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Nature and Origins of Phosphorus Compounds in Isolated Cell Walls of *Staphylococcus aureus*

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Preparations of purified cell walls from *Staphylococcus aureus* were shown to contain small amounts of phospholipid and glycerol teichoic acid. Since these are components of the cell membrane, it is probable that the wall itself contains no lipid, but does retain fragments of membrane because of physical connections between wall and membrane. In walls of *S. aureus* strain 52A5, which completely lacks ribitol teichoic acid, the only phosphorylated compound identified as a genuine wall component was a phosphorylated derivative of murein that gave rise to muramic acid phosphate on acid hydrolysis. Muramic acid phosphate was also identified in hydrolysates of walls from *S. aureus* H and strain 52A2.

An early distinction made between the wall composition of gram-positive and gram-negative bacteria was the marked difference in lipid content (21). The lipid content reported for the walls of gram-positive organisms has ranged from 1 to 3% in *Micrococcus lysodeikticus*, *Bacillus subtilis*, *Sarcina lutea* (20), *Staphylococcus aureus* (6) and, more recently, *B. stearothermophilus* (7). The question arises whether this small quantity of lipid is a true wall component.

During the investigation of a mutant of *S. aureus* H (strain 52A5) which completely lacked ribitol teichoic acid (21), it was found that isolated walls of both the parent and the mutant contain a small but significant amount of glycerol. This necessitated an investigation to determine whether the glycerol was a normal component of the wall or was derived from another source. The following report documents our findings that the lipids associated with purified walls of *S. aureus* are those characteristic of the membrane.

MATERIALS AND METHODS

Bacterial strains. The isolation and maintenance of the parent strain, *S. aureus* H (Str^r), the mutant 52A2 (lacking *N*-acetylglucosamine substituents on the ribitol teichoic acid), and of the mutant 52A5 (completely without ribitol in the cell wall) were described previously (3, 22).

Materials. *Escherichia coli* and calf intestinal alkaline phosphatases and potato acid phosphatase were from the Sigma Chemical Co. (St. Louis, Mo.). *Chalaropsis* muramidase was the crystalline enzyme, kindly given by J. G. Hash (9). Lysostaphin was a gift from P. Tavormina (Mead Johnson & Co.). Phosphatidyl glycerol and cardiolipin were gifts from J. P. Ballesta and were isolated from *B. megaterium* by the method of Dankert et al. (5).

Preparation of cell walls. Cell walls were prepared as described previously (22). The procedure involved mechanical disruption of the cells with glass beads, treatment of the broken cell preparation with deoxyribonuclease and ribonuclease, isolation of cell walls in a fraction sedmenting between 1,100 and 7,500 × *g*, and repeated washings with 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, *p*H 7.2. At this stage of purification, the walls are referred to as crude walls. For use in enzyme studies, crude cell walls were suspended in 0.05 M Tris-hydrochloride buffer, *p*H 8.0, containing 0.001 M mercaptoethanol to give about 30 mg (dry weight) per ml.

Further purification of the walls was carried out by suspension in water, heating for 15 min at 100 C, digestion with trypsin, repeated washings with water, homogenizing with chloroform-methanol, and washing with water (22). Walls given this treatment are referred to as purified walls. Analyses of the walls have been published elsewhere (22). Less than 1% of the ninhydrin-positive material obtained after hydrolysis were amino acids other than those of the mucopeptide.

Preparation of ^{32}P-labeled cell walls. Cells were grown for about six generations in a medium containing 0.5% yeast extract (Difco), 0.5% Phytone (BBL), 0.1 M Tris-hydrochloride, 0.2% glucose, and 0.01% $K_2H^{32}PO_4$ (2 μCi per μmole), *p*H 7.6. Cell walls were prepared as described above.

Membrane fraction. The membrane fraction was the particulate enzyme fraction sedimented between 12,000 and 100,000 × *g* (22).

Column chromatography. Cell walls were hydrolyzed in 1 N NaOH for 3 hr at 100 C. The hydrolysate was

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deionized with Dowex 50-H^+, and the filtrate was neutralized to *p*H 7.0 with dilute NaOH. Hydrolysates of ^{32}P-labeled walls were chromatographed on diethylaminoethyl Sephadex A25 (DEAE-Sephadex) after the method of Critchley et al. (4) with DEAE cellulose. The column of DEAE-Sephadex (0.5 cm^2 by 25 cm) was eluted with 200 ml of a gradient (0.05 to 0.5 M) of ammonium bicarbonate (*p*H 8.7), the flow rate was 0.4 ml per min, and 2-ml fractions were collected. Partial alkali hydrolysates [obtained by the action of 0.1 N $Ba(OH)_2$ on cell walls for 20 min at 37 C] were chromatographed in the same manner with an additional elution by 1 M ammonium bicarbonate to elute residual poly(glycerophosphate).

Autolysis of crude cell walls. Crude cell walls (0.5 g) were suspended in 100 ml of 0.1 M ammonium acetate, *p*H 6.8, and incubated for 18 hr at 37 C. The slightly opalescent solution was centrifuged at 30,000 × *g* for 15 min, and the clear supernatant fluid was fractionated on a column (2.2 by 105 cm) of Sephadex G-25 in water with water as eluant.

Enzymatic digestion of purified cell walls. Purified cell walls (1 g) were suspended in 100 ml of 0.1 M sodium citrate buffer, *p*H 4.7, together with 1 mg of *Chalaropsis* muramidase. The mixture was incubated for 18 hr at 37 C and adjusted to *p*H 7.6; then 10 ml of 1.0 M Tris-hydrochloride buffer, *p*H 7.6, was added together with 1 mg of lysostaphin, and the mixture was incubated again for 19 hr at 37 C.

Assays of wall and membrane enzymatic activities. Cytidine diphosphate ribitol: acceptor phosphoribitoltransferase and uridine diphosphate *N*-acetylglucosamine: poly(ribitol phosphate) *N*-acetylglucosaminetransferase were assayed as described previously (22). The incorporation of radioactivity from uridine diphosphate *N*-acetylmuramyl-(L-[^{14}C]lysine) pentapeptide into disaccharide lipid intermediate and mucopeptide was measured under conditions similar to those described by Park and Chatterjee (15). The reaction mixture contained 0.5 mM adenosine triphosphate, 10 mM $MgCl_2$, 100 mM NH_4Cl, 40 mM Tris-hydrochloride, *p*H 8.0, 0.8 mM mercaptoethanol, 0.5 mM uridine diphosphate *N*-acetylglucosamine, 0.1 mM uridine diphosphate *N*-acetylmuramyl-(L-[^{14}C]lysine) pentapeptide (30 counts per pmole), and enzyme, in a volume of 0.1 ml. The mixture was incubated for 30 min at 25 C, and the reaction was stopped by adding 2 ml of ice-cold 0.3 N perchloric acid. The mixture was centrifuged for 10 min at 18,000 × *g;* the pellet was washed twice with 2.5 ml of 0.3 N perchloric acid and once with water, and finally was suspended with 0.2 ml of 1 M NH_4OH. Controls were done by stopping the reaction at zero time. The radioactivity of the suspended pellet was determined by counting in ethoxyethanol-containing scintillation fluid (22). To determine the proportions of lipid intermediate and mucopeptide, the suspended pellet was chromatographed on paper by use of solvent A (1). The chromatogram was cut up, and 2-cm lengths were counted (22).

Analytical methods. Descending paper chromatography was on Whatman no. 1 paper with the following solvents: A, *iso*butyric acid-1 M NH_4OH (5:3, v/v); B, butan-1-ol-glacial acetic acid-water (3:1:1, v/v/v); C, propan-1-ol-concentrated ammonia-water (6:3:1, v/v/v). Paper electrophoresis was carried out by use of Whatman no. 3 MM paper with a constant voltage of 50 v/cm for 80 min with the following buffer systems: 1 M acetic acid-pyridine, *p*H 3.5; 1.2 M pyridine-acetic acid, *p*H 6.5. Thin-layer chromatography employed Chromar Sheet 500 (Mallinckrodt Chemical Works) and solvent D, chloroform-methanol-water (90:28:3, v/v/v). Glycols were detected by a periodate-benzidine dip (8); phosphate compounds, by a $FeCl_3$-sulfosalicylic acid dip (19); and amino compounds, by a ninhydrin dip (0.5% ninhydrin in acetone, heat at 80 C for 15 min). Radioactive compounds were located with a Nuclear-Chicago Actigraph III chromatogram scanner or by cutting the paper into sections and counting these in scintillation fluid.

Gas-liquid chromatography of fatty acids was performed by use of a Varian Aerograph 1700 series, with a 5-ft (1.5-meter) column containing 3% S.E. 30 on 100/120 Variport #30. Fatty acid methyl esters were prepared by methanolysis of lyophilized material in the presence of boron trichloride (Applied Science Laboratories Inc., State College, Pa.).

Protein was estimated by the method of Lowry et al. (11). The procedure for measuring the protein content of crude cell wall preparations was as follows: 0.4 ml of wall suspension was mixed with 2.0 ml of the alkaline copper tartrate reagent, after 15 min at 23 C the mixture was centrifuged for 5 min at 3,000 × *g*, 1.2 ml of the supernatant fluid was then mixed with 0.1 ml of Folin-Ciocalteau reagent, the mixture was left for 30 min at 23 C, and the extinction was determined in the standard manner.

Other methods, described previously, were those for acid hydrolysis of cell walls and for the determination of glycerol, ribitol, phosphate, radioactivity (22), and D-alanine (13).

RESULTS

The search for traces of ribitol teichoic acid in purified walls of *S. aureus* strain 52A5 led to the discovery of small amounts of glycerol and glycerophosphate. These were first detected by paper chromatography (solvents B and C) and then confirmed by enzymatic assays. Strain 52A5, as well as strain 52A2 and the parent strain (*S. aureus* H), all contained 0.05 to 0.1 μmole of glycerol per mg of wall. The occurrence of glycerol and glycerophosphate suggested that the walls might contain glycerol teichoic acid or phospholipid. Both glycerol teichoic acid and phospholipid were found in the purified walls and were characterized as follows.

Glycerol teichoic acid. Glycerol teichoic acid may be identified by alkali hydrolysis to diglycerol triphosphate, which is the characteristic product formed from 1,3-linked poly(glycerophosphate) (10). ^{32}P-labeled, purified cell walls of strain 52A5 were hydrolyzed in 1 N NaOH for 3 hr at 100 C. Column chromatography of the hydrolysate on DEAE-Sephadex separated the [^{32}P]-phosphate compounds into four principal fractions. The first fraction, containing 66% of the ^{32}P, was identified by paper chromatography

(solvent C) and paper electrophoresis (*p*H 6.5) as a mixture of α- and β-glycerophosphates with a trace of inorganic phosphate. Treatment with alkaline phosphatase gave rise to inorganic phosphate and glycerol, which were identified by paper chromatography and enzymatic assay. The second fraction, with 9% of the phosphate, was identified in a similar manner as glycerol diphosphates. The third fraction, about 3% of the phosphate, was identified as diglycerol triphosphate. Paper chromatography with solvent C gave $R_{\alpha\text{-glycerophosphate}}$ 0.18, digestion with alkaline phosphatase gave rise to inorganic phosphate ($R_{\alpha\text{-glycerophosphate}}$ 0.24) and glycerophosphorylglycerol ($R_{\alpha\text{-glycerophosphate}}$ 2.05) which, on heating in 1 N HCl for 10 min at 100 C, gave glycerol ($R_{\alpha\text{-glycerophosphate}}$ 3.20) and glycerophosphate. The fourth fraction (about 16% of the phosphate) contained no glycerol, was immobile in solvent C, and appeared to be a phosphorylated derivative of murein since acid hydrolysis gave rise to muramic acid phosphate (*see below*).

Baddiley and co-workers (4, 23) reported that the intracellular glycerol teichoic acids of *Lactobacillus arabinosus* and *L. buchneri*, with chain lengths of 18 and 14, respectively, yielded about 7% of the teichoic acid phosphate as diglycerol triphosphate under the same conditions of alkali hydrolysis. If it is assumed that there would be the same yield from glycerol teichoic acid present in the purified wall preparations from *S. aureus* 52A5, it may be deduced that about half the glycerophosphate in the wall was glycerol teichoic acid.

Glycerol teichoic acid was identified in the walls of strains H and 52A2 after removal of the ribitol teichoic acid by oxidation with periodic acid. Periodate oxidation destroyed less than 10% of the glycerol in these walls, or those of strain 52A5. Suspension of the oxidized walls from strain 52A5 in 0.1 N $Ba(OH)_2$ for 20 min at 37 C solubilized about 55% of the glycerol and 40% of the phosphate. DEAE-Sephadex column and paper chromatography and electrophoresis (*p*H 6.5) of the solubilized compounds from all three strains showed comparable amounts of glycerophosphorylglycerol, glycerol mono- and diphosphates, and oligoglycerophosphates.

Phospholipid. A large amount of glycerophosphorylglycerol (15% of the phosphate extracted) was found in the $Ba(OH)_2$ hydrolysates of 52A5 cell walls, described above, suggesting that purified walls contained phosphatidylglycerol in addition to glycerol teichoic acid. When the purified walls were again shaken at 23 C with chloroform-methanol (2:1, v/v), the phospholipid was not readily extracted (e.g., 15% of the glycerol in the wall was extracted after 15 min). Refluxing for 4 hr extracted 60 to 70% of the glycerol and 40% of the phosphate. Thin-layer chromatography of the extract with solvent D showed two main spots with the same R_F values as authentic phosphatidylglycerol and cardiolipin, which are the principal lipids of *S. aureus* (24). Fatty acids from the wall were identified by gas chromatography and found to be typical acids of *S. aureus* lipids (Table 1; 12).

Homogenization of purified walls with 0.5% Triton X-100 or 2% sodium dodecyl sulfate removed about 90% of the fatty acid (determined by gas chromatography) and about 70% of the glycerol. Strong buffers and salt solutions (e.g., 6 M LiCl) failed to remove significant amounts of glycerol or fatty acid. When cell walls of *S. aureus* H and strain 52A5 were digested exhaustively by lysostaphin and then by *Chaloropsis* muramidase (see Materials and Methods), 95% of the wall was solubilized, as judged by the decrease in optical density. The small amount of insoluble material that remained (collected by centrifuging for 10 min at 30,000 $\times$ *g*) contained 90% of the fatty acid present in the starting material and 60% of the glycerol.

Muramic acid phosphate. Two of the phosphorus-containing compounds of strain 52A5 cell wall preparations were identified as phospholipid and glycerol teichoic acid. A third compound was identified as a phosphate derivative of murein. This compound was isolated by column chromatography of alkali-hydrolysates (*see above*). A phosphate derivative of murein was also identified among the products of autolysis of crude cell walls. ^{32}P-labeled crude walls of strain 52A5 were permitted to autolyze as described in Materials and Methods. Insoluble material (7% by weight), removed by centrifugation, had the gel-like appearance of sedimented membranes. The clear supernatant fluid, which contained

TABLE 1. *Fatty acid composition of purified cell walls and of material solubilized by heating crude cell walls*[a]

Fatty acid	*S. aureus* 52A5		*S. aureus* H	
	Purified walls	Material solubilized by heating	Purified walls	Material solubilized by heating
C_{15} branched ..	25.5	188	30.5	172
C_{17} branched ..	12.8	120	2.5	148
C_{18}	7.2	54	9.0	46
C_{18} unsaturated	14.6	58	18.7	110
C_{20}	8.9	72	23.5	62
Total	69.0	492	84.2	538

[a] Nanomoles per milligram (dry weight).

85% of the ^{32}P, was fractionated on a column of Sephadex G-25. The radioactivity eluted with the void volume amounted to 24% of the ^{32}P in the original wall. No fatty acids and only small amounts of glycerol (8% of the total) were detected in this peak. Treatment with alkaline phosphatases from *E. coli* or calf intestinal mucosa or with acid phosphatase from potato did not yield inorganic phosphate. Acid hydrolysis (6 N HCl for 90 min at 105 C) gave one principal phosphate-containing compound which traveled on paper electrophoresis (*p*H 6.5) with 70% of the mobility of inorganic phosphate and gave a positive ninhydrin reaction. Analysis of this material after elution from the paper gave muramic acid and phosphate in the ratio of 0.8 to 1.0. *E. coli* alkaline phosphatase removed the phosphate from this compound and released free muramic acid, which was identified by paper chromatography in solvent B ($R_{glucose}$ 1.39) and quantitated on an amino acid analyzer.

A similar phosphorylated derivative of murein, which gave rise to muramic acid phosphate on acid hydrolysis, was found in the soluble fraction obtained by exhaustive digestion of purified walls of strains H and 52A5 by lysostaphin and *Chalaropsis* muramidase.

Enzymatic activities of unheated isolated walls. Since glycerol teichoic acid and phospholipid are components of the protoplast membrane, the presence of these substances in preparations of purified cell walls suggested that fragments of membrane remained adhering to the wall. To confirm the presence of membrane in the crude cell walls (taken at a stage of preparation prior to the heating step; see Materials and Methods), the wall preparations were examined for the presence of membrane-bound enzymes.

Table 2 shows that the specific activities of unheated walls and membranes were similar for phosphoribitoltransferase and *N*-acetylglucosaminetransferase. The activity was slightly higher in the wall preparation for incorporation of radioactivity from uridine diphosphate *N*-acetylmuramyl-(L-[^{14}C]lysine)pentapeptide. Chromatography in solvent A showed that most of the counts incorporated in the latter system traveled near the solvent front, indicating the formation of disaccharide lipid intermediate, and some 10 to 15% remained at the origin as expected for murein (1).

Materials solubilized by heating isolated walls. The heating step (15 min at 100 C) included in the purification of isolated walls was employed primarily to inactivate the wall autolytic system. An appreciable amount of material was brought into solution during the heating, about 16% of the weight of walls of strain H and somewhat more (24%) of the weight of walls of strain 52A5.

Analyses of the solubilized material from strains H and 52A5 showed the presence of murein components (Table 3), and the material from strain H also contained ribitol, indicating the presence of ribitol teichoic acid. Protein was indicated by a positive reaction in the Lowry protein assay and also by the presence of a wide range of amino acids in acid hydrolysates. Extraction of the solubilized material with chloroform-methanol (2:1, v/v) took approximately 20% of the phosphorus into chloroform solution. Chromatography of the extracted material on thin-layer plates with solvent D showed two main

TABLE 2. *Comparison of enzymatic activities of wall and membrane preparations*

Expt	Fraction	Protein content of wall (mg/mg)	Phosphoribitol-transferase[a]	*N*-acetylglucosamine-transferase[a]	*N*-acetylmuramyl-pentapeptide incorporation[b]
1	Wall	0.07	0.70	0.59	1.75
	Membrane	—	0.26	0.12	0.19
2	Wall	0.11	0.52	0.27	0.61
	Membrane	—	0.61	0.32	0.43
3	Wall	0.12	0.20	0.01	0.45
	Membrane	—	0.15	0.19	0.24
4	Wall	0.06	0.39	0.09	—
	Membrane	—	1.07	0.34	—
5	Wall	0.10	0.25	0.02	0.31
	Membrane	—	0.34	0.11	0.22

[a] Expressed as nanomoles per minute per milligram of protein.

[b] Expressed as nanomoles per 30 min per milligram of protein.

TABLE 3. *Composition of material solubilized by heating crude cell walls*

Component	Amt (μg) per mg (dry weight) of material solubilized		Percentage of dry wt of crude wall	
	Strain 52A5	Strain H	Strain 52A5	Strain H
Murein[a]	180	80	5	1
Ribitol teichoic acid[b]	<1	75	0	1
Protein	195	210	5	3
Phospholipid[c]	250	260	6	4
Nucleic acid[d]	400	100	10	2

[a] Calculated from the muramic acid content.

[b] Calculated from the ribitol content.

[c] Calculated from the fatty acid content, assuming an average phospholipid molecular weight of 960.

[d] Calculated from the extinction at 260 nm, assuming molecular extinction of 10^4.

spots with the same R_F values as authentic phosphatidyl glycerol and cardiolipin. Fatty acids were identified by gas chromatography and found to be typical of *S. aureus* (Table 1; 12, 24). Thus, some of the glycerol and phosphate in hydrolysates of the solubilized material was derived from phospholipids but some was also derived from membrane glycerol teichoic acid, as indicated by the presence of ester-linked D-alanine in the material obtained from strain 52A5 (16).

Solubilization by other reagents. A variety of buffers, detergents, and other reagents were tested for their ability to remove residual phosphorus compounds from the crude walls isolated by differential centrifugation from cells that had been uniformly labeled with ^{32}P. The labeled compounds include the wall (ribitol) teichoic acid, membrane (glycerol) teichoic acid, phospholipid, nucleic acid, and other compounds. Table 4 shows that heating was one of the most effective ways of removing ^{32}P-compounds from isolated walls. Since the walls of strain H contain ribitol teichoic acid and the walls of 52A5 do not, the proportion of ^{32}P extracted from 52A5 (about 60%) was generally greater than from walls of strain H (about 15%).

TABLE 4. *Effectiveness of various treatments on extraction of phosphorus compounds from crude cell walls*[a]

Treatment[b]	^{32}P solubilized	
	From *S. aureus* 52A5 walls	From *S. aureus* H walls
Water, 15 min, 100 C	60	11
Trichloroacetic acid, 5%, 30 min, 0C	4	2
Trichloroacetic acid, 5%, 5 min, 100 C	68	89
NaOH, 0.1 N, 60 min, 0 C	60	15
NaCl, 3.2 M	45	21
Sodium perchlorate, 0.2 M	41	—
Urea, 6 M	27	16
Sodium deoxycholate, 2%, 60 min.	68	26
Triton X-100, 0.1%, 30 min.	55	—
Phenol, 80%	35	14
Butan-1-ol	19	6

[a] Percentage of total ^{32}P in crude walls.

[b] Lyophilized crude cell walls were suspended in the reagent at a concentration of 2 mg/ml. The extraction took place at 25 C and for 10 min unless indicated otherwise.

DISCUSSION

S. aureus cell walls that had been subjected to rigorous purification contained small amounts of phospholipid and glycerol teichoic acid. Both of these substances are characteristic of the bacterial membrane (2, 16, 18). The association of membrane with cell walls, isolated by differential centrifugation and well washed, was clearly indicated by the presence of enzymes for teichoic acid and murein synthesis. Heat and treatment with detergents were the most effective methods found for freeing crude wall preparations of associated membrane. It was difficult to remove the last traces of lipid by extraction with organic solvents. Autolysis or digestion of purified walls with muralytic enzymes seemed able to dissolve away the true wall, leaving an insoluble residue of membrane. It seems likely, therefore, that the walls do not contain any lipid, but rather that wall preparations contain traces of membrane and that the lipids reported in cell wall preparations from other gram-positive bacteria (6, 7, 20) may also have been the remains of contaminating membrane. As shown in Table 3, crude cell wall preparations contained 4 to 6% lipid, and a recent paper (14) also reported about 5% lipid in crude cell walls; i.e., well-washed walls not treated with detergent, heat, or proteolysis may actually contain more than 10% by weight of membrane.

It is possible that the residue of membrane found in preparations of *S. aureus* cell walls was simply trapped by the collapse of the rigid wall during disruption of the cells and not completely removed by subsequent purification procedures. Alternatively, Rogers (17) has recently summarized the morphological evidence indicating connections between bacterial wall and membrane and suggests that connecting bridges may form when intermediates in wall synthesis are bound simultaneously to membrane components and to wall. The fragments of membrane found in our wall preparations may thus be physically joined to the wall by such connections, and this speculation seems worthy of further investigation.

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