Mechanism of the Inhibitory Action of Linoleic Acid on the Growth of *Staphylococcus aureus*

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Linoleic acid, but not stearic acid, inhibited the growth of *Staphylococcus aureus* NCTC 8325. Growth inhibition was associated with an increase in the permeability of the bacterial membrane. The presence of a plasmid conferring resistance to penicillin (PC plasmid, e.g. pI₂₅₈blaI⁻) increased the growth inhibitory and membrane permeability effects of linoleic acid. Under growth inhibitory conditions, linoleic acid was incorporated into the lipid of both PC plasmid-containing and PC plasmid-negative bacteria and there was little difference between these cultures in the uptake or fate of linoleic acid. Experiments using a glycerol auxotroph of *S. aureus* suggested that free linoleic acid, rather than lipid containing this acid, inhibits growth. Linoleic acid probably inhibits growth by increasing the permeability of the bacterial membrane as a result of its surfactant action, and the presence of the PC plasmid increases these effects.

INTRODUCTION

The growth of Gram-positive bacteria is usually inhibited by long-chain fatty acids to a greater extent than that of Gram-negative bacteria (Nieman, 1954). Galbraith & Miller (1973*a, b, c*) have shown that long-chain fatty acids are bactericidal for Gram-positive bacteria, causing lysis of osmotically stabilized protoplasts, leakage of 260 nm-absorbing material and protein from both bacteria and protoplasts, and inhibition of both respiration (oxygen uptake) and amino acid uptake. Similar results have been reported for *Bacillus subtilis* (Sheu & Freese, 1973; Freese *et al.*, 1973; Sheu *et al.*, 1975).

Both groups have suggested (Galbraith *et al.*, 1971; Galbraith & Miller, 1973*c*; Sheu & Freese, 1973; Sheu *et al.*, 1975) that the large difference in the fatty acid sensitivities between Gram-positive and Gram-negative bacteria may result from the outer membrane preventing fatty acids reaching the inner, fatty acid-sensitive cytoplasmic membrane of Gram-negative bacteria. Nikaido (1976) has shown that the outer membrane of *Salmonella typhimurium* is an effective barrier against hydrophobic substances whereas the cytoplasmic membrane of spheroplasts from it is very permeable. The impermeability of the outer membrane appears to be related to the structure of the lipopolysaccharide molecules. 'Deep rough' mutants which have lost 80 to 90% of the carbohydrate portion of the lipopolysaccharide molecule have greatly increased sensitivity towards certain hydrophobic antibiotics and dyes (Roantree *et al.*, 1969; Schlecht & Schmidt, 1970; Schmidt *et al.*, 1969).

Butcher *et al.* (1976) have shown that the growth of *Staphylococcus aureus* is inhibited by long-chain *cis* unsaturated fatty acids (UFAs) and that the presence of a plasmid conferring resistance to penicillin (PC plasmid) enhanced the sensitivity of *S. aureus* to UFAs. The

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aims of the present work were to investigate further the effects of UFAs on *S. aureus* and to examine how the PC plasmid increases the UFA sensitivity of the bacterium.

METHODS

Bacteria. *Staphylococcus aureus* NCTC 8325 was used for most of these studies and was designated 8325(N) to indicate that it does not contain a known plasmid. *Staphylococcus aureus* 8325(pI₂₅₈) was constructed by transduction selecting for erythromycin resistance from *S. aureus* 258(pI₂₅₈) (Mitsuhashi *et al.*, 1965) to *S. aureus* 8325(N). A strain producing penicillinase constitutively has been isolated from 8325(pI₂₅₈) after ethyl methanesulphonate mutagenesis (K. G. H. Dyke & H. Hackling, unpublished) and designated 8325-(pI₂₅₈blaI⁻). Isogenic 8325(N) and 8325(pI₂₅₈blaI⁻) strains (except for the pI₂₅₈blaI⁻ PC plasmid) were constructed by transduction selecting for cadmium ion resistance from 8325(pI₂₅₈blaI⁻) to a culture derived from a single colony of 8325(N), according to the method of Johnston & Dyke (1971) using phage 53 of the International Set of Typing Phages. This strain is resistant to cadmium ions, erythromycin, arsenate ions and produces penicillinase constitutively; it is assumed to carry the entire plasmid. *Staphylococcus aureus* 147-92 and 147-92 S4glyc were kindly given by Dr L. Mindich (Department of Microbiology, Public Health Research Institute of the City of New York, N.Y. 10016, U.S.A.). *Staphylococcus aureus* 147-92 S4glyc is a glycerol auxotroph requiring 20 µg glycerol ml⁻¹ for normal growth (Mindich, 1971).

Growth of bacteria. CY medium (Novick, 1963) was used except in experiments involving the glycerol auxotroph when SNMM medium was used (Mindich, 1971). Growth was followed by measuring the absorbance at 675 nm in a 1 cm path-length cuvette in a Unicam SP600 spectrophotometer. All experiments were conducted at 30 °C.

Growth inhibition was measured as follows. A culture of stationary phase bacteria was diluted into fresh warm medium to give an *A*₆₇₅ of 0.05 to 0.1. The bacteria were grown with shaking for three generations to approximately mid-exponential phase and then diluted to an *A*₆₇₅ of 0.2 with fresh warm medium. The diluted culture was added to flasks containing twice the desired concentration of fatty acid in CY medium plus 2% (v/v) ethanol, to give a final *A*₆₇₅ of 0.1. The mean generation time (MGT) was measured and growth inhibition was calculated from:

$$\text{Growth inhibition (\%)} = \left(1 - \frac{\text{MGT, control culture}}{\text{MGT, treated culture}}\right) \times 100$$

Broth media were solidified by adding 1.5% (w/v) agar (Difco Bacto). Strains carrying the PC plasmid were maintained on CY agar containing 13.3 µg cadmium acetate ml⁻¹.

Measurement of macromolecular syntheses. DNA, RNA, cell wall, protein and membrane synthesis were measured by monitoring the incorporation of [2-¹⁴C]thymidine, [5-³H]uracil, [2-³H]glycine, L-[U-¹⁴C]-phenylalanine and [2-³H]glycerol, respectively, into material precipitated by ice-cold 5% (w/v) trichloroacetic acid (TCA). Since glycine is incorporated into both cell wall and protein, but phenylalanine only into protein (Hancock & Park, 1958), the study of incorporation of these two precursors allows the measurement of protein synthesis and cell wall synthesis. In conditions of growth, Hancock & Park (1958) found that 36% of the glycine was incorporated into protein and 64% into mucopeptide. Samples were mixed with an equal volume of ice-cold 10% (w/v) TCA, and after 60 to 120 min at 0 °C they were filtered through glass-fibre filters (Whatman GF/C, 2.5 cm diam.). The filters were washed with 4 × 5 ml ice-cold 5% (w/v) TCA and then with 4 × 5 ml ethanol. The filters were placed in glass vials, dried *in vacuo* for 1 h at 80 °C and radioactivity was determined.

Uptake and incorporation of [1-¹⁴C]linoleic acid. (i) **Uptake into whole bacteria.** Samples of bacteria, growing exponentially, were rapidly filtered through Millipore filters (2.5 cm diam., 0.45 µm pore size) and washed with 20 sample volumes of 0.1 M-phosphate buffer, pH 7.0, containing 1% (v/v) Triton X-100. The filters were then placed in glass vials, dried and counted.

(ii) **Incorporation into macromolecules.** The method used was essentially that described above for incorporation of other precursors into TCA-precipitated material, except that the precipitates were washed with 4 × 5 ml ice-cold 5% (w/v) TCA containing 0.5% (w/v) Brij-35, followed by ethanol.

The inclusion of detergents in the washes is necessary to remove linoleic acid which is bound to the filters. Control experiments with bacterial samples whose lipids and proteins were radioactively labelled showed that neither macromolecular species was removed by the detergent washes.

Radioactivity was measured after adding 2.5 ml scintillation fluid [2-(4'-*tert*-butylphenyl)-5-(4"-biphenyl)-1,3,4-oxadiazole, 5 g l⁻¹ in toluene] to each sample vial, using a Wallac LKB liquid scintillation counter (model 81000). To determine the radioactivity of liquid or aqueous samples, 100 µl portions were placed on GF/C filters that were then dried and counted as previously described.

Oxygen uptake measurements. The oxygen uptake of bacterial suspensions was measured using a Clark-

type oxygen electrode (Rank Bros, Bottisham, Cambs.). The apparatus had a volume of 3 ml and was maintained at 30 °C. A suspension (3 ml) of exponentially growing bacteria (as described for growth inhibition studies) in CY medium (A_{675} 0.2) was placed inside the electrode and the rate of oxygen uptake was measured. Additions were made through a fine hole in the top of the electrode. Linoleic acid was added in a small volume of ethanol. The electrode was calibrated with air-saturated buffer at 30 °C, assumed to contain oxygen at a concentration of $0.47 \mu\text{g atom ml}^{-1}$ (Kaye & Laby, 1966).

Extraction of lipid from bacteria. The lipids of *S. aureus* were extracted by the method of White & Frerman (1967).

Thin-layer chromatography (t.l.c.). Separations were carried out on silica gel G t.l.c. plates (Merck, 20×20 cm, $250 \mu\text{m}$ thick thin-layer) which had been previously activated for 30 min at 100 °C. Samples, together with appropriate standards, were applied to the thin layers. After development, components were visualized by exposure to iodine vapour and the spots were traced. Two different developing solvents were used for methyl linoleate extract analyses: (a) hexane/diethyl ether/acetic acid (70:30:2, by vol.); (b) hexane/ethanol (3:1, by vol.). Membrane lipid extracts were developed with $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$ /acetic acid (65:25:4:1, by vol.). After autoradiography, the lipid separations were analysed by using the spray reagents of Dittmer & Lester (1964).

Autoradiography of t.l.c. plates was performed by using Kodak 'Non-screen' X-ray film placed on top of thoroughly dried plates. Exposure time was usually 5 to 7 d. For some experiments radioactive areas, identified by autoradiography, were scraped off and counted.

Leakage of 260 nm-absorbing material from bacteria. This was measured using a method modified from that described by Salton (1951). Bacteria grown to mid-exponential phase (A_{675} 0.8) as previously described were harvested by centrifugation (5000 g for 15 min), washed three times in ice-cold 50 mM-Tris/HCl buffer, pH 7.5, and resuspended in similar buffer (A_{675} about 15; 6 mg dry wt ml^{-1}). The bacterial suspensions were diluted five fold into buffer at 30 °C containing various concentrations of fatty acids, added in a small volume of ethanol (final concn, 1%, v/v). After the appropriate time, 5 ml of these suspensions was pipetted into centrifuge tubes, cooled rapidly to 0 °C and centrifuged. One ml of the supernatant was then carefully removed and after dilution into absolute ethanol, the A_{260} was measured. Dilution with ethanol was necessary to avoid precipitation of linoleic acid at high concentrations in aqueous solution. The total amount of 260 nm-absorbing material present in the bacteria was measured by diluting bacterial samples fivefold into buffer and boiling for 15 min. The samples were then cooled, centrifuged and A_{260} of the supernatant was measured as described above.

Chemicals. All chemicals used were the best grade commercially available. Linoleic acid and stearic acid were obtained from Sigma and were used without further purification. Solvents were redistilled before use. All radiochemicals were obtained from The Radiochemical Centre, Amersham.

RESULTS

Effect of linoleic and stearic acids on growth

Linoleic acid ($\text{C}_{18:2}$) inhibited the growth of *S. aureus* whereas the fully saturated stearic acid ($\text{C}_{18:0}$) at similar concentrations did not (Fig. 1). Strain 8325($\text{pI}_{258}\text{blaI}^-$) was more sensitive to the effects of linoleic acid than strain 8325(N).

Effect of linoleic acid on macromolecular synthesis

The effect of linoleic acid on the incorporation of the following radioactive precursors into macromolecules was studied: RNA synthesis, [$5\text{-}^3\text{H}$]uracil ($20 \mu\text{g}$, $1 \mu\text{Ci ml}^{-1}$); DNA synthesis, [$2\text{-}^{14}\text{C}$]thymidine ($10 \mu\text{g}$, $0.05 \mu\text{Ci ml}^{-1}$); protein synthesis, L-[$\text{U-}^{14}\text{C}$]phenylalanine (10 mg , $0.1 \mu\text{Ci ml}^{-1}$); cell wall synthesis, [$2\text{-}^3\text{H}$]glycine ($20 \mu\text{g}$, $1 \mu\text{Ci ml}^{-1}$); membrane synthesis, [$2\text{-}^3\text{H}$]glycerol ($1 \mu\text{Ci ml}^{-1}$). Linoleic acid affected similarly the synthesis of protein, cell wall, RNA, DNA or membrane in both *S. aureus* 8325(N) and 8325($\text{pI}_{258}\text{blaI}^-$). Results are shown for protein synthesis (Fig. 2). The inhibition was proportional to the inhibition of growth as measured by the increase in A_{675} .

Effect of linoleic acid on oxygen uptake

Since linoleic acid did not preferentially affect any of the types of macromolecular synthesis studied, the linoleic acid-sensitive reaction may be common to all these syntheses, the most obvious being the supply of energy. Linoleic acid inhibited the uptake of oxygen

Fig. 1. Effect of linoleic and stearic acids on the growth of *S. aureus* 8325(N) and 8325(pI₂₅₈blaI⁻). Cultures of stationary phase bacteria were diluted into fresh warm CY medium to an A_{675} of 0.1 and grown at 30 °C for three generations. The cultures were then diluted to 5×10^7 colony-forming units ml⁻¹ with CY medium and linoleic or stearic acid was added [final ethanol concn 1% (v/v)]. Growth inhibition was measured as described in Methods. Linoleic acid: ○, 8325(N); ●, 8325(pI₂₅₈blaI⁻). Stearic acid: △, 8325(N); ▲, 8325(pI₂₅₈blaI⁻).

Fig. 2. Effect of linoleic acid on macromolecular synthesis in *S. aureus* 8325(N) (a) and 8325(pI₂₅₈blaI⁻) (b). Cultures of stationary phase bacteria grown at 30 °C were diluted into fresh warm CY medium (supplemented with phenylalanine, 10 μg ml⁻¹) to an A_{675} of 0.1 and grown for three generations. The cultures were then diluted to an A_{675} of 0.05 and 1 μCi L-[U-¹⁴C]phenylalanine ml⁻¹ was added. The incorporation of radioactivity into ice-cold 5% (w/v) TCA-precipitated material was followed for approximately 40 min until the A_{675} was about 0.1. At this point (arrowed) linoleic acid was added [final concn 5 μg ml⁻¹ for 8325(N) and 3 μg ml⁻¹ for 8325(pI₂₅₈blaI⁻); final ethanol concn 1% (v/v)] to one flask, and the same volume of ethanol was added to a control flask. Growth and incorporation of precursor into TCA-precipitated material was followed for a further 120 min. Growth inhibition was 36% for 8325(N) and 38% for 8325(pI₂₅₈blaI⁻). ○, Control; ●, with linoleic acid.

by suspensions of *S. aureus* in the mid-exponential phase of growth (Fig. 3). The extent of this inhibition correlated with growth of both 8325(N) and 8325(pI₂₅₈blaI⁻) as did the inhibition of synthesis of macromolecules.

Uptake of linoleic acid

An explanation for the increased sensitivity of PC plasmid-containing bacteria was sought by studying the uptake of [1-¹⁴C]linoleic acid into both whole bacteria and TCA precipitates. There was little difference in the initial rate of uptake into either whole bacteria or TCA precipitates (Fig. 4). Calculation of K_m and V_{max} values by the method of Eisenthal & Cornish-Bowden (1974) revealed a small difference between the uptake system of *S. aureus* 8325(pI₂₅₈blaI⁻) [K_m 1.98 μM; V_{max} 0.18 μg linoleic acid min⁻¹ (mg dry wt bacteria)⁻¹]

Fig. 3. Inhibition by linoleic acid of oxygen uptake and growth. Cultures of stationary phase *S. aureus* 8325(N) and 8325(pI₂₅₈blaI⁻) grown at 30 °C were diluted into fresh warm CY medium to an A_{675} of 0.1. The cultures were grown for three generations, and then diluted to an A_{675} of 0.2. Samples (3 ml) of these cultures were added to a Clark-type oxygen electrode, maintained at 30 °C, and the rate of oxygen uptake was measured. Linoleic acid in a small volume of ethanol was then added [final ethanol concn 1% (v/v)] and the new rate of oxygen uptake was measured. Growth inhibition was determined as described in Fig. 1. Growth inhibition: ○, 8325(N); ●, 8325(pI₂₅₈blaI⁻). Oxygen uptake inhibition: □, 8325(N); ■, 8325(pI₂₅₈blaI⁻).

Fig. 4. Uptake of linoleic acid by *S. aureus* 8325(N) (a) and 8325(pI₂₅₈blaI⁻) (b). Cultures of stationary phase bacteria grown at 30 °C in CY medium were diluted into fresh warm CY medium to an A_{675} of 0.1 and grown at 30 °C for three generations. The cultures were then diluted with fresh CY medium to an A_{675} of 0.1, and linoleic acid was added together with 0.1 µCi [1-¹⁴C]linoleic acid ml⁻¹. [In experiments where low concentrations of linoleic acid (0.25 and 0.125 µg ml⁻¹) were used, [1-¹⁴C]linoleic acid was present at 0.05 and 0.025 µCi ml⁻¹, respectively.] Uptake into whole bacteria or acid-precipitated material was measured at 30 °C for 6 to 10 min. Duplicate 1 ml samples of culture were pipetted either on to a Millipore filter (0.45 µm pore diam.) followed by washing with 20 vol. 0.1 M-phosphate buffer, pH 7.0, containing 1% (v/v) Triton X-100, or into 1 ml 10% (w/v) ice-cold trichloroacetic acid (TCA). The TCA-precipitated material was collected on a Whatman GF/C filter, washed with 4 × 5 ml ice-cold 5% (w/v) TCA containing 0.5% (w/v) Brij-35 and 4 × 5 ml ethanol. The filters were dried *in vacuo* and counted in a liquid scintillation counter. ○, Uptake into whole bacteria; ●, incorporation into macromolecules.

Table 1. *Extraction of lipid from S. aureus 8325(N) and 8325(pI₂₅₈blaI⁻) grown in the presence of [1-¹⁴C]linoleic acid at growth inhibitory concentrations of linoleic acid*

Staphylococcus aureus 8325(N) and 8325(pI₂₅₈blaI⁻), growing exponentially, were grown for one generation in the presence of [1-¹⁴C]linoleic acid (0.5 μ Ci ml⁻¹) at growth inhibitory concentrations of linoleic acid [10 μ g ml⁻¹ for 8325(N) and 4 μ g ml⁻¹ for 8325(pI₂₅₈blaI⁻)]. The labelled bacteria (approx. 2.4 mg dry wt) were mixed with bacteria grown in the presence of unlabelled linoleic acid (approx. 20 mg dry wt) and subjected to the extraction procedure of White & Frerman (1967), except that the bacteria were washed in 0.1 M-phosphate buffer, pH 7.0, containing 1% (v/v) Triton X-100 and then twice in 0.05 M-phosphate buffer, pH 7.6. The results of two independent fractionations are shown.

	8325(N)		8325(pI ₂₅₈ blaI ⁻)	
	Expt 1	Expt 2	Expt 1	Expt 2
Growth inhibition (%)	58	50	30	42
Total c.p.m. present	678 640	652 000	2 750 400	2 526 200
C.p.m. extracted as lipid (%)	91	89	92	94
C.p.m. remaining in aqueous phase (%)	7.6	7.6	3.6	3.7
Recovery of c.p.m. (%)	98.6	96.6	95.6	97.7

and 8325(N) [K_m 2.25 μ M; V_{max} 0.168 μ g linoleic acid min⁻¹ (mg dry wt bacteria)⁻¹]. Thus, the PC plasmid-containing bacteria took up linoleic acid at a slightly higher rate than the plasmid-negative bacteria.

A large amount of linoleic acid was incorporated by *S. aureus* (approximately 0.9% by weight per generation, assuming a mean generation time of 50 min). To locate the incorporated radioactivity, *S. aureus* was grown in the presence of growth inhibitory concentrations of [1-¹⁴C]linoleic acid for the time necessary for the A_{675} to double, and the lipids were then extracted by the method of White & Frerman (1967). At least 89% of the total radioactivity incorporated was recovered in the lipid fraction (Table 1).

Analysis of the [1-¹⁴C]linoleic acid-labelled lipid fractions from both *S. aureus* 8325(N) and 8325(pI₂₅₈blaI⁻) by t.l.c. revealed that radioactivity was present in lipids characteristic of *S. aureus* (Fig. 5). No free linoleic acid was detected and there were no obvious differences between the lipids derived from 8325(N) and 8325(pI₂₅₈blaI⁻). The identity of the various species in each fraction was determined by comparison with standard lipids on the same t.l.c. plate and by spraying the plates with various diagnostic reagents (Dittmer & Lester, 1964). The probable identity of the labelled species is as follows: Spot 1, neutral lipid, possibly glycolipid; 2, phosphatidic acid; 3, cardiolipin; 4, phosphatidylglycerol; 5, *O*-lysyl-phosphatidylglycerol; 6, unknown. Spots 2 and 3 gave positive reactions for the presence of phosphorus, so cannot be free linoleic acid.

The extracted radioactive lipid was subjected to acid-catalysed methanolysis, followed by extraction of methyl esters in hexane and analysis of hexane fractions by t.l.c. (Fig. 6). More than 98% of the radioactivity applied was present in the component having the same R_f as methyl linoleate. Similar results were obtained using a second solvent system (hexane/diethyl ether/acetic acid; 70:30:1, by vol.). Thus, [1-¹⁴C]linoleic acid incorporated into the lipid of *S. aureus* 8325(N) and 8325(pI₂₅₈blaI⁻) was essentially still linoleic acid.

The growth inhibitory component: linoleic acid itself or lipid into which linoleic acid has become incorporated

Under the conditions of growth inhibition used, linoleic acid is extensively incorporated into neutral lipids. The possibility that the incorporation into lipid is a prerequisite for the growth inhibitory action of linoleic acid was studied with the aid of a glycerol auxotroph S4 of *S. aureus* 147-92 (Mindich, 1971). If glycerol is removed from the growth medium, this mutant grows at the usual rate for one generation and then growth slows and subsequently stops. DNA, RNA and protein synthesis are all normal during the period of 'normal' growth, but incorporation of fatty acids into lipid stops almost completely im-

Fig. 5

Fig. 6

Fig. 5. T.l.c. analysis of lipid extracted from *S. aureus* 8325(N) and 8325(pI₂₅₈blaI⁻) after growth in the presence of [1-¹⁴C]linoleic acid under growth inhibitory conditions. To a culture of exponentially growing *S. aureus* 8325(N), linoleic acid was added (final concn 10 µg ml⁻¹) and incubation at 30 °C was continued until the *A*₆₇₅ had doubled. Similarly, *S. aureus* 8325(pI₂₅₈blaI⁻) was grown in 4 µg linoleic acid ml⁻¹. Growth was inhibited 50% for 8325(N) and 42% for 8325(pI₂₅₈blaI⁻). The bacteria were then harvested. Lipids extracted from the bacteria were concentrated by evaporation of excess CHCl₃, and 50 µl of each extract was applied to an activated silica gel G plate together with other standards: I, phosphatidic acid; II, cardiolipin; III, lysolecithin; IV, unlabelled linoleic acid; V, [1-¹⁴C]linoleic acid; VI, 8325(N) lipid; VII, 8325(pI₂₅₈blaI⁻) lipid; VIII, phosphatidylglycerol; IX, phosphatidylethanolamine; X, phosphatidylcholine; XI, phosphatidylserine. The plate was developed in CHCl₃/CH₃OH/H₂O/acetic acid (65:25:4:1, by vol.) for 12 cm, then dried and exposed to iodine vapour. After tracing the visible spots, the plate was autoradiographed. Finally, the plate was sprayed with 0.25% (w/v) ninhydrin and then molybdenum blue reagent (Dittmer & Lester, 1964). Shaded spots correspond to radioactive material as determined by autoradiography.

Fig. 6. T.l.c. analysis of material produced by methanolysis of ¹⁴C-labelled lipid. Lipid extracted from *S. aureus* 8325(N) and 8325(pI₂₅₈blaI⁻) after growth in the presence of [1-¹⁴C]linoleic acid was subjected to acid-catalysed methanolysis. After partition of the resulting material between hexane and saturated NaCl solution, samples of the hexane fractions [8325(N), II, and 8325(pI₂₅₈blaI⁻), III] were applied to activated silica gel G plates together with standards of linoleic acid (I) and methyl linoleate (IV). The plate was developed in hexane/ethanol (3:1, by vol.) and then exposed to iodine vapour. Spots were marked and the plate was autoradiographed. Radioactive areas of gel were scraped off and counted. Shaded spots correspond to radioactive material as determined by autoradiography.

mediately on glycerol starvation (Mindich, 1971). Therefore, a study of the effect of linoleic acid on growth immediately after glycerol starvation should indicate whether or not linoleic acid has to be incorporated into lipid prior to inhibiting growth.

First, the incorporation of [1-¹⁴C]linoleic acid into TCA-precipitated material by 147-92 S4glyc was studied. There was very little incorporation of linoleic acid in the absence of glycerol whereas glycerol-supplemented cultures, that exhibited the same growth inhibition, incorporated linoleic acid into membrane lipid (Fig. 7a). After 60 min in conditions of glycerol deprivation, the incorporation was only 6% of that with glycerol supplementation.

The presence of the PC plasmid potentiated the growth inhibitory properties of linoleic acid on the 147-92 strains. Like 147-92 S4glyc, 147-92 S4glyc(pI₂₅₈blaI⁻) incorporated only about 8% of linoleic acid into lipid during glycerol starvation compared with that under conditions of glycerol supplementation (Fig. 7b).

During the initial period following glycerol deprivation, linoleic acid inhibited the growth of both 147-92 S4glyc and 147-92 S4glyc(pI₂₅₈blaI⁻) (Fig. 8). These experiments strongly suggest that it is linoleic acid itself that is the growth inhibitory species.

Fig. 7. Dependence of $[1-^{14}\text{C}]$ linoleic acid incorporation on the presence of glycerol. Cultures of stationary phase *S. aureus* 147-92 *S4glyc* (a) and 147-92 *S4glyc*($pI_{258}blaI^-$) (b) grown at 30°C in SNMM medium plus $20\text{ }\mu\text{g}$ glycerol ml^{-1} were diluted into fresh warm SNMM plus glycerol to an A_{675} of 0.05. These cultures were grown at 30°C for three generations, then harvested by centrifugation, washed with warm SNMM without glycerol and finally resuspended in warm SNMM without glycerol. Samples of these cultures were added to flasks of SNMM containing $[1-^{14}\text{C}]$ linoleic acid ($5\text{ }\mu\text{g}\text{ ml}^{-1}$) with or without glycerol, to give an A_{675} of about 0.05. Incorporation of $[1-^{14}\text{C}]$ linoleic acid into TCA-precipitated material was followed as described in Methods. $\circ$, With glycerol; $\bullet$, without glycerol.

Fig. 8. Effect of linoleic acid on growth following glycerol deprivation of *S. aureus* 147-92 *S4glyc* and 147-92 *S4glyc*($pI_{258}blaI^-$). Cultures were grown as described in Fig. 7, and flasks of SNMM with or without glycerol ($20\text{ }\mu\text{g}\text{ ml}^{-1}$) and with or without linoleic acid ($5\text{ }\mu\text{g}\text{ ml}^{-1}$) were inoculated to give an A_{675} of about 0.05. Growth was followed at 30°C . $\circ$, Controls; $\bullet$, with linoleic acid. (a) 147-92 *S4glyc* with glycerol (growth inhibition 38%); (b) 147-92 *S4glyc* without glycerol (initial growth inhibition 34%); (c) 147-92 *S4glyc*($pI_{258}blaI^-$) with glycerol (growth inhibition 45%); (d) 147-92 *S4glyc*($pI_{258}blaI^-$) without glycerol (initial growth inhibition 50%).

Fig. 9

Fig. 10

Fig. 9. Release of 260 nm-absorbing material from *S. aureus* by linoleic acid. Leakage of 260 nm-absorbing material from *S. aureus* 8325(N) (○, ●) and 8325(pI₂₅₈blaI⁻) (□, ■) was measured as described in Methods. Linoleic acid (final concn 50 μg ml⁻¹) was added in a small volume of ethanol (1%, v/v); ethanol (to 1%, v/v) was added to control suspensions. Arrows I and II indicate the amount of 260 nm-absorbing material released per mg dry wt bacteria after 15 min boiling, from 8325(N) and 8325(pI₂₅₈blaI⁻), respectively. ○, □, Controls; ●, ■, with linoleic acid.

Fig. 10. Dependence of release of 260 nm-absorbing material from *S. aureus* on the linoleic acid concentration. The release of 260 nm-absorbing material from suspensions of *S. aureus* 8325(N) (○) and 8325(pI₂₅₈blaI⁻) (●) was measured as described in Fig. 9. Ethanol was present in all suspensions at 1% (v/v) final concentration. The release of material, expressed as a percentage of the material released by boiling, was measured after 15 min incubation at 30 °C. The results of three independent experiments are shown.

Release of 260 nm-absorbing material by linoleic acid

The growth inhibitory properties of linoleic acid may be due to its strong surfactant activity (Dervichian, 1954). Salton (1951) has previously shown that *S. aureus* is particularly susceptible to the cationic detergent cetyltrimethylammonium bromide, which causes the release of 260 nm-absorbing material; linoleic acid may behave in a similar way.

Leakage of 260 nm-absorbing material from *S. aureus* 8325(N) and 8325(pI₂₅₈blaI⁻) was measured by the method of Salton (1951), except that mid-exponential phase bacteria were used instead of stationary phase bacteria, and all experiments were conducted in 50 mM-Tris/HCl buffer, pH 7.5, instead of distilled water. Linoleic acid caused a rapid loss of 260 nm-absorbing material from both 8325(N) and 8325(pI₂₅₈blaI⁻) (Fig. 9). After the very rapid initial loss, the release was linear for 20 min, and by 30 min linoleic acid had resulted in the loss of material equivalent to that caused by boiling. It is interesting to note that 8325(N) had more 260 nm-absorbing material per mg dry wt bacteria than did 8325(pI₂₅₈blaI⁻). This release of 260 nm-absorbing material was dependent on the linoleic acid concentration; it was maximal at linoleic acid concentrations greater than 50 μg ml⁻¹ (Fig. 10). Strain 8325(pI₂₅₈blaI⁻) lost a higher proportion of this material (85 to 100%) compared with that released by boiling than did 8325(N) (68 to 85%). Consistent with the growth inhibition studies (Fig. 1), stearic acid at a similar concentration did not cause release of 260 nm-absorbing material from *S. aureus* (Table 2).

If linoleic acid is behaving as a surfactant in this system, it may be altering the interfacial tension between the bacterial membrane and the bulk aqueous phase of the growth medium. In a model system, Peters (1931) studied the changes of the interfacial tension between a solution of stearic acid in benzene and an aqueous buffer solution. Increasing the pH of the aqueous phase from 5.5 did not significantly change the interfacial tension until the pH was 7.5 to 8.0. The effect of pH on the growth inhibition of *S. aureus* was therefore studied. Since the growth was being measured, the pH of the growth medium could not be varied far

Table 2. Release of 260 nm-absorbing material from *S. aureus* by stearic and linoleic acids

Fatty acid*	Release [A_{260} (mg dry wt) $^{-1}$] after 15 min	
	8325(N)	8325(pI ₂₅₈ blaI $^{-}$)
Control (none)	0.76	0.52
Linoleic acid	1.57	1.21
Stearic acid	0.71	0.52

* Fatty acids (final concn 50 μ g ml $^{-1}$) were added in a small volume of ethanol (final concn 1 %, v/v). This concentration of ethanol was present in the control suspensions.

Fig. 11. Effect of pH on growth inhibition produced by linoleic acid. Cultures of stationary phase *S. aureus* 8325(N) (○) and 8325(pI₂₅₈blaI $^{-}$) (●) grown at different pH values at 30 °C were diluted into fresh warm CY medium at the same pH to an A_{676} of 0.1 and grown for three generations. The growth inhibition produced by 3 μ g linoleic acid ml $^{-1}$ was then determined as described in Fig. 1.

from neutrality. Between pH 6.0 and 8.0, the mean generation time of control cultures did not alter significantly, and the pH of the growth medium did not change over the duration of the experiment from that set at the beginning. At pH 6.0 and 6.5, linoleic acid did not inhibit growth (Fig. 11). Between pH 7.0 and 8.0, growth inhibition became evident, with the highest inhibition observed at pH 7.4 (pH of CY medium) and 8.0. This pH effect was similar for both 8325(N) and 8325(pI₂₅₈blaI $^{-}$), except that the latter was more inhibited.

DISCUSSION

Linoleic acid is rapidly taken up and incorporated into the phospholipids of *S. aureus* 8325(N) and 8325(pI₂₅₈blaI $^{-}$). This incorporation is high, equivalent to approximately 0.9 % of the bacterium's dry weight after a generation of growth (about 16 % of the fatty acid composition). Since the membranes of *S. aureus* have a low UFA content [principally monoenoic and only about 2.8 % of the fatty acid composition (White & Frerman, 1968)], the fivefold increase in the UFA content of the bacterial membrane phospholipids after growth in linoleic acid-containing medium might be expected to alter the permeability properties of the bacterial membrane. As the incorporated linoleic acid is not significantly metabolized, linoleic acid-containing lipid could certainly be deleterious to the bacterium. However, experiments using the glycerol auxotroph of *S. aureus*, 147-92 S4glyc, have shown that incorporation of linoleic acid into membrane lipid is not essential for growth inhibition. The period of growth following glycerol deprivation was still inhibited to the same degree by linoleic acid as were glycerol-supplemented cultures, even though the former cultures incorporated less than 10 % of the amount of linoleic acid incorporated by glycerol-supplemented cultures. Thus, free linoleic acid itself seems to be the growth inhibitory substance and not linoleic acid-containing membrane lipid.

Linoleic acid does alter the permeability of *S. aureus* membranes, since there is rapid leakage of 260 nm-absorbing material from treated bacteria. This material is probably similar to that previously identified as consisting of nucleotides and amino acids, and representing 'pool' material (Salton, 1951). In agreement with the growth inhibition studies, stearic acid does not produce a similar permeability increase. It is possible that the primary effect of linoleic acid may be to increase the membrane permeability to low molecular weight solutes, e.g. ions, and that the observed 260 nm-absorbing material leakage is a secondary effect.

The altered permeability could explain the inhibition of synthesis of macromolecules and oxygen uptake. One possibility is that the organization of proteins such as permeases and cytochromes within the membrane is disrupted by linoleic acid and this results in cessation of synthetic activity. Although it is known that long-chain fatty acids can uncouple oxidative phosphorylation (Pressman & Lardy, 1956; Hüllsmann *et al.*, 1960; Borst *et al.*, 1962), it is possible for an uncoupler of oxidative phosphorylation to cause inhibition of oxygen uptake of bacteria when energy is necessary for the uptake of carbon sources and metabolites. For *S. aureus* grown on glucose as its carbon source, energy is necessary for glucose uptake via the phosphotransferase system (see Postma & Roseman, 1976). Under the experimental conditions used, linoleic acid behaves as an inhibitor of oxidative phosphorylation although it may be, in addition, an uncoupler.

Linoleic acid may increase membrane permeability as a result of its surfactant properties. The pH dependence of growth inhibition by linoleic acid supports this idea since linoleic acid may be decreasing the interfacial tension at the bacterial lipid membrane-aqueous medium interface. As the pK_a of linoleic acid is about 5.0 (Smith & Tanford, 1973), alteration of the pH between 6.0 and 8.0 will not substantially alter the degree of ionization of the fatty acid but the interfacial tension can vary widely in this pH range in model systems, as shown by Peters (1931). Altenbern (1977*a*) has recently reported a similar pH dependence of growth inhibition by linoleic acid.

Stearic acid may not inhibit growth because, at 30 °C, it is a much poorer surfactant than linoleic acid (see Dervichian, 1954). Only at temperatures of 70 °C or higher do linoleic and stearic acids have similar surfactant properties. Further evidence supporting these ideas is that UFAs caused greater expansions of monolayers of *S. aureus* lipid than did saturated fatty acids under similar conditions (Gale & Llewellyn, 1971).

The leakage experiments have indicated a possible mechanism that may account for the difference in linoleic acid sensitivity between 8325(N) and its PC plasmid-containing derivative. The permeability of 8325(pI₂₅₈*blaI*⁻) membranes is affected more than those of 8325N such that pool material is lost at a higher rate. This is the only observation so far that could account for the differential linoleic acid sensitivity. Some component – either the PC plasmid itself or a component determined by the plasmid – appears to affect the membranes of 8325(pI₂₅₈*blaI*⁻) bacteria so that they are more sensitive to the effects of linoleic acid. Such a component appears to be the membrane-bound penicillinase protein itself (D. L. A. Greenway & K. G. H. Dyke, unpublished).

The uptake studies have highlighted a number of interesting points. (1) The kinetic properties of uptake of linoleic acid by 8325(N) and 8325(pI₂₅₈*blaI*⁻) are similar but not identical. The PC plasmid may specify a different or altered uptake system since the K_m is lower (i.e. higher affinity) and the V_{max} is higher than that of the corresponding plasmid-negative strain. It seems unlikely that these differences are sufficient to account for the differing UFA sensitivities of the two strains, especially when considered with the properties of an isolated linoleic acid resistant mutant LAR-1 (D. L. A. Greenway & K. G. H. Dyke, unpublished). (2) The lack of metabolism of linoleic acid by *S. aureus* suggests that this bacterium has a very limited ability to metabolize such compounds by, for example, β -oxidation, and this may account for their high level of incorporation into membrane phospholipids. Similar observations have been made by others with [1-¹⁴C]oleic acid

(Hedström, 1975) and [^{14}C]stearic acid (Gale & Folkes, 1967). This finding apparently excludes the possibility that the difference in linoleic acid sensitivity between 8325(N) and 8325(pI₂₅₈blal⁻) results from 8325(N) bacteria having the ability to metabolize linoleic acid to some less toxic form. (3) Incorporation of linoleic acid into membrane lipid may be a kind of detoxification mechanism. This may explain the discrepancy between the lowest concentration required to produce growth inhibition (about $3\text{ }\mu\text{g ml}^{-1}$) and the K_m for linoleic acid uptake (about $0.5\text{ }\mu\text{g ml}^{-1}$). The maximum rate of linoleic acid incorporation (i.e. V_{max}) occurs at a concentration of about 1.5 to $3.0\text{ }\mu\text{g ml}^{-1}$, so that at this concentration linoleic acid is efficiently incorporated into membrane lipid. At higher concentrations (greater than $3\text{ }\mu\text{g ml}^{-1}$), this incorporation appears insufficient to prevent linoleic acid from interacting with its sensitive site. *Staphylococcus aureus* is known to be able to tolerate quite large variations in its fatty acid composition without any apparent deleterious effect (Váczi *et al.*, 1967; Altenbern, 1977b).

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