23% of lipid in the 'small particle' (membrane) fraction from *Staph. aureus* Duncan. In the present work the protoplast membranes were found to contain 22–25% of lipid, whereas the L-form membranes contained 25–36%. Similar differences have been reported for membranes of L-forms induced from *Strep. pyogenes* when compared with the protoplast membrane of the parent bacteria (James *et al.* 1965; Cohen & Panos, 1966). A further difference in the analysis of the two types of membrane is the increased carbohydrate content of the L-form. This was particularly obvious when the membrane of the L-form induced from *Staph. aureus* H was compared with bacterial membrane. Subsequent investigation showed that the material measured as carbohydrate was all glycolipid. These findings contrast with those of Gilby *et al.* (1958), who found an average of 19% carbohydrate, as mannose polymer, in the defatted protoplast membrane of *M. lysodeikticus*, and Weibull & Bergstrom (1958), who found 1–10% hexose, as a glucose polymer, in the membrane fraction from *B. megaterium*. Mitchell & Moyle (1951) reported the carbohydrate content of *Staph. aureus* Duncan membranes as 1·7%, but did not state whether it was glycolipid. The carbohydrate content of *Strep. pyogenes* L-form was four times as great as that of the protoplast membrane, all being present as glycolipid (Cohen & Panos, 1966).

Lipid and lipid phosphorus have been suggested as specific constituents of the bacterial membrane (Kolb, Weidner & Toennies, 1963). This has been established for *M. lysodeikticus* (Macfarlane, 1961*a*) and *Strep. faecalis* (Vorbeck & Marinetti, 1965) by direct analysis. The lipid content of subcellular fractions prepared from the staphylococci and their induced L-forms has been examined. In all preparations 89–94% of the lipid was found in the membrane fraction. The remaining lipid was found in the cytoplasmic fractions, none being detected in the bacterial cell wall. The small quantities of cytoplasmic lipid might have been due to contamination of this fraction with membrane fragments. However, the overall distribution of lipid within the cell shows that *Staph. aureus* resembles, in this respect, the other species of Gram-positive cocci examined.

Analysis of the total lipid extracted from the protoplast and L-form membranes confirmed the differences already noted on examination of the whole membranes. The carbohydrate content of the L-form lipids showed substantial increases over that found in the protoplast-membrane lipid. This was particularly marked in *Staph. aureus* H and its L-form, where the difference was threefold. The carbohydrate recovered from the lipid accounted for, on average, 91% of that previously found in the membrane. The recovery of phosphorus was much lower; the two protoplast-membrane lipids accounted for 51% (*Staph. aureus* H) and 61% (*Staph. aureus* 100) of the total phosphorus as lipid phosphorus. The recovery from the L-form-membrane lipids was slightly higher, being 57% (*Staph. aureus* H, L-form), 72% (*Staph. aureus* 100, L-form L_1) and 83% (*Staph. aureus* 100, L-form L_{20}) respectively. These improved recoveries in the L-form membranes may be due to the lower content of RNA in the membrane preparations. The total fatty acid analysis of the extracted lipids showed that most of the fatty acids present were of the saturated branched-chain type, C_{15} and C_{17} predominating. These results were in good agreement with the findings of Macfarlane (1962*a*) and Houtsmuller & van Deenen (1965).

The major glycolipid from both protoplast and L-form membranes has been characterized as diglucosyl glyceride by examination of hydrolytic products. Macfarlane (1962*a*) reported the occurrence of glucosyl glyceride in *Staph. aureus* Duncan, and diglucosyl diglyceride has been found in various strains of staphylococci including *Staph. aureus* H (Brundish *et al.* 1965; Polonovski, Wald & Petek, 1965).

The phospholipid content of the membranes has been identified as a mixture of diphosphatidylglycerol (G-P-G-P-G), phosphatidylglycerol (G-P-G) and lipoamino acids. Macfarlane (1962*a*) and Houtsmuller & van Deenen (1965) have reported the isolation of similar components from the phospholipids of *Staph. aureus*. However, in both of these investigations the lipoamino acids represented the major component. This is almost certainly due to the growth phase of the organism and the conditions of lipid extraction used. In the present experiments the major phospholipid component of the protoplast membranes was phosphatidylglycerol (G-P-G) together with the lipoamino acids. On the other hand, the L-forms contained diphosphatidylglycerol (G-P-G-P-G) as the major component, although there was some variation in amount.

The ninhydrin-positive material present in both L-form and protoplast lipids has been tentatively identified as amino acid derivatives of phosphatidylglycerol. The major amino acid present in all lipid extracts was lysine, although a smaller amount of alanine was present in the protoplast membrane. Macfarlane (1962*b*) has reported the occurrence of *O*-amino acid esters of phosphatidylglycerol in *Clostridium welchii* and *Staph. aureus*. Houtsmuller & van Deenen (1965) characterized lysylphosphatidylglycerol from *Staph. aureus* and suggested the presence of small quantities of an *O*-alanyl ester in addition. As pointed out by Macfarlane (1964), lipoamino acids are not found even in all Gram-positive bacteria, and their significance remains uncertain.

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