# THE SYNTHESIS OF LIPOTEICHOIC ACID CARRIER[1]

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Summary: Staphylococcus aureus strain H was pulse-labeled with [2-$^3$H]-glycerol and then incubated in medium containing unlabeled glycerol. During the chase period radioactivity is transferred from phosphatidylglycerol to form the polyglycerolphosphate chain of the lipoteichoic acid carrier.

Most gram positive organisms contain a cell wall teichoic acid, which is a derivative of polyglycerolphosphate or polyribitol phosphate (1). In addition, these cells contain a lipoteichoic acid in the cell membrane (2) which functions as a carrier for the synthesis of the cell wall polymers and has been designated as lipoteichoic acid carrier or LTC[2] (3-5). In Staphylococcus aureus H LTC is a polyglycerolphosphate 12 units long, which at its hydrophobic end contains glucose and fatty acids (4,6).

The mechanism of biosynthesis of lipoteichoic acids is unknown. Our laboratory has been unable to demonstrate the synthesis of lipoteichoic acids from CDPglycerol in a variety of microorganisms. Recently Kennedy and coworkers (7,8) have shown that in E. coli phosphatidylglycerol is the precursor of glycerolphosphate units linked to a polyglucose backbone. In analogy with this interesting observation, evidence is presented that in S. aureus H phosphatidylglycerol is a precursor of LTC. After completion of this work, a report by Emdur and Chiu reported similar findings in S. sanguis (9).

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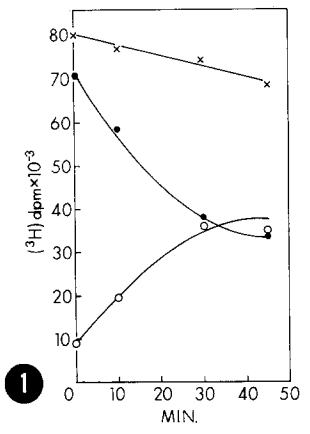
[2]The abbreviations used are LTC = lipoteichoic acid carrier.

MATERIALS AND METHODS

S. aureus H was grown at 37° in 50 ml of Difco antibiotic medium 3 to $OD_{600}$ = 0.4, 25 μC of [2-$^{3}$H]glycerol (New England Nuclear) were added to the culture. After incubation for 2 minutes at 37°C, the cells were collected by filtration on a 4 cm diameter 0.45 μm Millipore filter and washed with antibiotic medium 3 at 37° containing 4 mM glycerol. The washed cells were suspended in 50 ml of antibiotic medium 3 containing 4 mM glycerol at 37°. At the times indicated in the experiments, 10 ml of the culture were filtered on a 2.5 cm 0.45 μ Millipore filter and washed with 0.05 M Tris-HCl, pH 8.0, 1 mM glycerol. The cells were suspended in 1 ml of 37 mM Tris-HCl, pH 8.0, 5 mM spermidine, 1 mM 2-mercaptoethanol containing 0.5 mg of Lysostaphin (Schwartz-Mann) and were incubated at 15° for 5 min, and lyophilized. The dry powder was extracted successively with 4 and 1 ml of $CHCl_3$-methanol (2/1). The residue was dried under a stream of nitrogen and extracted with 1 ml of 37 mM Tris-HCl, pH 8.0, 5 mM spermidine, 1 mM 2-mercaptoethanol, 0.1% Triton X100.

In some experiments cells were grown in 1% Peptone with identical results. In these experiments cells were labeled at $OD_{600\ nm}$ = 0.3 in order to keep cells in logarithmic growth throughout the experiment.

Lipids were separated by thin-layer chromatography on Silica Gel G (Analtec) plates activated in acetone using $CHCl_3$-methanol-$H_2O$ (65/25/4) as the solvent. For elution 1 cm areas were scrapped off the plates and extracted with 2 ml of the same solvent for 1/2 hour at 25°. Aliquots of the supernatant fluid were evaporated under nitrogen in counting vials and the radioactivity determined using 3a70 (Research Products International) as the counting fluid. The recovery was 80-90%. SDS gel electrophoresis of lipoteichoic acid carrier was carried out by the method of Laemmli (10,4) using 20% gels. Polyribitolphosphate polymerase was purified from S. aureus H as described (4,5).



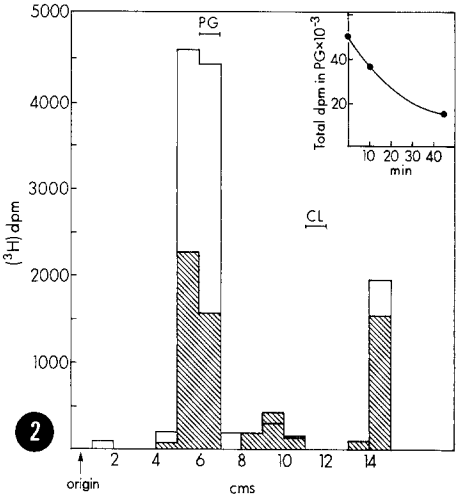



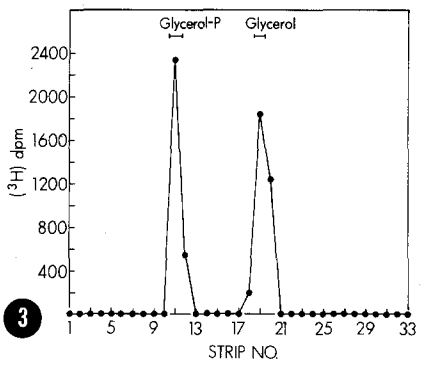

Fig. 1. Pulse labeling of staphylococcus aureus H. Cells were labeled in 1% Peptone with [2-$^{3}$H]glycerol as described under Methods. ● = Radioactivity extracted by chloroform-methanol. O = Radioactivity extracted by 0.1% Triton. X = Total radioactivity. The residue after Triton extraction contained negligible radioactivity.

Fig. 2. Chromatography of the lipid extract. An aliquot of the chloroform-methanol extracted material (Fig. 1) was chromatographed on Silica Gel G. Open bars = sample after 10 min chase. Cross hatched bars = samples after 45 min chase. The insert shows a plot of the total loss of radioactivity from phosphatidylglycerol with time. PG = standard phosphatidylglycerol. CL = standard cardiolipin.

## RESULTS AND DISCUSSION

In Fig. 1 we show that in a pulse chase experiment radioactivity from [2-$^{3}$H]glycerol is transferred from material that can be extracted by chloroform-methanol (2/1) into material which is not extracted by solvent but can be extracted in buffer containing 0.1% Triton-X100. The total radioactivity in the cells remains essentially constant.

The loss of radioactivity from the lipid phase represents primarily the loss of material with the chromatographic mobility of phosphatidylglycerol (Fig. 2). The radioactive component with the mobility of phosphatidylglycerol was eluted from the thin-layer plates. After saponification in 0.1 N NaOH in methanol for 10 min at 37°, it gave rise to a compound with the chromatographic behavior of glycerolphosphorylglycerol (11), and after acid hydrolysis in 1 N HCl in 50% methanol, gave rise to



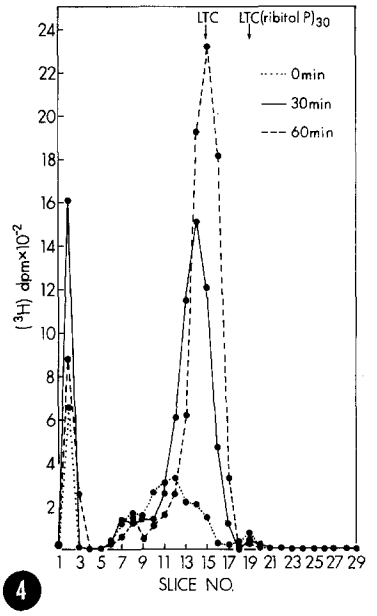




Fig. 3. Acid hydrolyses of phosphatidylglycerol. Phosphatidylglycerol eluted from silica Gel G plate was hydrolyzed in 1 N HCl in 50% methanol for 3 hours at 100° and chromatographed in isobutyric acid/$NH_4$/$H_2O$ (66/1/33) on Whatman 3 MM paper. Standards of glycerol P and glycerol are indicated by the bars. Radioactivity was determined in 1.5 cm strips of paper, which were incubated in counting vials in 1 ml of 0.2 N HCl for 30 min before counting. Strip No. 1 is the origin.

Fig. 4. Acrylamide gel electrophoresis of Triton extract. The samples are from an experiment in which cells were grown in antibiotic medium 3 and chased for the times indicated. Equal aliquots of the Triton extract were analyzed by disc gel electrophoresis. The arrows indicate the position of standard of LTC and LTC-(ribitol P)$_{30}$ electrophoresed on parallel gels. Slices were 2 mm thick and slice 1 is in the position of the tracking dye.

equal quantities of glycerol and glycerolphosphate (Fig. 3). Dephosphorylation of the phosphatidylglycerol with 60% HF (12) gave rise to diglyceride and glycerol. The labeling was somewhat assymetric and 58% of the radioactivity was found in glycerol and the remainder in the diglyceride.

The Triton extract was examined by polyacrylamide disc gel electrophoresis (Fig. 4). During the chase period there is a time dependent increase in the radioactivity of a compound with the electrophoretic mobility of LTC and a small and variable increase in radioactivity in material which moves with the tracking dye. Dephosphorylation of the

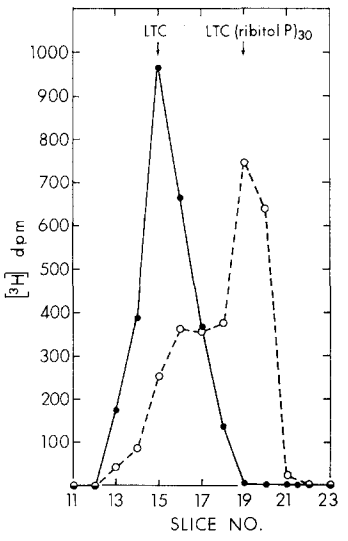



Fig. 5. Conversion of LTC to LTC-polyribitolphosphate. 50 µl (4,000 dpm) of Triton extract from cells labeled in Peptone and chased for 60 minutes were incubated with 0.1 µmole of CDP ribitol, 25 nmoles of cardiolipin and 1 unit of polyribitolphosphate polymerase for 16 hours at 25° and analyzed by disc gel electrophoresis as in Fig. 4. ● = Original sample. O = sample incubated with CDP ribitol and enzyme.

Triton extracted material obtained after 60 minutes chase (Fig. 4) with 60% HF yielded 80% of the radioactivity as free glycerol as expected for the structure of LTC. Glycerol was identified by paper chromatography using butanol/pyridine/$H_2O$ (6/4/3) as the solvent.

Incubation of the Triton extract with CDP ribitol and polyribitol phosphate polymerase converted 50% of the radioactivity to a compound with the electrophoretic mobility of LTC-(ribitol P)$_{30}$ (Fig. 5). The inability to convert all of the presumptive LTC to LTC polyribitol-phosphate may indicate the presence of a second radioactive component in the Triton extract. It should be noted, however, that polyribitol-phosphate polymerase is unable to reinitiate polymerization using LTC which contains only a few ribitol phosphate units (L. Glaser, unpublished observations) and it is likely that such partially loaded LTC chains would be present in a total membrane extract.

The data presented show that phosphatidylglycerol is a precursor

of at least a portion of the polyglycerolphosphate chain of LTC and not only a precursor of the hydrophobic end of the molecule. It is, of course, not proven but appears likely that this is a direct transfer. LTC represents only 5-6% of the total glycerolphosphate in the membrane and simple turnover of phosphatidylglycerol and random resynthesis would not give rise to the observed transfer of radioactivity from phosphatidylglycerol to LTC. The quantitative aspects of this reaction are of some interest. Thus in the experiment shown in Fig. 2 between 10 and 45 minutes chase there was a loss of 20,000 dpm [$^3$H] from phosphatidyl glycerol, while simultaneously by disc gel electrophoresis there was a gain of 14,000 dpm in LTC. Assuming recovery in all samples to be similar, and since 60% of the radioactivity in phosphatidylglycerol is in the glycerol moiety, these data would be consistent with the assumption that only the glycerolphosphate moiety of phosphatidylglycerol is used in the synthesis of LTC. Clearly enzymatic data in cell free systems will be required to establish the mechanism of this reaction. It is of interest that both in E. coli (7) and in S. aureus phosphatidylglycerol acts as a precursor of polymeric glycerolphosphate although these polymers have very different chemical structures.

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