

The Incorporation of Glycerol and Lysine into the Lipid Fraction of *Staphylococcus aureus*

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1. Incubation of washed cells of *Staphylococcus aureus* with [1-¹⁴C]glycerol results in the incorporation of glycerol into the lipid fraction of the cells. The rate of incorporation is increased by the presence of glucose and amino acids. The presence of amino acids increases incorporation into the fraction containing *O*-amino acid esters of phosphatidylglycerol. 2. Glycerol, incorporated into washed cells by incubation with glycerol, glucose and amino acids, is rapidly released from the lipid fraction when cells are incubated at low suspension densities in buffer. 3. Of nine amino acids tested, only lysine is significantly incorporated into the lipid fraction. The incorporation is increased by the presence of glycerol, glucose and other amino acids, especially aspartate and glutamate. 4. The incorporation of lysine is increased by the addition of puromycin at concentrations that inhibit protein synthesis. Chloramphenicol does not increase the incorporation of lysine but abolishes the enhancing effect of puromycin. 5. The enhancing effect of puromycin is accompanied by a similar increase in the incorporation of lysine into the fraction soluble in hot trichloroacetic acid. 6. Lysine is incorporated into the lipid fraction that contains *O*-amino acid esters of phosphatidylglycerol and corresponds in properties to phosphatidylglyceryl-lysine. 7. Lysine is rapidly released from the lipid of cells incubated in buffer only at low suspension densities. 8. Incubation of cells with the phosphatidylglyceryl-lysine fraction does not lead to the appearance of free lysine or to incorporation into the fraction insoluble in hot trichloroacetic acid.

Macfarlane (1962*a*) has shown that the lipid fraction of a number of species of bacteria contains, in addition to neutral lipid, phospholipids of which, in Gram-positive organisms, di- and mono-phosphatidylglycerol are the main components. Later studies (Macfarlane, 1962*b*, 1964) showed that in *Clostridium welchii* and *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*) a high proportion of the phosphatidylglycerol exists in the form of *O*-amino acid esters. Although it was first thought that a range of amino acids was present in this lipid form, later work has shown the presence of only three amino acids: lysine, ornithine or alanine being found in specific cases (Macfarlane, 1964; Houtsmuller & Van Deenen, 1963). Houtsmuller & Van Deenen (1964) have found that the amount of phosphatidylglyceryl-lysine formed in *S. aureus* and *Bacillus cereus* increases as the pH falls during growth. A number of workers have reported the presence of bound amino acids in the lipid fraction of bacterial and mammalian tissues and have suggested that the lipid-amino acid complex may

be involved in protein synthesis (e.g. Hendler, 1959, 1962; Hunter & Goodsall, 1960), and Macfarlane (1962*b*) suggests that the *O*-amino acid esters of phosphatidylglycerol have properties consistent with those described for such complexes. Hunter & James (1963) have produced evidence that the lipid-amino acid complexes of *Bacillus megaterium* include phosphatidylglycerol esters.

Gale & Folkes (1958*a,b*) described an Incorporation Factor that markedly increased the incorporation of amino acids by preparations of disrupted staphylococcal cells. Kuehl, Demain & Rickes (1960) found that the main component of an Incorporation-Factor preparation was glycerol. Gale & Folkes (1962) found that glycerol was unable to replace the Incorporation Factor in either disrupted staphylococcal cell preparations or pre-incubated suspensions of *Escherichia coli*; in both cases, however, incubation of cell preparations with glycerol led to the formation of active material. A survey has therefore been undertaken of the glycerol-containing substances in bacterial cells,

and the present paper deals with the nature and metabolism of such substances in the lipid fraction of *S. aureus* Duncan.

METHODS

Organism. The organism used was *S. aureus* Duncan, as used for studies on the Incorporation Factor by Gale & Folkes (1958*a,b*). The lipid fraction of this strain has been investigated by Macfarlane (1962*b*). The organism was grown in a liquid medium containing salts, glucose, arginine and Marmite (Gale, 1951) dispensed in 150 ml. quantities in Roux bottles that were incubated on their sides for 15 hr. at 25°. Organisms were harvested by centrifugation, washed once and suspended in distilled water. The suspension density was determined turbidimetrically on a Hilger spectrophotometer previously calibrated in terms of dry weight of suspension against turbidity. Suspensions were initially adjusted to 2.0 mg. dry wt./ml. To decrease the amino acid 'pool' in some experiments, cells were incubated for 30 min. at 37° and a suspension density of 0.3 mg./ml. in buffered saline and harvested on the centrifuge before use in experimental systems.

Incubation conditions. Cell suspensions, at a final density of 0.2 mg. dry wt./ml., were incubated at 37° in 15 ml. centrifuge tubes containing, in a total volume of 5.0 ml.: 2.5 ml. of buffered saline, pH 6.5 (22 mM-KH₂PO₄-70 mM-Na₂HPO₄-8.5 mM-MgSO₄-50 mM-NaCl), glycerol (0.1 mM), glucose (3 mM) and, where indicated, 0.1 ml. of the amino acid mixture A consisting of 18 amino acids each at a concentration of 4 mg. of L-isomer/ml. (Gale & Folkes, 1955). In experiments where ¹⁴C-labelled amino acids were added, the corresponding unlabelled amino acids were omitted from the mixture. Experiments were stopped as required by the addition of 1.0 ml. of 5% (v/v) acetic acid to each tube. Where larger amounts of material were required for fractionation purposes, experiments were scaled up 12- or 24-fold and incubation was carried out in 100 ml. centrifuge tubes.

Radioactive substrates. The ¹⁴C-labelled glycerol and amino acids were obtained from The Radiochemical Centre, Amersham, Bucks. [¹⁴C]Glycerol was used at specific activity 1.57 mc/m-mole; amino acids were L-isomers uniformly labelled with ¹⁴C at specific activities ranging from 6 to 209 mc/m-mole. Radioactivity was estimated with a conventional end-window Geiger-Müller tube and scaler giving a sensitivity of 2 × 10⁵ counts/min./μc. Samples were prepared for counting as described by Gale & Folkes (1962).

Fractionation of cells. After acidification, cells were centrifuged down and lipid was extracted from the pellet as described by Macfarlane (1962*b*). Pellets containing 1 mg. dry wt. of cells were suspended in 1.0 ml. of methanol and incubated at 37° for 15 min.; the cells were centrifuged down and the methanol supernatant was collected quantitatively. The pellet was resuspended in 1.0 ml. of chloroform, incubated for a further 15 min. at 37° and again centrifuged down. The chloroform and methanol extracts were combined and washed twice with 2 ml. of 0.3% NaCl; the chloroform layer was separated, measured and sampled for counting.

After lipid extraction, the cell pellets were further separated into fractions soluble and insoluble in hot 5%

(w/v) trichloroacetic acid according to the procedure of Roberts, Abelson, Cowie, Bolton & Britten (1955); samples were freed from trichloroacetic acid by washing three times with ether before drying for counting. In certain experiments, radioactivity in the fraction soluble in cold 5% trichloroacetic acid was estimated on replicate cell samples before lipid extraction.

Chromatography and radioautography. Column chromatography of lipid samples were carried out on silicic acid as described by Macfarlane (1962*a,b*). Approx. 1.5 g. of silicic acid (Mallinckrodt) was stirred to form a slurry in light petroleum (b.p. 40-60°), poured into a tube and allowed to settle to form a column approx. 5 cm. × 0.8 cm. Lipid samples, dissolved in light petroleum, were applied to the column and washed on with petroleum. The column was then eluted successively with chloroform containing 0, 6, 15, 40 and 80% (v/v) of methanol. Eluates (2.0 ml.) were collected, the radioactivity was determined and elution was continued with each solvent until no further radioactivity appeared in the eluate. For further examination, eluates within each solvent fraction were pooled. Fractions obtained by elution with 100% chloroform are referred to below as fraction A, with 6% methanol in chloroform as fraction B, with 15% methanol in chloroform as fraction C, with 40% methanol in chloroform as fraction D, and with 80% methanol in chloroform as fraction E.

Paper chromatography of lipid samples was carried out on Whatman no. 1 paper impregnated with silicic acid (Marinetti, Erbland & Kochen, 1957) and developed with di-isobutyl ketone-acetic acid-water (40:20:3, by vol.) (Macfarlane, 1962*b*). Water-soluble preparations were developed either in descending chromatograms with ether-ethanol-water-aq. ammonia (sp.gr. 0.88) (50:40:10:1, by vol.) as solvent, or in a two-dimensional system with, in one direction, butan-2-ol-formic acid-water (7:1:2, by vol.) and, in the second direction, phenol-aq. ammonia (sp.gr. 0.88)-water (800:3:200, by vol.) (Roberts *et al.* 1955).

After being dried, chromatograms were exposed for 7-10 days to Ilford Industrial X-ray film and the distribution of radioactivity was determined by inspection of the radioautograph. Amino acids were located by spraying the papers with 0.1% ninhydrin in acetone and heating at 100° for 5 min.; glycerol was located by the periodate-Schiff spray of Baddiley, Buchanan, Handschumacher & Prescott (1956).

Treatment with 1-fluoro-2,4-dinitrobenzene. Lipid fractions were treated with fluorodinitrobenzene under the conditions described by Macfarlane (1962*b*). After treatment, the reaction mixture was placed on a silicic acid column and excess of reagent, together with 2,4-dinitrophenol, eluted with chloroform before column chromatography as described above.

RESULTS

Incubation of washed cells of *S. aureus* with [¹⁴C]glycerol resulted in incorporation of radioactivity into the cell pellet. Of the radioactivity precipitated by cold 5% trichloroacetic acid, approx. 25% was located in the lipid fraction, 55% in the fraction soluble in hot trichloroacetic acid and 20% in the fraction insoluble in hot trichloroacetic acid. Hydrolysis of the last fraction in 6 N-hydrochloric acid for 18 hr. at 105° followed

by two-dimensional radiochromatography showed that 90% of the radioactivity was located in a single spot, ninhydrin-positive, corresponding in position to that of alanine. No glycerol was identified in this fraction. The radioactivity in the fraction soluble in hot trichloroacetic acid was found to be distributed over a range of substances that are not discussed in the present paper, which deals only with the radioactivity incorporated into the lipid fraction.

Incorporation of glycerol into the lipid fraction

Conditions affecting the rate of incorporation. Fig. 1 shows progress curves for the incorporation of radioactivity into the lipid fraction of *S. aureus* incubated in the presence of [^{14}C]glycerol. In the presence of 3 mM-glucose the initial rate of incorporation of glycerol became maximal at 0.5 mM and half-maximal at approx. 0.05 mM. In most experiments quoted below, the initial concentration of glycerol was 0.1 mM. With concentrations less than 0.1 mM-glycerol, there was a loss of radioactivity from the lipid fraction after 30–60 min. incubation at 37°.

The rate of incorporation was increased by the presence of glucose and the amino acid mixture. Optimum incorporation in 60 min., under the conditions used, was obtained in the presence of an initial concentration of 3 mM-glucose and the amino

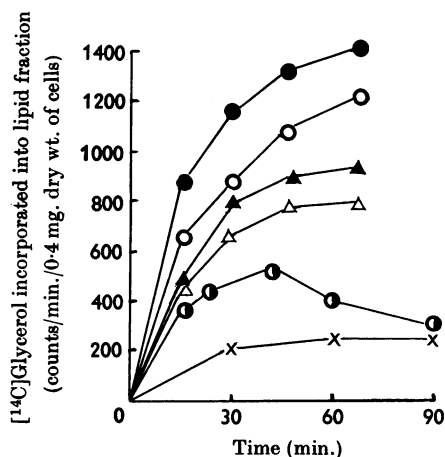


Fig. 1. Incorporation of radioactivity into the lipid fraction of *S. aureus* incubated with [^{14}C]glycerol. Washed cells were incubated at 37° in the presence of buffered saline, pH 6.5, and [^{14}C]glycerol (specific activity 1.57 mc/m-mole) (0.1 mM) at final suspension density 0.2 mg. dry wt./ml. with additions as follows: Δ , none; \blacktriangle , amino acid mixture (0.1 ml./5 ml.); \circ , glucose (3 mM); \bullet , glucose (3 mM) and amino acid mixture; \times , glucose (3 mM), amino acid mixture and 2,4-dinitrophenol (2 mM); \bullet , glycerol (0.01 mM), glucose (3 mM) and amino acid mixture.

acid mixture. The presence of 2 mM-2,4-dinitrophenol inhibited the incorporation by 90–95%. The addition of 30 μg . of chloramphenicol, penicillin, streptomycin, puromycin or chlortetracycline/ml. had little or no effect on the rate of incorporation; with chloramphenicol, puromycin and chlortetracycline an increase of 10–15% in the rate of incorporation was obtained in some experiments but this was not consistent. The rate of incorporation was not markedly sensitive to pH; a broad optimum was obtained between pH 7 and 8, the rates at pH 6 and 5 being 76 and 61% respectively of that at pH 7.

Nature of the radioactive material. A sample of lipid extracted from cells incubated with [^{14}C]glycerol in the presence of glucose and amino acids was evaporated to dryness *in vacuo* in a small tube and the resulting gum suspended in 0.3 ml. of 6 N-hydrochloric acid. The tube was sealed and heated at 105° for 48 hr. Excess of hydrochloric acid was removed in a stream of air, and the hydrolysate was taken up in 2 drops of water, evaporated to dryness, dissolved in about 30 μl . of water, spotted on paper and developed in the two-dimensional chromatographic system. Only one radioactive spot appeared, in the position corresponding to that of glycerol; spraying the paper with the periodate-Schiff reagent of Baddiley *et al.* (1956) showed that the position of the radioactivity corresponded exactly to that of glycerol. The radioactivity of lipid extracts obtained under these conditions therefore provides a measure of the incorporation of glycerol and, with the scaler equipment used, 1000 counts/min. correspond to 3.54 μmoles of glycerol. When compared with the results obtained for this organism by Macfarlane (1962a), the amount of glycerol incorporated/hr. into the lipid fraction, under the conditions of Fig. 1, corresponds to the formation of 20–25% of the total lipid of the cell.

Cells (48 mg. dry wt.) were incubated for 30 min. at 37° and a final suspension density of 0.3 mg./ml. in buffered saline diluted 1:2 with distilled water. The cells were harvested, divided into two equal portions and resuspended at a final suspension density of 0.2 mg. dry wt./ml. in buffered saline containing [^{14}C]glycerol (0.1 mM) and glucose (3 mM); 2.0 ml. of the amino acid mixture was added to one portion. After 60 min. incubation, the suspensions were acidified and the cells centrifuged down. The lipids were extracted, recovered and separated on a silicic acid column. The total radioactivity was greater in extracts from cells incubated in the presence of the amino acid mixture (see Fig. 1), and Table 1 shows that the greater part of the extra glycerol was present in fraction D of the eluted material. Some 1–2% of the radioactivity remained on the column after elution with chloroform containing 40% of methanol and this residual

Table 1. *Fractionation of lipid from Staphylococcus aureus*

Cells were pretreated by incubation at a final suspension density of 0.3 mg. dry wt./ml. in dilute buffer for 30 min. at 37°. Cells were then centrifuged down and samples taken for glycerol incorporation: for each sample 24 mg. dry wt. of cells was incubated at 0.2 mg. dry wt./ml. in buffered saline, [^{14}C]glycerol (specific activity 1.57 mc/m-mole) (0.1 mM), glucose (3 mM) and the amino acid mixture as below (each amino acid at a final concentration of 80 $\mu\text{g./ml.}$). After 60 min. at 37° cells were centrifuged down, the lipid was extracted and fractionated on silicic acid with the solvents shown.

Lipid fraction	Solvent	Nature of lipid*	Radioactivity recovered in fraction			
			With amino acids		Without amino acids	
			(counts/min.)	(% of total)	(counts/min.)	(% of total)
A	Chloroform	Neutral lipid	3616	10.5	5636	21.5
B	6% Methanol in chloroform	Phosphatidic acids, glycolipid and phosphatidylglycerols	5127	15	5481	21
C	15% Methanol in chloroform		10953	32	8246	31.5
D	40% Methanol in chloroform		14364	42	6240	24
		amino acid esters				

* Macfarlane (1962a,b) and this paper.

material was eluted by raising the methanol concentration to 80%.

Using similar fractionation procedures with the same organism, Macfarlane (1962a,b) found that fraction A consisted of neutral lipid and that fractions C and D contained phosphatidylglycerol and its amino acid esters; small amounts of glycolipid were present in fractions B and C. These findings were confirmed for the present experiments by showing that fractions B, C and D contained organic phosphorus and that material eluted in fraction D was ninhydrin-positive. The combined eluates from each fraction were evaporated to small volume and developed on silica-impregnated paper in di-isobutyl ketone-acetic acid-water. Radioautography showed that fractions C and D gave a well-developed spot (R_f 0.5) corresponding in position to that of phosphatidylglycerol. Fraction C gave an additional spot with R_f 0.3; on hydrolysis in 6 N-hydrochloric acid for 16 hr. at 105° it gave the same radioactive products as those obtained from phosphatidylglycerol, and it was probably diphosphatidylglycerol. Fraction D gave a slower-running spot (R_f 0.15-0.2), ninhydrin-positive and containing glycerol and organic phosphorus: this corresponded in behaviour to the *O*-amino acid esters of phosphatidylglycerol described by Macfarlane (1962b). Fraction D was dissolved in benzene and treated with fluorodinitrobenzene as described by Macfarlane (1962b). After incubation, the solution was run through the silicic acid column; excess of reagent and free dinitrophenol, but not radioactivity, were eluted with chloroform. The yellow colour and 54% of the radioactivity on the column were eluted by chloroform containing 10% (v/v) of methanol; the remaining radioactivity from the column required 40% methanol in chloro-

form for elution, as did the untreated fraction D. Radiochromatography of this last material showed that the phosphatidylglycerol component was still present, but the slower-running ninhydrin-positive component had been removed. On warming a chloroform solution of the DNP-lipid with 2% (w/v) sodium carbonate solution, at 60° for 3 min., the yellow colour was extracted into the aqueous phase; after acidification with hydrochloric acid and shaking, the yellow colour was re-extracted into the chloroform layer. Fractions C and D were separately dissolved in a mixture of equal volumes of chloroform and 0.1 N-potassium hydroxide in methanol and incubated at 37° for 60 min. This removed the amino acid residues from fraction D and the fatty acid residues from both fractions. The hydrolysates were evaporated to dryness and the residues were extracted with water and examined by radiochromatography in ether-ethanol-ammonia and in the two-dimensional system. Approx. 90% of the radioactivity of both fractions was located in a single component running in the position corresponding to that of glycerylphosphorylglycerol (Macfarlane, 1962b). Two minor radioactive components (R_f 0.42 and 0.65) were present.

Effect of the addition of single amino acids. The addition of the amino acid mixture to the incubation medium increased the incorporation of glycerol into fraction D of the lipid (Table 1). Table 2 shows the effect of adding lysine, aspartate or glutamate singly to the incubation medium. Lysine alone had relatively little effect on the total incorporation into lipid but resulted in some redistribution of glycerol, in favour of fraction D, between fractions C and D. The addition of either aspartate or glutamate increased the total incorporation and the proportion

Table 2. *Effect of amino acids on the distribution of glycerol in the lipid fraction of Staphylococcus aureus*

The conditions were as given for Table 1.

Amino acid added	Radioactivity of lipid (counts/min.)	Radioactivity recovered (%)			
		Fraction A	Fraction B	Fraction C	Fraction D
None	19700	22.5	21.5	32	24
Lysine	21300	22	21	26	31
Aspartate	24150	22	21	22	35
Glutamate	27500	18	21	28	33
Complete mixture	31750	14	12	31	43

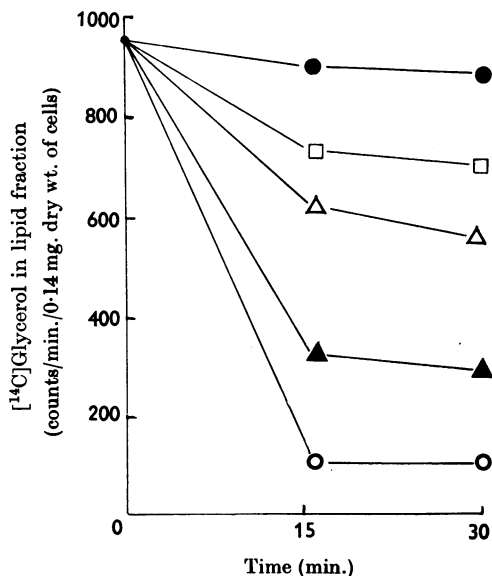


Fig. 2. Effect of suspension density on the release of [^{14}C]glycerol from the lipid fraction of *S. aureus*. Washed cells of *S. aureus* were incubated for 60 min. at 37° and final suspension density 0.2 mg. dry wt./ml. with [^{14}C]glycerol (0.1 mM), glucose (3 mM) and amino acid mixture. Cells were centrifuged down and resuspended in buffered saline at final suspension density of: ●, 1.0; □, 0.3; △, 0.1; ▲, 0.03; ○, 0.01 mg. dry wt./ml.

of radioactivity eluted in fraction D. The acidic amino acids thus had effects resembling, but not as great as, that of the amino acid mixture.

Release of radioactivity from lipid fraction. Cells labelled by incubation with [^{14}C]glycerol, glucose and the amino acid mixture were centrifuged down and resuspended in buffered saline either alone or containing unlabelled glycerol (0.3 mM), glucose (3 mM) and the amino acid mixture. Samples were taken at intervals, the cells were centrifuged down and the lipid was extracted. The radioactivity of the lipid fraction decreased during the second

incubation, and the rate of decrease was unaffected by the presence of unlabelled glycerol, with or without glucose and amino acids. The rate of release of radioactivity from cells suspended in buffer proved to be variable and, for cells suspended at 0.2 mg. dry wt./ml. in buffered saline, the release of radioactivity in 30 min. at 37° varied between 5 and 35% of that present at the start of the second incubation period. A major factor influencing the release proved to be suspension density: Fig. 2 shows results with a suspension in which rapid release was obtained. The loss of radioactivity, up to 90% of that initially present, was consistent when cells were suspended at a density of 0.03 mg. dry wt./ml. or less. The release was rapid, ceased after about 30 min. and was unaffected by the presence of unlabelled glycerol, glucose or amino acids. Parallel experiments in which [^{14}C]glycerol was present during the second incubation showed that incorporation of glycerol into the lipid fraction took place normally under these conditions so that, at low suspension densities, there was a rapid turnover of the lipid fraction. Fractionation of the lipid on silicic acid showed that the loss of radioactivity applied to all fractions. Only when some 90% of the radioactivity had been released did there appear to be any significant alteration of the distribution between fractions: at this stage 70% of the remaining radioactivity was located in fraction C.

Incorporation of lysine into the lipid fraction

Incorporation of radioactivity from amino acids. Cells were suspended at a final concentration of 0.2 mg. dry wt./ml. in glycerol (0.1 mM), glucose (3 mM) and a ^{14}C -labelled amino acid: (1) without further addition; (2) in the presence of the amino acid mixture (less the labelled amino acid); (3) with the amino acid mixture and 50 μg . of puromycin/ml.; (4) with the mixture and 30 μg . of chloramphenicol plus 30 μg . of penicillin/ml. The reaction was stopped after 30 or 60 min. and the lipid fraction extracted and counted. The amino acid giving the highest incorporation of radioactivity

Table 3. Incorporation of radioactivity from ^{14}C -labelled amino acids into the lipid fraction of *Staphylococcus aureus*

Washed cells of *S. aureus* were incubated for 30 min. at 0.2 mg. dry wt./ml. in buffered saline, pH 6.5, glycerol (0.1 mM), glucose (3 mM) and uniformly ^{14}C -labelled amino acid (0.02 mM) as below. In each case additions were made as follows: (1) none; (2) the amino acid mixture omitting the radioactive amino acid; (3) as (2) plus 50 μg . of puromycin/ml.; (4) as (2) plus 30 μg . of chloramphenicol and 30 μg . of penicillin/ml. For comparison purposes the radioactivity in the lipid fraction was calculated as $\text{m}\mu\text{-moles}$ of 'amino acid' incorporated/mg. dry wt. cells.

Amino acid	Specific activity (mc/m-mole)	'Amino acid' incorporated into lipid ($\text{m}\mu\text{moles/mg. dry wt. of cells}$)			
		(1)	(2)	(3)	(4)
Lysine	7.1	1.16	1.73	3.36	2.20
Aspartate	6.25	0.72	0.030	0.033	0.031
Glycine	4.52	0.62	0.025	0.027	0.050
Phenylalanine	22.0	0.41	0.060	0.065	0.062
Glutamate	8.6	0.13	0.026	0.029	0.021

into the lipid fraction was lysine (Table 3). The incorporation was increased by the presence of the amino acid mixture, and still further increased by puromycin, whereas the mixture of chloramphenicol and penicillin had little effect. Other amino acids tested gave rise to much less radioactivity in the lipid fraction and, in the examples shown in Table 3, this incorporation was decreased by the presence of the amino acid mixture whether antibiotics were present or not. In addition to the amino acids shown in Table 3, the incorporation of [^{14}C]-alanine, -histidine, -proline and -arginine was examined but incorporation of radioactivity was less than that obtained for [^{14}C]-glutamate.

Lipid fractions labelled by incubation of cells with [^{14}C]-lysine, -aspartate or -glutamate were extracted, hydrolysed for 48 hr. at 105° with 6 N-hydrochloric acid and the water-soluble products chromatographed in the two-dimensional system. Radioautography showed that [^{14}C]-lysine-labelled lipid (from cells incubated with or without other amino acids) gave rise to one radioactive spot, ninhydrin-positive, identical with that of lysine marker. The hydrolysate of lipid labelled with [^{14}C]-aspartate gave one major spot, ninhydrin-positive, corresponding to that of lysine, and two faint unidentified spots in the approximate positions of methionine and the leucines. Similarly the radioautogram of material obtained from lipid labelled from [^{14}C]-glutamate gave one major spot corresponding to that of lysine. In neither case could any significant darkening of the film be

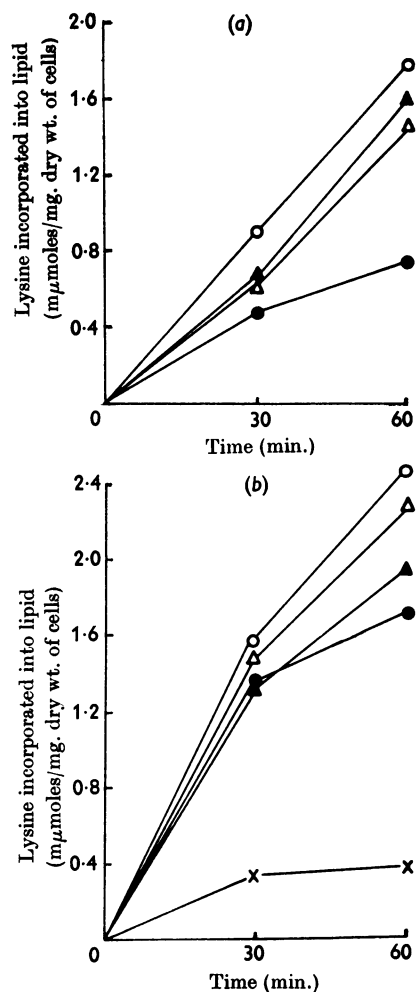


Fig. 3. Course of incorporation of [^{14}C]-lysine into the lipid fraction of *S. aureus*: (a) when lysine was the only amino acid present; (b) in the presence of the amino acid mixture. Washed cells were incubated at final suspension density 0.2 mg. dry wt./ml. in buffered saline, glycerol (0.1 mM), glucose (3 mM) and [^{14}C]-lysine (specific activity 7.1 mc/m-mole) (0.02 mM). ●, Lysine alone; ○, lysine and glycerol; Δ, lysine and glucose; ▲, lysine, glycerol and glucose; ×, lysine, glycerol, glucose and 2,4-dinitrophenol (2 mM).

observed in regions corresponding to aspartate or glutamate. The original preparations of labelled aspartate and glutamate were tested by two-dimensional chromatography for contaminants but no lysine contamination was detected.

Conditions affecting the rate of incorporation of [^{14}C]-lysine into the lipid fraction. Fig. 3 shows the course of incorporation of lysine into the lipid

fraction of *S. aureus*. Some incorporation took place when cells were incubated with lysine in buffer alone but the rate was markedly increased by the presence of 0.1 mM-glycerol or 3 mM-glucose. Glycerol alone increased the rate of incorporation to a greater extent than glucose alone or together with glycerol. The presence of the amino acid mixture approximately doubled the rate of incorporation in all cases. 2 mM-2,4-Dinitrophenol inhibited the incorporation by 85–90%. In most experiments an initial concentration of 0.02 mM-lysine has been used; the initial rate of incorporation (over 10–15 min.) was increased by 50% with 0.1 mM-glycerol but the subsequent course varied markedly with the experimental conditions, especially whether protein synthesis was taking place or not. If the initial concentration of lysine was 0.01 mM or less, incorporation into the lipid fraction ceased after 20–30 min. incubation in the presence of glucose and the amino acid mixture; continued incubation did not lead to any significant decrease in the radioactivity of the lipid fraction. Houtsmuller & Van Deenen (1964) have shown that the amount of phosphatidylglyceryl-lysine formed during growth of *B. cereus* or *S. aureus* increases as the pH of the medium falls. The rate of incorporation of lysine by washed cells in the presence of glycerol was found to vary little with pH over the range pH 7.8–5.0 and to have a broad optimum around pH 6.5.

Nature of the lipid-lysine fraction. The lipid obtained from cells incubated with [¹⁴C]lysine, glycerol and glucose was fractionated on silicic acid. All the radioactivity was eluted from the column in fraction D. The lipid in fraction D was recovered and examined by radiochromatography on silica-impregnated paper developed in di-isobutyl ketone-acetic acid-water. The radioactivity was located in one slow-running ninhydrin-positive spot corresponding to that of *O*-amino acid esters of phosphatidylglycerol. Mild alkaline hydrolysis liberated phosphatidylglycerol and free lysine and the DNP derivative behaved as expected. The fractionation procedure was repeated with lipid labelled with [¹⁴C]lysine in the presence of the amino acid mixture and puromycin: the radioactive material behaved in the same fashion.

Effect of other amino acids and puromycin. The increase in lysine incorporation brought about by the presence of other amino acids and puromycin might reflect changes in protein synthesis by the cells. However, this appears improbable since the presence of chloramphenicol and penicillin, which together would prevent incorporation of lysine into protein and cell-wall mucopeptide, are without significant effect. Expt. A in Table 4 shows the effect on lysine incorporation into lipid of adding aspartate and glutamate to the incubation medium.

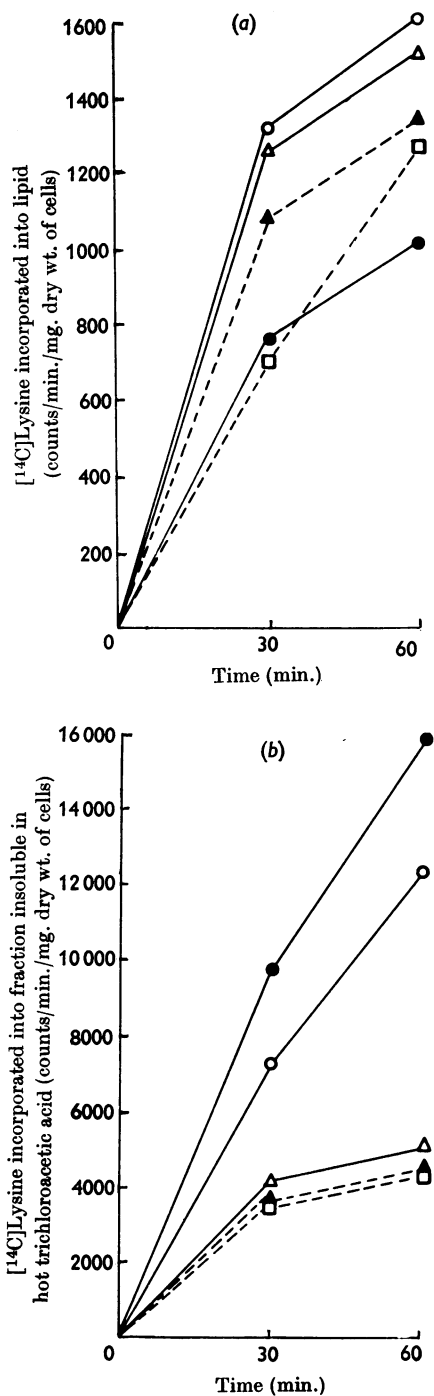


Fig. 4. Effect of puromycin on incorporation of [¹⁴C]lysine into *S. aureus* (a) lipid fraction and (b) fraction insoluble in hot trichloroacetic acid. The conditions were as given for Fig. 3(b). Concentration of puromycin: ●, 0; ○, 30; △, 100; ▲, 300; □, 1000 µg./ml.

Both of the acidic amino acids enhanced lysine incorporation to the extent shown by the amino acid mixture. Aspartate and glutamate together were no more effective than was glutamate alone. Amino acids that, added singly, were without effect on lysine incorporation included glycine, leucine, phenylalanine, arginine and proline.

Fig. 4 shows the effect of puromycin on the incorporation of lysine into lipid or the fraction insoluble in hot trichloroacetic acid (protein and mucopolypeptide): concentrations of 30–100 μg . of puromycin/ml., which strongly inhibited lysine incorporation into the fraction insoluble in hot trichloroacetic acid, resulted in a marked stimulation of incorporation into the lipid fraction. Table 4 shows that the effect of puromycin was not depend-

ent on the presence of the amino acid mixture but was also shown in the presence of the acidic amino acids. The rate of lysine incorporation into lipid was almost as great in the presence of glutamate and puromycin as when the amino acid mixture and puromycin were present. Nathans & Lipmann (1961) found that puromycin inhibited the transfer of leucine from leucyl-s-RNA to ribosomes in *E. coli* and brought about deacylation of the s-RNA. Ehrenstein & Lipmann (1961) reported that chloramphenicol interfered with the action of puromycin and concluded that chloramphenicol has a site of action coming before that of puromycin in the sequence of events leading to peptide bond formation on the ribosome. Expt. B in Table 4 shows that chloramphenicol abolished the effect of

Table 4. *Effect of amino acids and antibiotics on the incorporation of lysine into the lipid of Staphylococcus aureus*

Washed cells were incubated for 30 min. at 0.2 mg. dry wt./ml. in buffered saline, [^{14}C]lysine (specific activity 7.1 mc/m-mole) (0.02 mM), glycerol (0.1 mM), glucose (3 mM) and additions as shown. Amino acids were added at 80 μg ./ml.; antibiotics at 30 μg ./ml.

[^{14}C]Lysine incorporated (counts/min./mg. dry wt. of cells)

Expt.	Antibiotics added	Amino acid(s) added ...	[^{14}C]Lysine incorporated (counts/min./mg. dry wt. of cells)				
			None	Aspartate	Glutamate	Aspartate + glutamate	Complete mixture
Expt. A	None		648	1118	1340	1240	1222
	Puromycin		828	1502	1548	1501	1715
Expt. B	None		774	1052			1119
	Puromycin		822	1503			1791
	Chloramphenicol		685	1058			1097
	Puromycin + chloramphenicol		751	1160			1088

Table 5. *Incorporation of [^{14}C]lysine into Staphylococcus aureus*

Cells were pretreated by incubation at 0.3 mg. dry wt./ml. in buffered saline for 30 min. at 37°. Cells were harvested and resuspended at 0.2 mg. dry wt./ml. in buffered saline containing [^{14}C]lysine (specific activity 7.1 mc/m-mole) (0.02 mM), glycerol (0.1 mM), glucose (3 mM) and additions as below. The reaction was stopped after 20 min. at 37° by rapid cooling (for cold-trichloroacetic acid extract) or acidifying to pH 3 with 5% (v/v) acetic acid for other fractions. Replicate samples used in each case for determination of radioactivity in cold-5% (w/v)-trichloroacetic acid extract, or for lipid together with fractions soluble and insoluble in hot trichloroacetic acid.

Additions to incubation mixture	dry wt. of cells	Radioactivity										
		(counts/min./mg. dry wt. of cells in 1)					(as % of 1)					
		(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
Amino acid mixture	+	+	+	+	+	+	+	-	-	-	-
Puromycin (50 μg ./ml.)	-	+	-	-	-	+	+	-	+	-	-
Chloramphenicol (30 μg ./ml.)	-	-	+	-	+	-	+	-	-	-	-
Penicillin (30 μg ./ml.)	-	-	-	+	+	+	-	-	-	-	-
Aspartate (3 mM)	-	-	-	-	-	-	-	-	-	+	+
Fraction												
Cold-trichloroacetic acid extract	8500	100	107	81	112	120	110	82	590	536	446	461
Lipid	1100	100	178	103	95	92	181	102	72	98	111	140
Soluble in hot trichloroacetic acid	2500	100	150	93	110	117	166	92	258	341	236	225
Insoluble in hot trichloroacetic acid	19600	100	68	24	75	12	63	28	25	18	21	16

puromycin in stimulating the incorporation of lysine into the lipid of *S. aureus*; this effect of chloramphenicol was the same whether incorporation took place with lysine alone or in the presence of aspartate or the amino acid mixture. It appears that the action of puromycin on lysine incorporation into lipid was a secondary effect arising from the action of the antibiotic on the ribosomal system, an action prevented by chloramphenicol. A survey was therefore carried out of the incorporation of lysine into other cell fractions under conditions that modify incorporation into lipid. The results of a typical experiment are shown in Table 5. No clear correlation can be seen between the values for incorporation of lysine into the lipid fraction and for other fractions under the experimental conditions used. If, however, the experiments involving the amino acid mixture are considered, there is a correlation between incorporation into the lipid and the fractions soluble in hot trichloroacetic acid. The addition of puromycin is followed by similar increases in the lipid fraction and fraction soluble in hot trichloroacetic acid, but this correlation does not hold good for the effect of aspartate in the absence of other amino acids.

Release of lysine from the lipid fraction. Incubation of cells in buffer at suspension densities less than 0.1 mg./ml. resulted in rapid disappearance of lysine from the lipid fraction, as expected from the results with glycerol reported above. At higher suspension densities, release of lysine on incubation in buffer with or without glucose was slow and possibly insignificant in experiments lasting 60 min. or less. Under these conditions an accelerated decrease in lipid radioactivity was brought about,

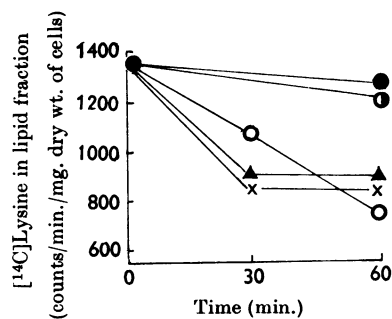


Fig. 5. Release of $[^{14}\text{C}]$ lysine from the lipid fraction of *S. aureus*. Cells were labelled by 60 min. incubation under the conditions given for Fig. 3(b) with lysine, glycerol and glucose. The cells were centrifuged down and resuspended at 0.2 mg. dry wt./ml. in buffered saline with additions as follows: ●, none; ○, glucose (3 mM); ▲, glucose and amino acid mixture (0.1 ml./ml.); ×, glucose and lysine (80 µg./ml.); ◐, glucose and amino acid mixture with lysine omitted.

as shown in Fig. 5, by a second incubation of labelled cells in the presence of glucose and unlabelled lysine (with or without the other amino acids of the mixture) or the amino acid mixture from which lysine had been omitted. The loss obtained on incubation with unlabelled lysine, with or without the amino acid mixture, ceased after 20–30 min.; parallel experiments with the amino acid mixture containing $[^{14}\text{C}]$ lysine showed that protein synthesis occurred under these conditions but that the rate at which lysine was released from the lipid fraction was less than 1% of that at which the amino acid was incorporated into protein. When the labelled cells were incubated with glucose and the amino acid mixture without lysine, lysine was liberated at a slow but constant rate from the lipid fraction. This rate was variable from experiment to experiment and was less than the initial rate at which radioactivity was lost when unlabelled lysine was present. Attempts to discover whether lysine lost from the lipid fraction was incorporated into the fraction insoluble in hot trichloroacetic acid showed that the extent of the labelling of this fraction during the first incubation (with $[^{14}\text{C}]$ lysine) was such that any possible gain (from lipid) in the second incubation was within the limits of experimental error. It was also found that cells tended to lose protein during the second incubation with the unbalanced amino acid mixture. The addition of puromycin did not prevent the loss of lysine from the lipid fraction under any of the conditions tested; in some experiments the rate of loss of radioactivity increased by 20–30% in the presence of puromycin.

The lipid fraction D, labelled with $[^{14}\text{C}]$ lysine, was dissolved in ethanol and added to suspensions of both intact and disrupted staphylococcal cells (Gale & Folkes, 1955) to test whether the lipid-lysine could give rise to free lysine in the soluble 'pool' of intact cells or act as a source of lysine for incorporation into the fraction insoluble in hot trichloroacetic acid of intact or disrupted cells. Tests were made with the lipid (at concentrations that did not inhibit the incorporation process) alone and with the addition of glucose, excess of unlabelled lysine and the amino acid mixture with and without lysine. In no case was any significant transfer of free lysine to the 'pool' obtained. In most experiments, incorporation of radioactivity into the fraction insoluble in hot trichloroacetic acid was negligible and in no case did such incorporation amount to more than 6% of the radioactivity supplied in the lipid fraction.

DISCUSSION

Macfarlane (1962b) has studied the nature of the lipid extract of the strain of *S. aureus* used in the present investigation. Though complete identifica-

tion of the fractions of lipid separated by silicic acid chromatography has not been undertaken in the present work, their chromatographic behaviour, solubilities, stabilities and the nature of their hydrolytic products indicate that the main components are the same as those described by Macfarlane (1962b). Thus the column fractions C and D contain phosphatidylglycerol, and fraction D also contains the *O*-amino acid esters. In agreement with Macfarlane (1964) and Houtsmuller & Van Deenen (1963) it has been found that the main amino acid associated with phosphatidylglycerol in this organism is lysine. The present experiments show that the phosphatidylglycerol compounds can be synthesized by washed non-growing cells in simple media and that the lipid fraction is in a dynamic state, especially when the cells are suspended at low suspension densities.

Attempts have been made in the past to associate the lipid-amino acid complex with protein synthesis. There appears to be no reason to believe that phosphatidylglyceryl-lysine, as synthesized under the experimental conditions used in the present work, has any important role as an intermediate in the incorporation of lysine into protein in *S. aureus*. No significant incorporation of lysine supplied in lipid form has been obtained with intact or disrupted cells; such a negative result could be attributed to failure of the lipid complex to reach the site of action but, even in intact cells incubated with a mixture of amino acids complete except for lysine, the loss of lysine from the lipid fraction occurs at a rate less than 1% of that of the normal incorporation of lysine into protein, and the presence of unlabelled lysine in the added mixture does not result in any 'chase' of labelled lysine from the lipid. The phosphatidylglycerol complex may be more important in bacterial than other cells (Macfarlane, 1964), and conclusions drawn from its behaviour in staphylococci do not necessarily apply to the lipid-amino acid complex of other tissues.

Lysine is one of the few amino acids that can enter the staphylococcus by diffusion rather than active transport through the membrane (Gale & Taylor, 1947), and the question arises whether phosphatidylglyceryl-lysine mediates this process. No free lysine was obtained in the 'pool' when cells were incubated with [¹⁴C]lysine-labelled lipid, and Fig. 5 shows that there is no sweeping out of the [¹⁴C]lysine from the lipid fraction when cells are incubated with excess of unlabelled lysine. The small loss of radioactivity that occurs is less than 1% of the lysine that would be transported through the membrane under these conditions. It again appears improbable that the lipid-lysine plays any intermediate role in lysine transport.

The increased incorporation of lysine into the lipid fraction when puromycin and the acidic amino

acids are added to the incubation medium raises interesting problems. Since chloramphenicol abolishes the effect of puromycin it seems probable that the effect of the latter on lipid incorporation is related to its action at the ribosomal level. Table 5 shows that the amount of lysine in the fraction soluble in hot trichloroacetic acid increases, in parallel with that in the lipid, in the presence of puromycin. It is possible that there is a lysine derivative in the fraction soluble in hot trichloroacetic acid that is involved in esterification of the phosphatidylglycerol, and that the amount of this derivative is increased when lysine incorporation into peptide bonds at the ribosome is inhibited by puromycin. The label in the fraction soluble in hot trichloroacetic acid is not rapidly diluted out when protein synthesis takes place under the conditions of Fig. 5. It is therefore improbable that it is the s-RNA fraction that is involved. Such an explanation cannot account for the enhancing action of the acidic amino acids. Table 2 shows that the presence of the acidic amino acids increases the proportion of glycerol derivatives eluted in fraction D, whereas lysine itself has comparatively little effect. Chromatography of fraction D shows that about half of the phosphatidylglycerol in that fraction is esterified with amino acids, and there must presumably be some difference in the structure of the unesterified material in fraction D from that in fraction C. An explanation of the effect of the acidic amino acids would be that they increase the synthesis of that form of phosphatidylglycerol that acts as acceptor for lysine. It has not so far been possible to demonstrate any labelling of the phosphatidylglycerol with aspartate or glutamate; it is always possible that these esters are too unstable to be isolated by the procedures at present adopted.

The present experiments have not revealed any role for phosphatidylglycerol and its amino acid esters. However, the conditions that result in a loss of the lipid fraction from the cell, namely incubation at low suspension densities, are those that result in a marked decrease in the rate of protein synthesis by certain bacterial cell preparations, a decrease that can be immediately reversed by the presence of Incorporation Factor or, after a lag period, by incubation with glycerol (Gale & Folkes, 1958a,b, 1962).

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