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Extraction, Characterization, and Cellular Localization of the Lipids of *Staphylococcus aureus*

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Satisfactory extraction and assay procedures have been developed for the lipids of *Staphylococcus aureus*. The following lipids have been characterized in detail: the vitamin K_2, which is shown to exist as isoprenologues with side chains of 35, 40, and 45 carbon atoms; monoglucosyldiglyceride and diglucosyldiglyceride, which account for all the carbohydrate in the lipid extracts; the lysyl ester of phosphatidyl glycerol, phosphatidyl glycerol, and cardiolipin, which account for 98% of the phosphate in the lipid extract. The extraction procedure removes 98% of the total bacterial fatty acids. Acidification of the medium before harvest and refluxing in isopropanol are critical in the extraction procedure for the maximal recovery of lysyl-phosphatidyl glycerol and the glucolipids. The lipids have been shown to be a part of the same membrane as the respiratory pigments.

Staphylococcus aureus has the capacity to grow anaerobically with the energy produced from the glycolytic pathway. While growing anaerobically, the enzymes of the Krebs cycle and the membrane-bound electron transport system are repressed (6, 46). Addition of oxygen to the growing culture causes the induction of the membrane-bound electron transport system (12). This formation of the multienzyme respiratory system is coordinated with the synthesis of neutral lipid, glycolipid, phospholipids, and other components.

The function of the electron transport system requires structural integrity, since any treatment with organic solvents destroys the activity. To study the process of membrane formation, the components of the membrane must be identified and the techniques for their assay developed. This paper gives assay procedures for the lipids and establishes that these lipids are a part of the respiratory membrane complex.

Staphylococcus aureus has been shown to have a membrane-bound electron transport system consisting of cytochromes b_1 and *a* and cytochrome oxidase *o* (47). These bacteria contain a vitamin K_2 derivative which is formed when the bacteria are grown with aeration (3). A glycolipid, diglucosyl diglyceride, contains the glucose extractable with lipid solvents (37). Lysyl-phosphatidyl glycerol (LPG), phosphatidyl glycerol (PG), and cardiolipin are the principal phospholipids of *S. aureus* (17, 29, 30) and in this paper are shown to constitute 98% of the total phospholipids. The structure of staphylococcal LPG has recently been established by the work of Bonsen et al. (5) and the biosynthesis of LPG from PG and L-lysyl transfer ribonucleic acid by Lennarz et al. (24). The presence of vitamin K_2 isoprenologues, and a glycolipid not previously found in *S. aureus*, are reported. The following paper (12) will give evidence that the formation of the membrane-bound respiratory system involves the simultaneous synthesis of lipids and respiratory pigments.

MATERIALS AND METHODS

Growth of bacteria. Staphylococcus aureus strain U-71 was provided by J. N. Baldwin. The medium used to grow the bacteria was either the semisynthetic medium containing acid-hydrolyzed casein (22) or this medium supplemented with 0.2% (w/v) Difco yeast extract. Supplements were added as recommended (14). The final concentrations were: 10 mM acetate, 1 mM uracil, 1 mM xanthine, 1 mM adenine, and 15 mM glucose. The tryptophan, iron, vitamins, and glucose were added after autoclaving the medium. The bacteria were preserved in 15% (v/v) glycerol in the growth medium at −60 C. Purity of the culture was checked by Gram stain, colonial morphology, and phase-contrast microscopy.

Dry weight. The relationship between dry weight of the bacteria and absorbance of the bacteria is illustrated in Fig. 1. Dry weight was obtained by removing 50-ml portions from a growing culture, centrifuging, washing twice in distilled water, placing them in a vacuum oven at 40 C, and drying to constant weight. The absorbance at 750 mμ in 13-mm round test tubes was determined with a Spectronic 20 spectrophotometer. Dilutions were made such that the absorbance was measured between 0.2 and 0.8 absorbance units.

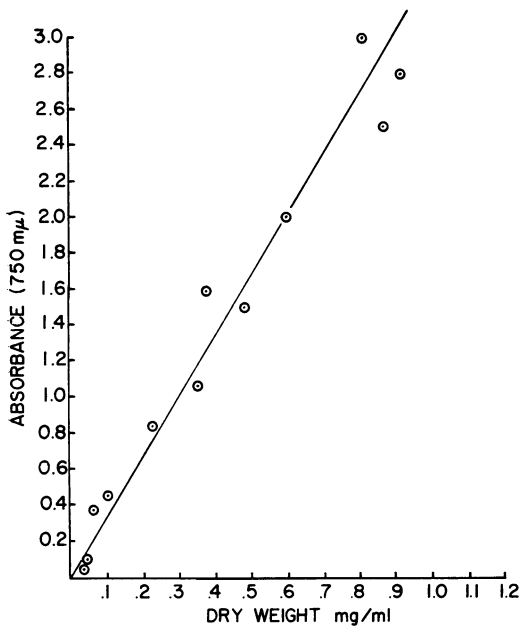

FIG. 1. *Relationship between the absorbance and the dry weight of Staphylococcus aureus. Portions (50 ml) of growing bacteria were withdrawn and the absorbance of the bacteria was measured in the growth medium in 13 mm diameter tubes at 750 mμ. The culture was diluted so that the absorbance measured between 0.2 and 0.8. The 50-ml portions were then centrifuged, the medium was decanted, the pellet was resuspended in distilled water and then centrifuged two times and dried to constant weight at 40 C in vacuo.*

Repeated experiments with aerobic or anaerobic cultures gave the same relationship shown in Fig. 1. Microscopic examination of the culture indicated no significant clumping. Lack of clumping during these growth conditions may explain the difference between the linear relationship found in this study and that reported by Rodgers (42). In each experiment reported in this paper, at least one dry weight determination was actually performed and the results agreed with those predicted from the data of Fig. 1. Protein was measured (28) after hydrolysis in 1 M NaOH with bovine serum albumin as standard.

Extraction of the lipid. Before harvesting, the medium was adjusted to *p*H 2.0 with concentrated HCl, and it was then centrifuged. The pellet was resuspended in 0.05 M phosphate buffer, *p*H 7.6, and was recentrifuged. The pellet from the second centrifugation was suspended in isopropanol (30 ml/100 mg, dry weight) and was refluxed for 20 min. After adding 100 ml of chloroform and 20 ml of methanol, the mixture was stirred for 20 min with a Teflon-covered magnetic stirrer. Where necessary, the bacteria were dispersed by a brief exposure to ultrasonic vibration. The extracts were then filtered through glass wool and the residue was re-extracted with chloroform-methanol (2:1). The combined chloroform-methanol extracts were partitioned against NaCl solution (10) and passed through Sephadex to remove nonlipid contaminants (53).

In later experiments, the bacteria were again harvested (after adjusting the *p*H of the growth medium to 2.0 with HCl), centrifuged, resuspended in buffer, centrifuged, and resuspended in 50 ml of isopropanol. The isopropanol suspension in a stainless-steel centrifuge tube was finely dispersed by exposure to ultrasonic vibration, and then was placed in a boiling-water bath for 5 min. The mixture was transferred to a glass separatory funnel; 25 ml of methanol, 30 ml of phosphate buffer, and 37.5 ml of chloroform were added with shaking after each addition. The one-phase mixture was allowed to stand for at least 2 hr at room temperature, and 37.5 ml of chloroform and 37.5 ml of water were added. After thorough mixing, the two phases were allowed to separate, and the lower phase was collected after filtration through a 20-cm disc of Whatman no. 1 filter paper. The lipid was then treated with Sephadex (53). This is a modification of the Bligh and Dyer (4) procedure.

Thin-layer chromatography (TLC). Thin-layer plates of 0.5-mm thick Silica Gel G were placed on glass plates with the Desaga applicator. The plates were activated at 110 C for 2 hr and stored in a desiccating cabinet for not longer than 24 hr before use. Lipids were detected with the following sprays: rhodamine 6-G and examination under ultraviolet light for total lipid (8), ninhydrin for amino nitrogen (8), acid molybdate for phosphate (8), diphenyl amine for glycolipid (52), and anisaldehyde-sulfuric acid for sugars (45). A modification of the method for detecting quinone as used for paper chromatography was suggested by R. L. Lester. The thin-layer plate is sprayed with 1% (w/v) KBH_4 in ethyl alcohol-water (1:1), dried for 3 min, and sprayed with aqueous 0.2% neotetrazolium. A red color develops immediately with naphthoquinones. Benzoquinones do not give the red color unless the plate is heated at 100 C for 5 min. Solvent systems in this paper are given as volume/volume unless otherwise specified.

Recovery of the lipids from thin-layer plates. After thin-layer chromatography, the plates were dried and sprayed lightly with rhodamine. A second plate, chromatographed with one-fourth of the sample, was sprayed with rhodamine, ninhydrin, and acid molybdate (8) or with diphenylamine for glycolipids (52). The lipid spots were then outlined on tracing paper and used to identify the spots on the rhodamine-sprayed plate. The individual spots on the plate sprayed with rhodamine were removed by vacuum and collected with a sealing tube with reduced ends (Corning 59580) fitted with a 3-cm coarse frittered disc. The lipid was eluted successively with 5 ml of chloroform-methanol (1:1, containing 4% water); 5 ml of chloroform-methanol (2:1, containing 2% water); and 4 ml of chloroform-methanol (1:1, containing 4% water) into a 25-ml mixing cylinder. Sufficient methanol and water were then added until the aqueous phase was 20% of the organic phase; the suspension was then mixed vigorously. After standing overnight at 4 C, the aqueous layer was removed and the lipid was recovered from the organic phase. By measuring phos-

phate, glycerol, amino nitrogen, or glucose, it was shown that <1% of the lipid remains in the water phase. Recovery in the organic phase was near 100%. Controls obtained by eluting areas in which lipids could not be detected were negligible. The elution procedure was developed by M. Steiner. Average recovery of the total phosphate from 16 plates was 99.7 ± 1%. The proportions of each of the phospholipids, as determined from the phosphate recovered from the thin-layer plates, agreed within 2% for 16 plates run with the same lipid mixture.

Analysis of the lipids. Glycerol was assayed by measuring formaldehyde production after acid hydrolysis (41) with α-glycerol phosphate as standard. Acyl ester was determined by the modified method (53) of Rapport and Alonzo (40) with tripalmitin as standard. Lipid glucose was determined with anthrone (38) as modified (53) with glucose as standard. Lipid phosphate was determined as described by Bartlet (2), after digestion of samples in 23% (v/v) perchloric acid at 200 C for 1 hr, as modified for the Technicon autoanalyzer by R. L. Lester. Glucose was determined colorimetrically (32) and enzymatically (18) after acid hydrolysis of the lipid. Lysine was measured with the ninhydrin reaction (23) after mild alkaline methanolysis with methyl lysine as standard. Fatty acids were determined after saponification, as described by Novák (33), with palmitic acid as standard. Vitamin K_2 was assayed by its difference spectrum in ethyl alcohol containing 1% 1 M ammonium acetate, *p*H 5.0 (27). A solution of oxidized quinone was compared to the hydroquinone formed on the addition of KBH_4, as described (55). Although the hydroquinone of vitamin K_2 is very autooxidizable, the assay is accurate if care is taken to reduce the quinone by at least four additions of KBH_4 and if the cuvette is stoppered. With these conditions, the quinone remained completely reduced for 10 min.

Paper chromatography. Paper chromatography was carried out with descending solvent system on acid-washed 589 paper (Schleicher and Schuell, Keene, N.H.). Schleicher and Schuell alumina-impregnated paper (288) or silica gel-impregnated paper (996) was used. Phospholipids were detected by the reaction of lipid-ferric chloride complexes with salicylsulfonic acid (51) as modified (50), or with acid molybdate as described by Hanes and Isherwood (15). Best results with the Hanes and Isherwood reaction were obtained by spraying the paper and drying at 110 C for 3 min, followed by acid hydrolysis in the dark for 12 hr at room temperature. A modification of the periodate-Schiff reaction (1) proved satisfactory for the dectection of vicinyl glycols. The papers were first dipped into 0.155 M sodium periodate in acetone-water (95:5), dried for 3 min, then dipped into a solution containing 0.01 M *o*-tolidine and 0.1 M acetic acid in acetone-water (95:5). The vicinyl glycols appear yellow on a green background. Amino nitrogen was measured as described (23). Reducing sugars were detected with aniline-phthalate (35) or with silver nitrate (48) fixed with photographic fixer (Eastman Kodak Co., Rochester, N.Y.).

Dinitrophenyl (DNP) derivatives. Derivatives were prepared as described (44).

Column chromatography. Lipids were fractionated with silicic acid chromatography as described by Vorbeck and Marinetti (49). Florisil (100 to 200 mesh) was used to purify the glycolipids. The Florisil was washed and packed into a 1-cm × 6-cm column (2 g) as described (21, 43). Neutral lipids were eluted with 40 ml of chloroform, and the glycolipids were eluted with 40 ml of chloroform-methanol (2:1). Deacylated phospholipids were separated with the columns of Dowex-1, 8X, 200 to 400 mesh in the formate form. The lipids were eluted by use of a gradient from 0.125 M to 0.657 M ammonium formate, *p*H 9.50, containing 0.02 M sodium borate. The columns were 6 mm × 81 cm. This chromatographic system was developed by R. L. Lester (Federation Proc. **22**: 415, 1963).

Deacylation by methanolysis of the lipids. Samples of lipid dried by vacuum evaporation were carefully dissolved in anhydrous methanol-toluene (1:1), and an equal volume of freshly prepared methanolic 0.2 M KOH was added. The mixture was then incubated at 0 C and the *p*H was adjusted to 7.0 with 1 M acetic acid. The time course of the reaction which deacylates both the phospholipids and glycolipids is shown in Fig. 2. One volume of chloroform and one volume of water were then added and the solution was mixed with a Vortex mixer for at least 5 min. The aqueous phase was then transferred and the chloroform phase was extracted with a second portion of water. The aqueous phases were combined and reduced in volume to 1 ml in a stream of nitrogen.

When a mixture containing all the lipids is extracted, less than 0.5% of the phosphate or carbohydrate remains in the chloroform phase, and the recovery from the aqueous phase approaches 100%. Hydrolysis at 37 C, or neutralization of the KOH with methyl formate, produces lipids which behave chromatographically like methylglycerol phosphate and cyclic glycerol phosphate. The appearance of these artifacts coincides with the disappearance of phosphatidyl glycerol. As much as 8% of the phospholipid may be degraded in this way. The mild alkaline methanolysis procedure was developed by R. L. Lester from the mild alkaline hydrolysis procedure of Dawson (7).

Enzyme assays. Glucose-6-phosphate dehydrogenase, 6-phospho-gluconate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, and fumarate were assayed as described (56). Cytochromes were assayed by difference spectroscopy and oxygen utilization was measured with an oxygen electrode, as described (54).

Materials. Materials were the best grade commercially available.

RESULTS

Characterization of the lipids. Extracts of lipids from *Staphylococcus aureus* were fractionated by use of silicic acid columns prepared as described by Vorbeck and Marinetti (49). An experiment showing this fractionation is illustrated in Table 1. The neutral lipids were removed as the chloroform fraction. This fraction contained 99% of the

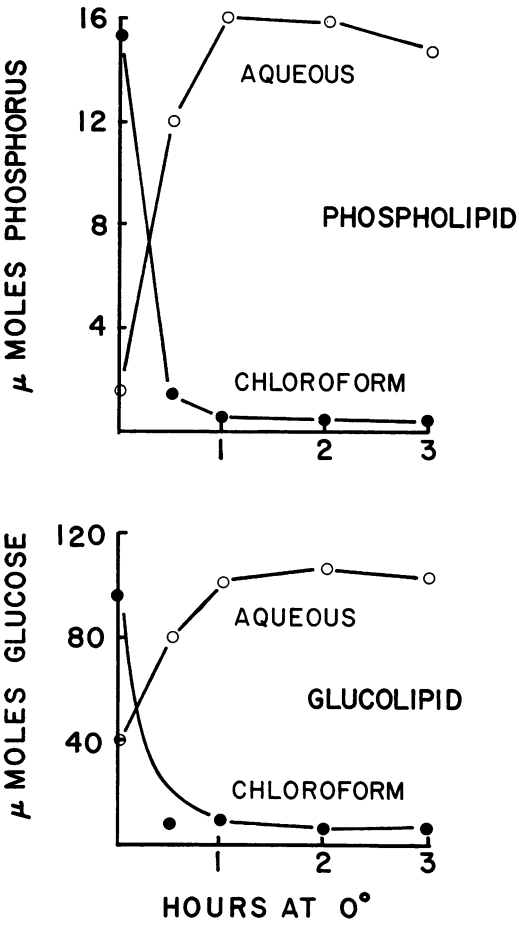

FIG. 2. *The time course of the mild alkaline methanolysis of staphylococcal lipid at 0 C. Mild alkaline methanolysis was performed as described in Materials and Methods. The aqueous and chloroform layers were sampled and phosphate (upper curve) or glucose (lower curve) was determined. The deacylation makes the glycerol-containing portion of the lipid soluble in water. The fatty acid esters were quantitatively recovered in the chloroform phase.*

vitamin K_2, 84% of the carotenoid lipids, 0.5% of the glycolipids, and 0.05% of the phospholipids. The chloroform-acetone and acetone fractions were pooled and contained less than 1% of the quinone, 15% of the carotenoid pigments, 98.2% of the glycolipid, and 0.15% of the phospholipids. The fractions containing methanol were pooled and contained 99.8% of the phospholipids and about 1.8% of the glycolipid with none of the neutral lipids.

Characterization of the quinone. The chloroform fraction from the column chromatography illustrated in Table 1 was rechromatographed on a silicic acid column. Quinone was quantitatively eluted in the first few fractions. The quinone was the only lipid detectable on thin-layer plates sprayed with rhodamine or detected with ultraviolet light. Tests for amino-nitrogen, glycolipid, and phospholipid were negative. The method of detecting quinones on thin-layer plates is described in Materials and Methods. A single red spot was seen at an R_F value of 0.35 when the staphylococcal quinone was chromatographed on thin-layer plates in a solvent system of chloroform-isooctane (2:1). With this method, the development of color occurred immediately, indicating the quinone is similar to a naphthoquinone. When the total staphylococcal lipid mixture was chromatographed in this system, there was only one entity which reduced neotetrazolium. The R_F values of the quinone obtained by chromatography of the mixture and of the purified quinone were the same. The spectrum of the quinone in isooctane revealed maxima at 327, 271, 262, 250, and 246 mμ. Minima were observed at 266, 256, 248, and 224 mμ. The spectrum of a solution corresponding to 5.3 μM vitamin K_2 [40] is replotted for a mM solution in Fig. 3. The extinction coefficient of synthetic vitamin K_2 [40] is 19.28×10^3 (19). The difference spectra of solutions of this quinone of known concentration were determined as described (55). The difference spectrum between the oxidized quinone and the hydroquinone reduced with KBH_4 is illustrated by the dashed line in Fig. 3. The extinction coefficient calculated from the difference spectrum between the maximum at 245 mμ and the minimum at 270 mμ was 31×10^3 for vitamin K_2 [40].

Several chromatographic systems can be used to separate isoprenologues of the vitamin K series. If staphylococcus quinone and synthetic vitamin K_2 isoprenologues of 25, 35, 45, and 50 carbon atom side chains are compared in several chromatographic systems (26, 34), the major quinone appears to be vitamin K_2 [40] with minor amounts of vitamin K_2 [35] and vitamin K_2 [45]; see Fig. 4.

Assay of the quinone. Difference spectra of the unresolved lipid can be used for the assay of this quinone using the same techniques described for 2-demethyl vitamin K_2 (55). This technique was satisfactory for the detection of the quinone without further purification. Five samples of the same staphylococcal lipid were assayed and gave values of 2.68 ± 0.20 μmoles vitamin K_2 [40] per g (dry weight). Adding a known amount of quinone to this lipid preparation was found to give an expected result within 3%. Results of the quinone assay by difference spectra of the total

TABLE 1. *Fractionation of staphylococcal lipids on silicic acid*[a]

Fraction	Solvent[b]	Vitamin K_2 (μmoles)	Carotenoid (concn at 430 mμ)	Glucose (μmoles)	Phosphate (μmoles)	Components[c]
1	A	6.05	0.43	0.25	0.1	Vitamin K_2 carotenoids
2	B	0.062	0.08	7.20	0.2	Glycolipid
3	C	<0.008	<0.01	37.4	0.1	Glycolipid
4	D	<0.001	<0.01	0.27	12.1	C, PG[d]
5	E	<0.001	<0.01	0.32	145.7	C, PG, LPG
6	F	<0.001	<0.01	0.22	27.4	PG, LPG
7	G	<0.001	<0.01	<0.01	3.3	LPG

[a] Staphylococcal lipid was extracted with the acid isopropanol method, treated with Sephadex (53), dried, and applied to a 10-g Unisil silicic acid column, 20 × 1.2 cm. The column was prepared in hexane and washed with diethyl ether and chloroform (49).

[b] The column was eluted with the following solvents, each of which was collected separately: A, 60 ml of chloroform; B, 60 ml of chloroform-acetone (1:1); C, 60 ml of acetone; D, 100 ml of chloroform, 10% methanol; E, 100 ml of chloroform, 30% methanol; F, 100 ml of chloroform, 50% methanol; F, 60 ml of methanol.

[c] Components were identified by thin-layer chromatography with chloroform-methanol-acetic acid-water (42:12:3.15:1) as solvent, followed by spraying of one plate with diphenylamine for glycolipid and of the others with rhodamine 6-G, ninhydrin, and acid molybdate. The bacteria extracted represent 2.5 g (dry weight).

[d] C = cardiolipin, PG = phosphatidyl glycerol, and LPG = lysyl-phosphatidyl glycerol.

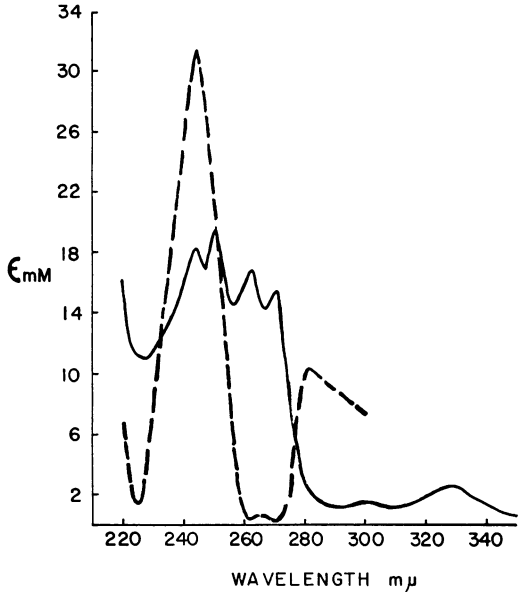

FIG. 3. *Spectra of the Staphylococcal quinone. The solid curve represents the spectrum of 5.3 μM vitamin K_2 from staphylococci measured in isooctane and replotted for 1 mM solution. The dotted curve represents the difference spectrum of staphylococcal quinone versus staphylococcal hydroquinone replotted for a 1 mM solution. The quinone was reduced as described in Materials and Methods in ethyl alcohol containing 1% (v/v) 1 M ammonium acetate, pH 5.0.*

lipid sample and with the quinone after purification were identical.

Characterization of glucolipids. The glucolipid fractions from silicic acid columns like those obtained in the experiment illustrated in Table 1 were chromatographed on Florisil columns (21) using the solvent system described by Rouser (43). The neutral lipids were eluted with chloroform and the glucolipids with chloroform-methanol (10:1). No phospholipids were detected in these eluates. Methanol or chloroform-methanol-water was necessary to elute the phospholipids. The pooled glucolipid fractions were then streaked on activated thin-layer plates and developed with a solvent of chloroform-methanol-acetic acid, 100:25:8 (36). The plates were divided into 1-cm strips which were removed individually, leaving the edges intact. The edges were then sprayed with diphenylamine. The major glucolipid appeared with an R_F value of 0.66 and the minor glucolipid with an R_F value of 0.93. The appropriate strips taken from the center of the thin-layer plate were eluted as described in the Materials and Methods.

Analysis of glucolipids. The ratio of glucose to acyl ester to glycerol of the major glucolipid was 1.91:1.67:1.00. The ratio expected for diglucosyldiglyceride would be 2:2:1. The ratio of glucose to acyl ester to glycerol of the minor glucolipid was 1.09:1.90:1.00. The ratio expected for monoglucosyldiglyceride would be 1:2:1.

Chromatography of glucolipids. Chromatography with silicic acid impregnated paper with a solvent of chloroform-methanol-acetic acid-water (50:10:2:0.25) showed that the major glucolipid moved with an R_F value of 0.69, and the minor glucolipid and authentic monogalactosyldiglyceride moved with an R_F value of 0.90. The glucolipid could be detected both with the silver nitrate-sodium hydroxide method (48)

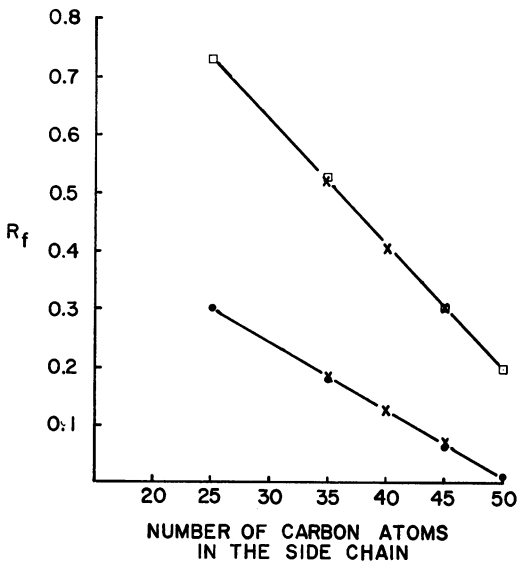

FIG. 4. *Chromatography of the staphylococcal quinone. The R_F values of quinones are plotted versus the number of carbon atoms in the side chains. Open symbols indicate synthetic vitamin K_2 25, 35, 45 and 50; × indicates staphylococcal quinone isoprenologues; □ indicates chromatography with vaseline-impregnated paper in dimethylformamide-water, 32:1 (35); ● indicates chromatography with Dow Corning 550-impregnated paper with a solvent of n-propanol-water, 3:1 (27). Staining was performed with neotetrazolium as described in Materials and Methods. The size and intensity of the staining of the spot corresponding to vitamin K_2 [40] showed that it was the major isoprenologue present.*

and with the periodate-Schiff (1) method. A comparison of the chromatographic behavior of di- and monogalactosyldiglyceride to the staphylococcal glucolipids in two chromatographic systems (25) is given in Table 2.

Characterization of deacylated glucolipids. The glucolipids were deacylated by mild alkaline methanolysis as described in Materials and Methods. The chromatographic behavior in two solvents indicated that the minor glucolipid behaved similarly to monogalactosylglycerol (Table 3).

Identification of the carbohydrate in the glucolipid. The purified, mixed, major and minor glucolipids were hydrolyzed with 3 M HCl in sealed glass tubes at 100 C for 2 hr. Recovery was about 89% on the basis of carbohydrate estimated with anthrone, with glucose as standard. The HCl was removed by repeated evaporations under a stream of nitrogen. The fractions from the column illustrated in Table 1 and a sample of total staphylococcal lipids were treated similarly. Thin-layer plates were prepared and subjected to chromatography under the conditions described

TABLE 2. *Thin-layer chromatography of the staphylococcal glucolipids*

Component	Solvent 1[a] (R_F value)	Solvent 2[b] (R_F value)
Digalactosyldiglyceride[c]	0.62	0.25
Major glucolipid[d]	0.67	0.35
Monogalactosyldiglyceride	0.78	0.51
Minor glucolipid	0.82	0.85

[a] Solvent 1 was chloroform-methanol-water (65:25:4).

[b] Solvent 2 was di-isobutylketone-acetic acid-water (8:5:1).

[c] The solvent system and R_F values of di- and monogalactosyl diglyceride are taken from Le-Page (25).

[d] Glucolipids were detected with the diphenylamine method (52).

TABLE 3. *Paper chromatography of the deacylated staphylococcal glucolipids*

Component	Solvent 1[a] (R_F value)	Solvent 2 (R_F value)
Glucose	0.39	0.42
Glycerol	0.57	0.67
Monogalactosylglycerol	0.40	0.65
Deacylated minor glucolipid	0.40	0.70
Deacylated major glucolipid	0.25	0.47

[a] Solvent system 1, isopropanol-acetic acid-water (3:1:1); solvent system 2, water-saturated phenol. R_F values are for descending paper chromatography in these systems. Silver nitrate (48) and periodate-Schiff (1) methods were used for detection.

by Stahl and Kaltenback (45). Only one moiety, which reacted with anisaldehyde-sulfuric acid, was present in the acid hydrolysates of the purified glucolipid, the total staphylococcal lipid, and the fractions from the column given in Table 1. The R_F value in this system for the single carbohydrate was 0.15. In the same system the R_F value for glucose was 0.15 and the R_F value for galactose was 0.12. Other common sugars could be excluded on the basis of chromatographic behavior. Descending paper chromatography with a solvent system of ethyl acetate, pyridine, and water as described by Smith (44) gave R_F values relative to glycerol of 0.50 for the unknown and glucose, and 0.42 for galactose. The hexoses could be detected by aniline phthalate (35), as well as by silver nitrate (48). Co-chromatography in this system with the unknown and galactose resulted in two spots separated by 2 cm. Co-chromatography with the unknown and glucose in this system resulted in one spot. The acid hydrolysis produced only a hexose and glycerol as

detected with the periodate-Schiff (1) or silver nitrate (48) methods on chromatograms. The only reducing hexose found in the total lipid was found exclusively in the same fractions as the glucolipid. The hexose concentration measured as reducing sugar (32) more closely approximates the glucose standard than does an equimolar galactose standard when measured with anthrone. The staphylococcal lipid carbohydrate was assayed with the Nelson technique (32). Assay of a second portion of the carbohydrate solution with the enzyme glucose oxidase (18) indicated that 104% of the carbohydrate was glucose. Under these assay conditions, chromatographically pure 1 mM galactose gave 8% of the reactivity with glucose oxidase that 1 mM glucose did. Consequently, the carbohydrate moiety of the glycolipid is glucose.

Assay of glucolipids. Since glucose was the only carbohydrate detectable in the lipid, the amount of glucose reflected the concentration of glucolipid. The fact that the glucose to acyl ester to glycerol ratios for the two lipids were compatible with the identification of the lipids by chromatography indicated that the anthrone reaction was a good measure of the lipid glucose. The two glucolipids were easily separated by TLC in the chloroform-methanol-acetic acid solvent (36). The areas near R_F values of 0.66 (diglucosyldiglyceride) and 0.95 (monoglucosyldiglyceride) were scraped off and eluted. Analysis for both glucose and glycerol indicated that about 30.4 $\pm$ 1% of the glucolipid was monoglucosyldiglyceride. Recovery of glucose from the thin-layer plates averaged 100 $\pm$ 3%.

Characterization of phospholipids. The phospholipid fractions from columns similar to those in Table 1 were pooled and applied as strips to thin-layer plates. Chromatography in one dimension with a solvent of chloroform-methanol-acetic acid-water (42:12:3.15:1) was performed. The plates were then covered with Saran wrap, except for 1-cm portions at the ends, and sprayed for phosphate (8). The phosphate was detected at R_F values of 0.20 for fraction I, 0.66 for fraction II, and 0.92 for fraction III. The material in the unstained portions of the thin-layer plates was scraped off and eluted as described in Materials and Methods. Portions of each phospholipid were rechromatographed in the two dimensional system described in Materials and Methods. The R_F values with the pyridine-containing solvent were 0.25 for fraction I, 0.66 for fraction II, and 0.77 for fraction III. The R_F values of these lipids in the second solvent system were: 0.02 for fraction I, 0.21 for fraction II, and 0.51 for fraction III. No glycolipid could be detected with diphenylamine. Each of the phospholipids appeared homogeneous when sprayed with rhodamine and acid molybdate. All the material reacting with ninhydrin appeared in fraction I.

Analysis of phospholipids. The glycerol-acyl ester-amino nitrogen-phosphate ratio was 0.98: 1.64:0.98:1.00 for fraction I. The ratio expected for lysyl ester of phosphatidyl glycerol would be 1:2:1:1. The ratio of glycerol to acyl ester to phosphate was 0.94:2.01:1.00 for fraction II. The ratio expected for phosphatidyl glycerol would be 1:2:1. The ratio of glycerol to acyl ester to phosphate was 1.45:1.72:1.00 for fraction III. The ratio expected for cardiolipin would be 1.5:2:1. No glucose could be detected in any fraction. Each analysis represents the average of five determinations. The acyl ester was determined with tripalmitin as standard. The ninhydrin reaction was measured after mild alkaline methanolysis with lysine as the standard. No detectable ninhydrin-reacting component remained in the chloroform phase after methanolysis.

Chromatography of phospholipids. Descending chromatography with Schleicher and Schuell silicic acid-impregnated paper was performed using a solvent system of chloroform-methanol-acetic acid-water (50:10:2:0.25). Fraction I had an R_F value of 0.36; fraction II had an R_F value of 0.72 and co-chromatographed with authentic phosphatidyl glycerol; and fraction III had an R_F value of 0.86 and co-chromatographed with authentic cardiolipin. Fraction I contained the ninhydrin-reacting material.

Identification of deacylated phospholipids. Each lipid fraction was deacylated by mild alkaline methanolysis. Ascending chromatography in two systems was used for identification. (See Table 4.) Fraction I gave two spots, one that reacted with ninhydrin which co-chromatographed with methyl lysine and a phosphate-reacting spot that co-chromatographed with glycerolphosphorylglycerol (GPG). Fraction II gave a single, phosphate-reacting spot that co-chromatographed with GPG. Fraction III gave a phosphate-reacting spot that co-chromatographed with diglycerolphosphorylglycerol (GPGPG). The phosphate was detected with the Hanes-Isherwood spray (15), and the Wade-Morgan dip (51). Each phosphate-reacting spot also reacted with the periodate-Schiff (1) reaction for vicinyl glycols.

The gradient chromatographic procedure developed by R. L. Lester was used to confirm the identification of the deacylated phospholipids. The elution pattern of deacylated phospholipids is reproducible. In fraction I, 87% of the total phosphate was recovered in a band with the same R_F values as GPG; in fraction II, 92% of the phosphate added to the column was recovered in

TABLE 4. *Paper chromatography of the deacylated phospholipids from Staphylococcus aureus*[a]

Component	Solvent A (R_F value)	Solvent B (R_F value)
Fraction I[b]		
+ Phosphate	0.17	0.38
+ Ninhydrin	0.58	0.61
Lysine	0.14	0.40
Methyl lysine	0.58	0.61
Fraction II	0.17	0.38
GPG[c]	0.17	0.38
Fraction III	0.03	0.17
GPGPG[d]	0.03	0.17

[a] Descending paper chromatography was run with solvent A, *n*-butanol-propionic acid-water (1.5:0.7:1.0), using the method of Ferrari and Benson (9), and with solvent B, water-saturated phenol.

[b] Phosphate was detected with the method of Hanes-Isherwood (15) by spraying, heating 3 min at 100 C, and keeping for 18 hr in the dark. All molybdate-reacting spots also reacted with periodate-Schiff for vicinyl glycols (1). Ninhydrin (44) was used to detect amino acids.

[c] GPG = glycerolphosphorylglycerol.

[d] GPGPG = diglycerolphosphorylglycerol.

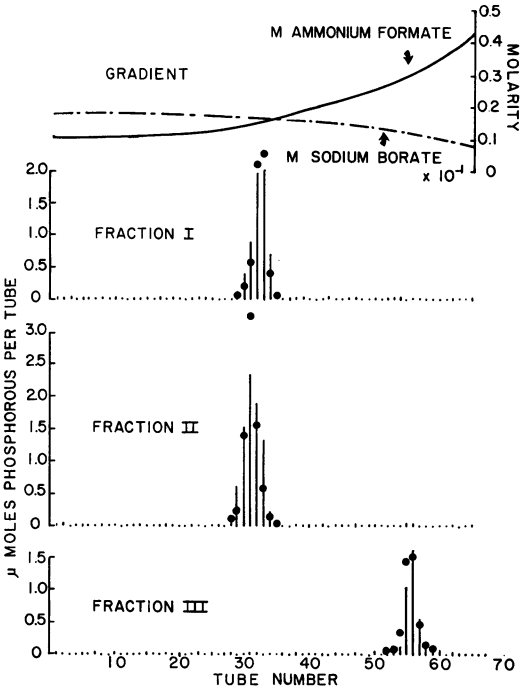

FIG. 5. *Column chromatography of the deacylated Staphylococcal phospholipids. Chromatography with columns 6 mm × 81 cm containing Dowex-1 (formate form) of each fraction of the phospholipid preparation was performed as described by R. L. Lester (Federation Proc. 22:415, 1963). The gradient used for elution is illustrated in the upper curve. Two chromatograms are illustrated for each fraction, one with the closed circles and the other with lines. The tube number for the phosphate from fractions I and II corresponded to that for authentic GPG. The tube number for the phosphate from fraction III corresponded to that for authentic GPGPG. The phosphate recoveries are given in the text.*

this band; in fraction III, 86% of the added phosphate was recovered as a band with the same R_F value as GPGPG (Fig. 5). A similar column analysis of the total deacylated staphylococcal lipid indicated that less than 2% of the phospholipid was not GPG or GPGPG.

Identification of the fragment of fraction I which reacts with ninhydrin. Fraction I contains the only ninhydrin-reacting group in the lipid. The phosphate- and ninhydrin-reacting groups move together in several chromatographic systems and exist at a molar ratio of 1:1. Care must be taken to remove all co-soluble amino acids before a meaningful interpretation of the presence of amino nitrogen in lipids can be evaluted. The Sephadex procedure of Wells and Dittmer (53) was used to purify the lipids used in this study. Both the purified fraction I and the total staphylococcal lipid were subjected to hydrolysis in 6 M HCl in sealed glass vials for 16 hr at 100 C. The HCl was removed by successive evaporations under nitrogen.

Chromatography in water-saturated phenol and the upper phase of *n*-butanol-acetic acid-water, 4:1:5 (9), showed one ninhydrin-reacting spot with R_F values of 0.4 and 0.12. Co-chromatography of the sample and lysine in these systems resulted in one spot. Dinitrophenyl derivatives of L-lysine and the unknown were prepared (44) and compared to known dinitrophenyl derivatives of L-lysine. Paper chromatography with *n*-butanol saturated with water (31) and in 1.5 M phosphate buffer, *p*H 5.95 (11), established that all three derivatives moved with the same R_F values (0.72 in the former and 0.21 in the latter). Co-chromatography of the unknown and dinitrophenyl derivatives of L-lysine resulted in one spot on thin-layer plates run with chloroform-*t*-butanol-acetic acid, 70:30:3, (R_F value of 0.60) and in the second dimension with a solvent of chloroform-methanol-acetic acid, 94:5:1 (R_F value of 0.45), as described by Randerath (39). It was discovered that overlaying the thin-layer plate with starch-impregnated tracing paper (blue fluorescence) and holding it over a long wavelength ultraviolet light intensifies the quenching of the dinitrophenyl derivatives about fivefold.

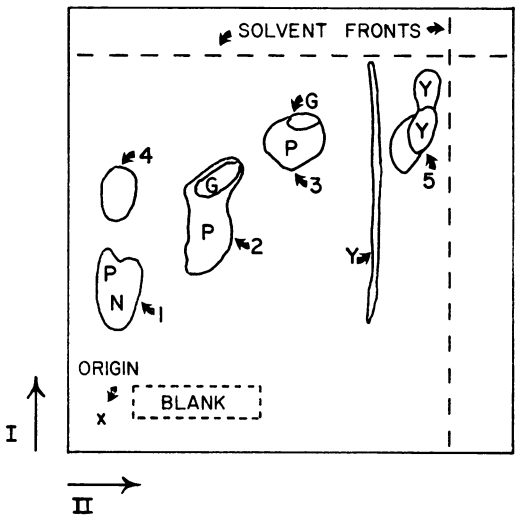

FIG. 6. *Diagrammatic representation of a thin-layer chromatogram used to separate the lipids of Staphylococcus. Thin layer plates of silica gel G were prepared as described in Materials and Methods and chromatographed in the first dimension with chloroform-methanol-pyridine-water (30:15:1:3) and in the second dimension with chloroform-methanol-acetic acid-water (45:7:3.15:0.5). Spots outlined with solid lines are rhodamine-positive, phospholipids are indicated as P; amino nitrogen-containing lipids are indicated as N; glucose containing lipids are indicated as G, and yellow neutral lipid spots are indicated as Y.*

Co-chromatography of the methyl ester of lysine with the methylated unknown in two systems, as shown in the preceding section, resulted in one spot.

Assay of phospholipids. A two-dimensional thin-layer chromatographic system was developed that will allow the separation of the phospholipids in the total staphylococcal lipid. A typical plate is shown in Fig. 6. Analysis of a thin-layer plate, run as in Fig. 6, indicated, after spraying with rhodamine, ninhydrin, and acid molybdate (8), that five major components were present. Two of the phosphate-containing spots also reacted with diphenylamine (52), indicating that glucolipid was present. The spots were eluted individually and analyzed for phosphate and glycerol (see Table 5). Spots 1, 2, and 3 contained 46, 30, and 25% of the phosphate and accounted for 100% of the phosphate applied to the plate. Spot 4 contained no amino nitrogen, glucolipid, or phosphate and accounted for 13% of the glycerol in the unfractionated lipid. When the total staphylococcal lipid was treated with Sephadex to remove the nonlipid contaminants, the only ninhydrin-reacting material was in spot 1. Spot 5 contained the vitamin K_2 [40], the carotenoids, and 11% of the total lipid glycerol. The glycerol to phosphorus ratios for spots 2 and 3 show the contamination by glucolipid. The lysine recovered from spot 1 represented 102% of that in the total lipid. The proportions of the three phospholipids as determined in the two-dimensional system were accurate to ± 2% on repetitive analysis of the same lipid. After deacylation by mild alkaline methanolysis, the lipid phosphorus of lysyl-phosphatidyl glycerol and phosphatidyl glycerol was GPG and the lipid phosphorus of cardiolipin was GPGPG. A sample of lipid chromatographed in triplicate showed 40 ± 2% as lysyl-phosphatidyl glycerol, 37 ± 3% as phosphatidyl glycerol, and 24 ± 2% as cardiolipin. After mild alkaline methanolysis, an analysis similar to that illustrated in Fig. 5 was performed with a Dowex column. The column indicated that 76% of the phosphate was GPG (77% expected) and 23% of the phosphate was GPGPG (24% expected). Hydrolysis of the lipid and analysis for the lysine gave a value of 1.9 μmoles of lysine, which would correspond to 39% of the total phosphate. Consequently, the analysis of phosphate eluted from thin-layer plates is a true representation of the proportions of the phospholipids. The concentration of lysine can be used as a measure of lysyl-phosphatidyl glycerol. The concentration of GPG and GPGPG can be used as a measure of the lysyl-phosphatidyl glycerol plus phosphatidyl glycerol and the cardiolipin.

TABLE 5. *Analysis of staphylococcal lipids eluted from thin-layer plates*[a]

Spot no.	Phosphate	Glycerol	Phosphate/glycerol
	μmole	*μmole*	*ratio*
1	0.61	1.72	1:2.8
2	0.39	1.51	1:3.9
3	0.32	0.90	1:2.8
4	<0.01	0.70	
5	<0.01	0.60	
Blank	<0.01	<0.01	
Recovery[b]	98	83	

[a] Spots eluted from Silica Gel G plates activated for 1 hr at 100 C were chromatographed in chloroform-methanol-water-pyridine (30:15:3:1) in the first dimension, dried in air for 1 hr, and then chromatographed in chloroform-methanol-water-acetic acid (90:14:1:6.3) in the other dimension. Plates were dried and sprayed with rhodamine 6-G. Lipids were detected with ultraviolet light and eluted as described in Materials and Methods. Spot 5 was shown to contain the quinone.

[b] Recovery shown in per cent phosphate and glycerol.

Assay of carotenoids. The carotenoids were assayed by their absorption at 430 mμ. This was the wavelength at the best-defined maximum in the visible region of the spectrum of the carotenoids. The acid treatment of the medium that is necessary for the preservation of lysyl-phosphatidyl glycerol caused isomerization of the carotenoids. Extraction of the staphylococci with neutral solvents in the dark and in the absence of oxygen liberated slightly more total carotenoids. Nine carotenoids can be separated on both paraffin-impregnated thin-layer plates (39) and alumina-impregnated paper (20). Their characterization will be presented elsewhere. After the acid treatment, 10 colored materials were found in the carotenoid fraction when assayed by TLC on paraffin-impregnated plates.

Extraction of lipids from staphylococcus. Various chloroform-methanol (2:1) extraction procedures were tried. The extraction procedure chosen was the one which liberates the lipid with the highest acyl ester-phosphorus ratios, the highest content of lipid glucose and vitamin K_2, and the maximal proportions of lysyl-phosphatidyl glycerol and cardiolipin.

This extraction procedure consisted of making the growth medium acid with HCl to prevent enzymatic hydrolysis of lysyl-phosphatidyl glycerol. The acidification of the medium prior to harvesting was found necessary by Macfarlane (29). When growing staphylococci were centrifuged and suspended in phosphate buffer, the lysyl-phosphatidyl glycerol was hydrolyzed, and the phosphatidyl glycerol concentration increased progressively during incubation at 37 C. Addition of acid to the medium was shown to prevent the increase in phosphatidyl glycerol at the expense of lysyl-phosphatidyl glycerol. The bacteria were then centrifuged, washed in buffer, centrifuged again, and then suspended in isopropanol and refluxed. The refluxing in isopropanol perhaps inactivates lipases and increases the yield of glucose in the lipid. The extraction was then completed with two portions of chloroform-methanol (2:1) Colorimetric analysis of the fatty acids released after saponification indicates that 92.6% of the total fatty acids were liberated after refluxing and the first chloroform-methanol extraction. An additional 5% of the total bacterial fatty acid was extracted in the second chloroform-methanol treatment. Only 1.4% of the total bacterial fatty acid was extracted from the residue of the first two chloroform-methanol extractions by further extraction with 30 ml of chloroform-methanol containing 0.8 ml of concentrated HCl. The nonlipid residue contained 2% of the total bacterial fatty acids.

TABLE 6. *Extraction of lipids from Staphylococcus aureus*[a]

Method	Lipid P	LPG[b]	PG	C	Lipid Glucose	MGD	DGD	Vitamin K_2
A	56.7[c]	7.95	31.9	15.99	11.9	2.31	9.60	2.20
B	56.4	9.15	38.6	8.75	11.5	1.75	9.70	0.90
C	57.3	11.00	36.6	8.75	11.1	3.08	7.02	0.88
D	68.1	18.4	29.4	20.4	17.8	5.40	12.40	2.40
E	56.3	14.2	22.5	19.4	17.8	5.40	12.40	2.70

[a] A staphylococcal culture was harvested in log phase of growth and was divided into five equal portions of 250 mg (dry weight). Extraction methods were: A, bacteria were harvested by centrifugation at 4 C, washed in 30 ml of 0.05 M phosphate buffer (*p*H 7.6), centrifuged, and resuspended in 30 ml of phosphate buffer. The suspension was added to a separatory funnel, 75 ml of methanol was added and mixed, and 37.5 ml of chloroform was added and mixed. After at least 2 hr, 37.5 ml of water and 37.5 ml of chloroform were added and mixed. The resulting lower phase was filtered through paper, partitioned against 0.7 M NaCl (10) two times, and analyzed as described in Materials and Methods. B, bacteria were treated as in A, except that the washed bacteria were suspended in 50 ml of isopropanol with exposure to ultrasonic vibration and then 25 ml of methanol were added. C, the extraction procedure of B was modified by heating the isopropanol suspension in a boiling water bath for 5 min. D, the extraction procedure of C was modified by making the culture media pH 2.0 with HCl before harvesting. E, the bacteria were harvested from acidified media, washed, resuspended, and boiled in isopropanol as in D. Then, 50 ml of methanol and 200 ml of chloroform were added, and the suspension was stirred with a Teflon-covered magnetic stirrer for 2 hr at 25 C. The mixture was centrifuged and re-extracted. The combined organic phases were filtered through glass wool and treated as described above. Recovery of the glycolipids and phospholipids from the thin-layer chromatography plates was 100.9 ± 4% and 97.0 ± 2%.

[b] Abbreviations used are: LPG, lysyl-phosphatidyl glycerol; PG, phosphatidyl glycerol; C, cardiolipin; MGD, monoglucosyldiglyceride; and DGD, diglucosyl diglyceride.

[c] Results are given in micromoles per gram (dry weight).

The most effective chloroform-methanol extraction of the bacterial pellet was compared to modifications of the more convenient Bligh and Dyer procedure (4). This is shown in Table 6. Again, with this extraction, the acidification of the medium and heating of the bacterial suspension in isopropanol were necessary for the maximal recovery of LPG, cardiolipin, and glucolipid. The acid treatment also increased the recovery of both the mono- and diglucosyl diglycerides. Apparently, the brief acid treatment did not cause significant hydrolysis of the glycosidic bond. The modified Bligh and Dyer (4) procedure was slightly more effective in extracting phospholipids than the chloroform-methanol extraction of the bacterial pellet.

Cellular localization of the lipid in staphylococcus. If the bacteria are ruptured in a pressure cell, a membrane fraction can be separated from the broken cell preparation by centrifugation. The ability to utilize oxygen in the presence of substrate is found exclusively in the membrane fraction. The cytochromes and the cytochrome oxidases that can be reduced with substrate are part of this membrane. The data in Table 7 indicate that essentially all the phospholipid, glucolipid, vitamin K_2, and carotenoids are part of the membrane complex. Fumarase in these bacteria seems to be a part of the membrane. Disintegration of the cells was effective, as nearly all the 6-phosphogluconate dehydrogenase, glucose-6-phosphate dehydrogenase, and glyceraldehyde-3-phosphate dehydrogenase activity of the broken cells was found in the supernatant portion after centrifugation, and none was found in the membrane fragments.

DISCUSSION

Quantitative extraction and assay of the lipids from *S. aureus* requires two precautions: (i) that the hydrolysis of lysyl-phosphatidyl glycerol be depressed by prompt acidification to *p*H 2.0 of the growth medium just prior to harvest, and (ii) that the lipid extract be refluxed in the absence

TABLE 7. *Cellular localization of the lipids and electron transport system in Staphylococcus aureus*[a]

Component[b]	Broken cells	Supernatant fraction	Pellet	Recovered[c]
				%
Lipid phosphate (1)	1.75	0.005	1.70	96
Lipid glucose (2)	0.46	0.002	0.45	101
Vitamin K_2 (3)	68.0	0.001	69	108
Carotenoid (4)	0.015	<0.005	0.015	100
Cytochrome *a* (5)	0.007	<0.0005	0.0068	95
Cytochrome *o* (6)	0.130	<0.001	0.140	104
Cytochrome b_1 (7)	0.015	<0.001	0.014	93
6-Phosphogluconate dehydrogenase (8)	158	129	<0.002	82
Glucose-6-phosphate dehydrogenase (8)	338	290	<0.002	86
Glyceraldehyde-3-phosphate dehydrogenase (9)	77	80.1	<0.002	104
Fumarase (10)	6.1	<0.01	6.0	99
Protein (11)	15.5	0.36	14.3	94

[a] Bacteria were grown in semisynthetic medium for 24 hr, harvested, and ruptured in a Ribi-Servall refrigerated pressure cell at 20,000 psi and 0 C. The broken cell preparation was centrifuged at 103,000 × *g* for 30 min. The supernatant fraction was decanted, and the pellet was resuspended in 0.05 M phosphate buffer, *p*H 7.0. Lipid extractions were performed as described in Table 6 and were freed of nonlipid contaminants with Sephadex (53). Cytochromes were estimated from the difference spectrum of a bacterial suspension with the respiratory pigments reduced (in the presence of 0.02 M L-lactate) and compared to a bacterial suspension with the respiratory pigments oxidized. Enzyme was measured as described in Materials and Methods.

[b] Results are expressed as (1) μmoles of phosphate; (2) μmoles of glucose; (3) mμmoles of vitamin K_2 [40]; (4) absorbancy at 430 mμ; (5) absorbancy increment between the maximum at 602 mμ and a line connecting 580 mμ and 620 mμ in the oxidized versus reduced difference spectrum; (6) absorbancy increment between the maximum at 412 mμ and the minimum at 433 mμ in the reduced and saturated with carbon monoxide versus reduced difference spectrum; (7) absorbancy increment between the maximum at 557 mμ and a line connecting 540 mμ and 580 mμ in the reduced versus oxidized difference spectrum; (8) reduced nicotinamide adenine dinucleotide phosphate appearance at 25 C in mμmoles per minute per milliliter; (9) reduced nicotinamide adenine dinucleotide appearance at 25 C in mμmoles per minute per milliliter; (10) decrease in absorbance at 300 mμ per minute per milliliter; (11) protein measured by the method of Lowry (28) with serum albumin as a standard.

[c] Per cent recovery indicates the activity in the broken cell preparation compared to the sum of activities in the resuspended pellet and the supernatant portion.

of water in isopropanol before the addition of chloroform. The refluxing is necessary to extract the maximal levels of mono- and diglucosyl diglycerides. The extraction of the bacteria with the one-phase chloroform-methanol-water system yields slightly higher values of phospholipids than the extraction of the bacterial centrifuge pellets with chloroform-methanol. The extraction procedure removes 98% of the total fatty acids in the bacteria.

The glucolipid contains glucose as the only carbohydrate, confirming the work of Polonovski et al. (37). Monoglucosyl diglyceride was also detected in *S. aureus*. The possibility that the acid treatment of the growth medium could lead to the formation of all of this lipid by hydrolysis of diglucosyl diglyceride is unlikely, since the monoglucosyl diglyceride is found in lipids extracted from bacteria at neutral *p*H.

Three phospholipids which correspond to 68 μmoles of lipid P per gram (dry weight) have been identified and characterized as lysyl-phosphatidyl glycerol, phosphatidyl glycerol, and cardiolipin. This confirms the earlier work (29, 30). These three lipids account for 98% of the lipid phosphate.

Vitamin K_2 has been detected in aerobically grown *S. albus* (3). This study established that vitamin K_2 is found in *S. aureus*. Three major isoprenologues of 35, 40, and 45 carbon side chains have been detected. Several more naphthoquinones of shorter side chains that make up less than 8% of the total quinone have also been detected (12). The finding of isoprenologues of vitamin K_2 in bacteria is not unique, since *Haemophilus parainfluenzae* contains isoprenologues of 2-demethyl vitamin K_2 of 25, 30, and 35 carbon side chains (27). In both these bacteria, the middle isoprenologue predominates (12, 27).

These lipids are part of the same membrane system that contains the cytochromes and cytochrome oxidase. The correlation between lipid metabolism and the formation of the electron transport system is given in the following paper (12).

Lysyl-phosphatidyl glycerol is a most interesting lipid, since it has a rapid turnover (13) and is formed from lysyl transfer ribonucleic acid (24). Houtsmuller and van Deenen (16, 17) show an increase in the relative proportion and absolute amount of lysyl-phosphatidyl glycerol when the bacteria are incubated at acid *p*H. Under these conditions, the total phospholipid is reduced to half the level found in *S. aureus* grown at neutral *p*H. These authors postulate a compensatory mechanism involving lysyl-phosphatidyl glycerol for growth at acid *p*H. However, in both this study and the studies of Macfarlane (29, 30), a significant proportion of the phospholipid is lysyl-phosphatidyl glycerol, even when the bacteria are grown at *p*H 7.0. This can be shown so long as the growth medium is promptly acidified to *p*H 2.0 before centrifugation.

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