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THE EFFECT OF LIPID PHASE TRANSITIONS ON THE ARCHITECTURE OF BACTERIAL MEMBRANES

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SUMMARY

1 Freeze-fracture faces of *Escherichia coli* membranes showed increasing particle aggregation when quenched from decreasing temperatures. Experiments on cells with different fatty acid composition demonstrated that the extent of aggregation is correlated with the degree of lipid phase transition

2 Particle aggregation, induced by lipid phase transition, could also be observed in *Streptococcus faecalis* membranes. However, no aggregation was seen in the fracture faces of some *Bacillus* species and *Staphylococcus aureus*, even far below the phase transition as detected by differential scanning calorimetry, and by breaks in the Arrhenius plots of membrane bound enzymic activities

3 The non-appearance of aggregation of the particles could be explained by the presence of branched-chain fatty acids in the membrane lipids of these bacteria

INTRODUCTION

In an earlier study [1] it was demonstrated that the particles, which can be seen on freeze-fracture faces of *Acholeplasma laidlawii* B membranes, aggregate upon cooling of the cells below the phase transition of the membrane lipids To explain this phenomenon it was suggested that cooling of the membrane below the temperature where solidification commences, results in a squeezing out of the protein groups that have penetrated into the hydrocarbon layer, finally resulting in a full separation of lipids and proteins. Particle aggregation has also been observed in the fracture faces of *Tetrahymena* membranes after cooling the cells [2] In addition a recent study on *Acholeplasma* showed that the extent of particle aggregation increases when more lipid becomes solid [3]

In order to answer the question as to whether the particle aggregation induced by lipid phase transition is a general membrane phenomenon of microorganisms we have now extended these studies to different bacteria From various studies it is al-

Abbreviation DCIP, dichlorophenolindophenol

ready known that the transition from the liquid–crystalline to gel state generally occurs in bacterial membranes In this study it is shown that this phase transition is not necessarily accompanied by aggregation of particles. Furthermore it will be shown that the non-appearance of particle aggregation may be explained by the presence of branched chain fatty acids in the membrane lipids of these bacteria

MATERIALS AND METHODS

Bacterial strains and growth media

Streptococcus faecalis, *Staphylococcus aureus*, *Bacillus subtilis* were grown in complex media [4] *Escherichia coli* K_{12} and K_{1060} were grown in minimal media with 0 3 % peptone [5] The growth temperature was 37 °C unless otherwise indicated

Preparation of the membranes

The *E coli* envelopes were prepared according to Birdsell and Cota-Robles [6] *S aureus* membranes were prepared with 100 μg lysostaphin/ml and those of *S faecalis* and the *Bacili* with 100 μg lysozyme/ml in buffered solutions of the same pH as the growth medium at the time of harvesting After the addition of 10 mM Mg^{2+} (final concn), 10 μg DNAase and RNAase per ml were added Whole cells were removed by centrifugation for 3 min at $5000 \times g$ Membranes were collected by centrifugation for 1 h at $35\,000 \times g$

Physical techniques

Freeze fracturing was performed as described before [7] Differential scanning calorimetric measurements on the membranes were carried out with the aid of Perkin–Elmer DSC-2 apparatus [8]

The succinate–dichlorophenolindophenol (DCIP) reductase activity was measured according to Esfahani et al [9], the NADPH oxidase activity according to Mavis et al [10] X-ray crystallography was performed with a Guinier camera

RESULTS AND DISCUSSION

Particle aggregation in E coli membranes

E coli is a suitable organism with which to study the possible relationship between particle aggregation and phase transition in biomembranes as methods are available to vary the fatty acid composition of the membrane lipids and consequently the temperature range of the lipid phase transition It is well-known that variations in the temperature of growth influences the ratio of saturated and unsaturated fatty acids The membrane lipids of *E coli* K_{12} cells grown at 13 °C have been shown to be considerably more unsaturated than those of cells grown at 37 °C [11] A more defined variation in the fatty acid pattern could be obtained by growing *E coli* K_{1060} (an unsaturated fatty acid requiring mutant generously supplied by Dr P Overath) in the presence of oleic and elaidic acid respectively [5] Several authors using different techniques such as differential scanning calorimetry [8, 12], X-ray crystallography [13, 14] and measurements of enzyme activities of membrane-dependent enzymes [14–23] have shown that variation in fatty acid composition results in different lipid phase transition temperatures

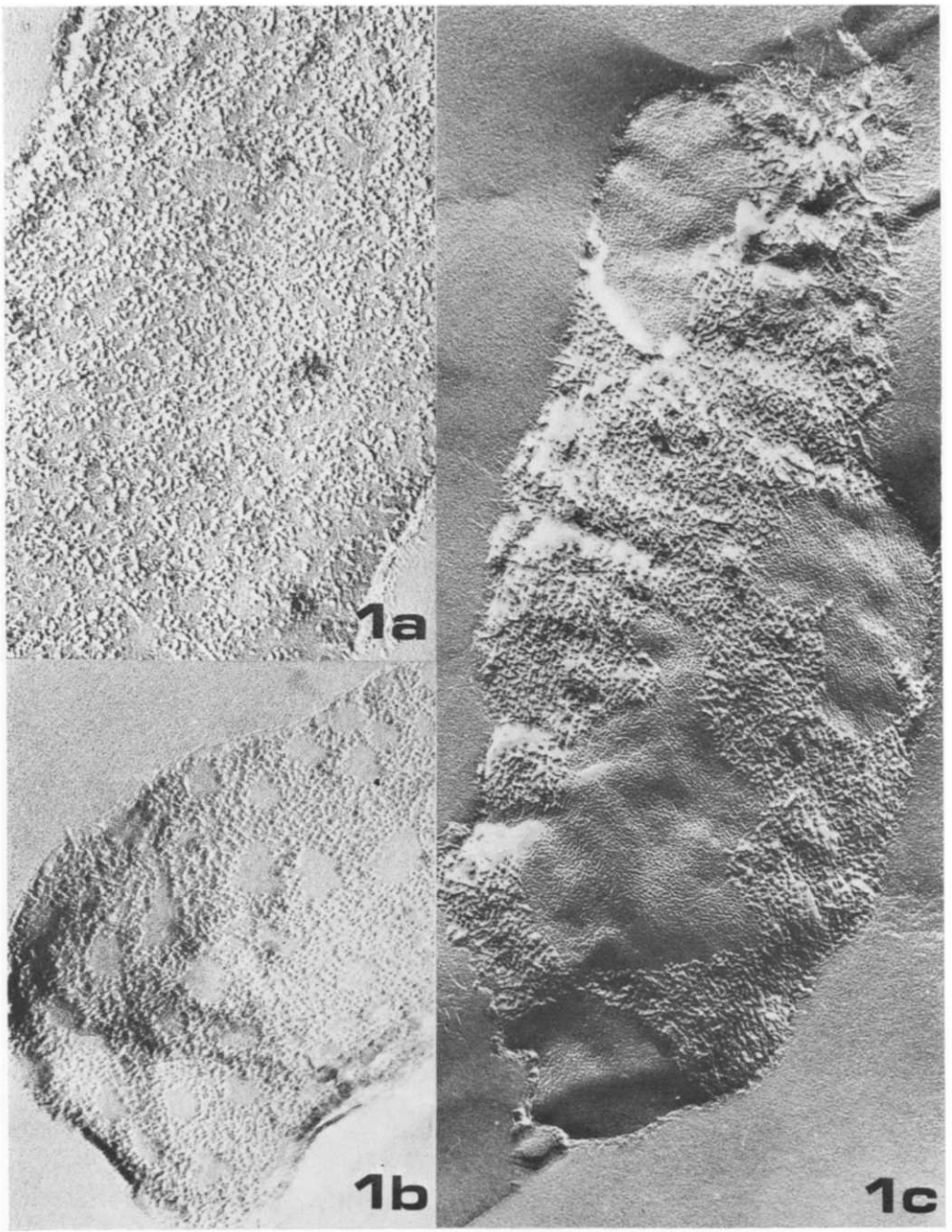

Fig 1 Typical structures, shown by freeze fracturing of membranes of *E coli* K_{1060} cells (a) Grown on oleate, quenched from 37 °C (−) (b) Grown on oleate, quenched from 22 °C or grown on elaidate, quenched from 37 °C (+) (c) Grown on oleate or elaidate, quenched from 0 °C (++) Magnification 120 000 ×

The freeze-fracturing experiments on various *E coli* cells grown under different conditions demonstrated, in agreement with the observations made on *Acholeplasma* [1, 3] and *Tetrahymena* [2], increasing aggregation of the particles when the membranes were gradually cooled to temperatures in or below the phase transition The effect of the fatty acid unsaturation was obvious in these studies For instance, the *E coli* K_{12} cells grown at 37 °C exhibited a definite aggregation at 20 °C, whereas cells grown at 13 °C showed no aggregation even at 10 °C Complete aggregation was reached at 5 and −5 °C for the cells grown at 37 and 13 °C, respectively

Fig 1 shows typical pictures obtained with the *E coli* 1060 cells when quenched from different temperatures It is noted that oleate grown cells showed a regular particle distribution at 37 °C while elaidate grown cells demonstrated some aggregation at this temperature At 22 °C the elaidate cells showed completed aggregation while only the onset of aggregation could be observed in the oleate cells

Differential scanning calorimetry is the most appropriate technique in the correlation of particle aggregation during lipid phase transition as this technique directly scans the melting of the fatty acid chains [12] The energy change undergone during the transition of the membrane lipids of oleic grown cells, i e with *cis* double bonds, appeared to be too small for detection on the instrument available, in spite of its being used at the highest sensitivity However, Fig 4 shows that it was possible to obtain results with cells grown on elaidic acid Between 28 and 47 °C the lipid phase transition takes place, while above 60 °C protein denaturation occurs The irreversibility of protein denaturation and the reversibility of the lipid transition is demonstrated by a second scan of the sample

From comparison of the temperature range over which the lipid phase transition takes place and the degree of aggregation at various temperatures, as shown in the pictures of Fig 1, it may be concluded that in *E coli* also the complete liquid–crystalline-gel transition must be attained to reach complete aggregation of the membrane particles It can be noted that with optical [27] and ESR [28] measurement the width of the transitions is only a few degrees (lower than 10°), while differential scanning calorimetry and also freeze etch data tend to the conclusion that the transition area is rather broad (about 20°)

As the differential scanning calorimetry technique failed to show the transition in *E coli* cells with *cis* double bonds we also studied the membrane enzymes, succinate–DCIP reductase [9] and NADPH oxidase [10] Kinks in the slopes of Arrhenius plots of membrane bound enzymic activities are generally accepted as being indicative of the lipid phase transition of the membrane [14–23] The influence of the fatty acid patterns is shown in Fig 5a

Lipid phase transition and membrane particles in other bacteria

As shown in Fig 2 particle aggregation is not limited only to Gram-negative bacteria The freeze-fracture pictures of *S faecalis*, having a fatty acid pattern of straight chain-saturated and unsaturated fatty acids, comparable to those of *E coli*, demonstrate increasing particle aggregation with decreasing temperature (The fatty acid composition of the lipids of *S faecalis* (expressed as percentage of the total fatty acids) was as follows 12 0, 1 6, 14 0, 6 1, 14 1, 2 7, 16 0, 18 9, 16 1, 7 1, 18 0, 2 9, 18 1, 46 6, 19 0, (a cyclopropane fatty acid derived from 18 1), 12 7, unidentified, 1 4) In contrast to this result we were unable to demonstrate any aggregation of the

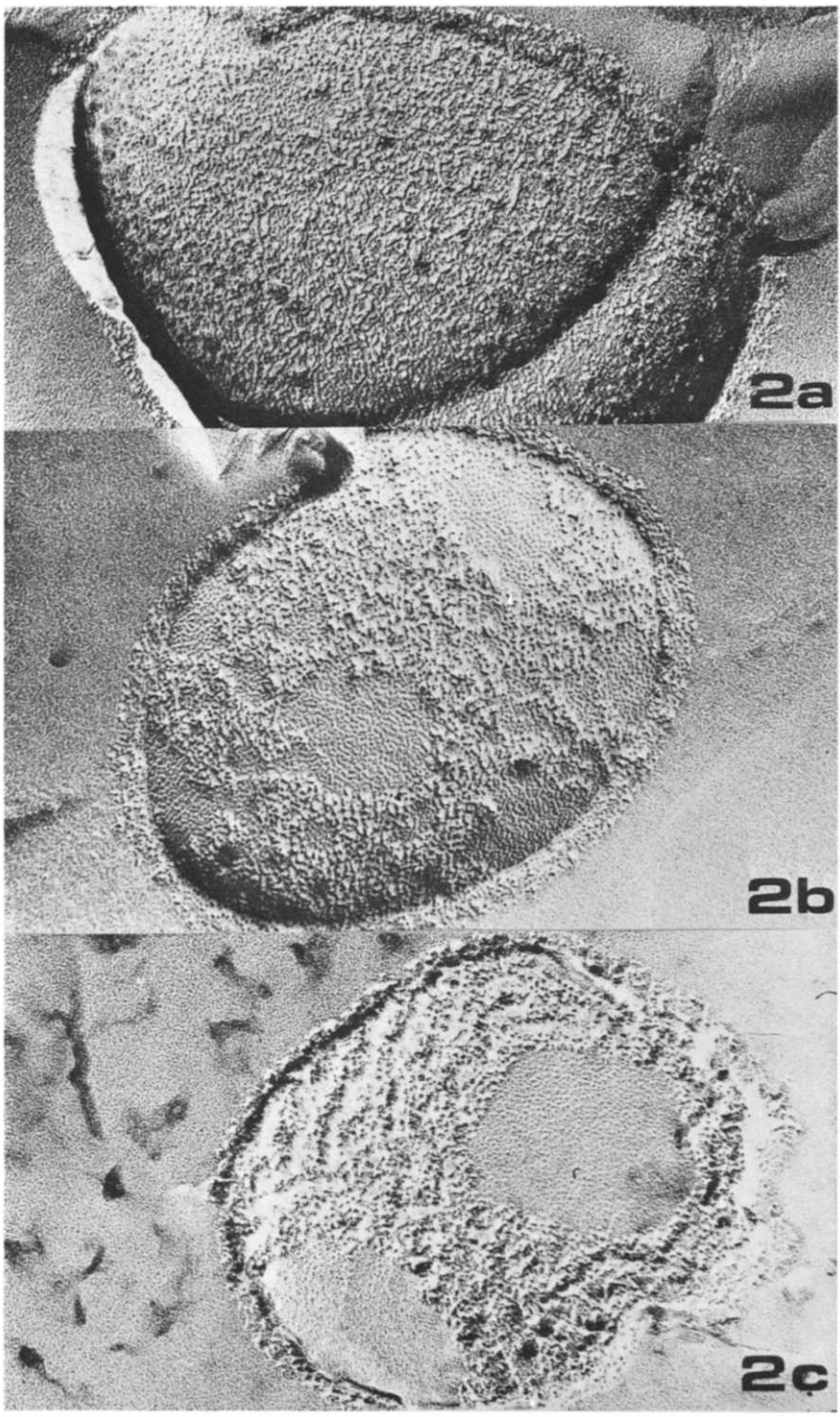

Fig 2 Freeze-fracturing faces of membranes of *S faecalis* quenched from 37 (a), 22 (b) and 0 °C (c) Magnification 120 000 ×

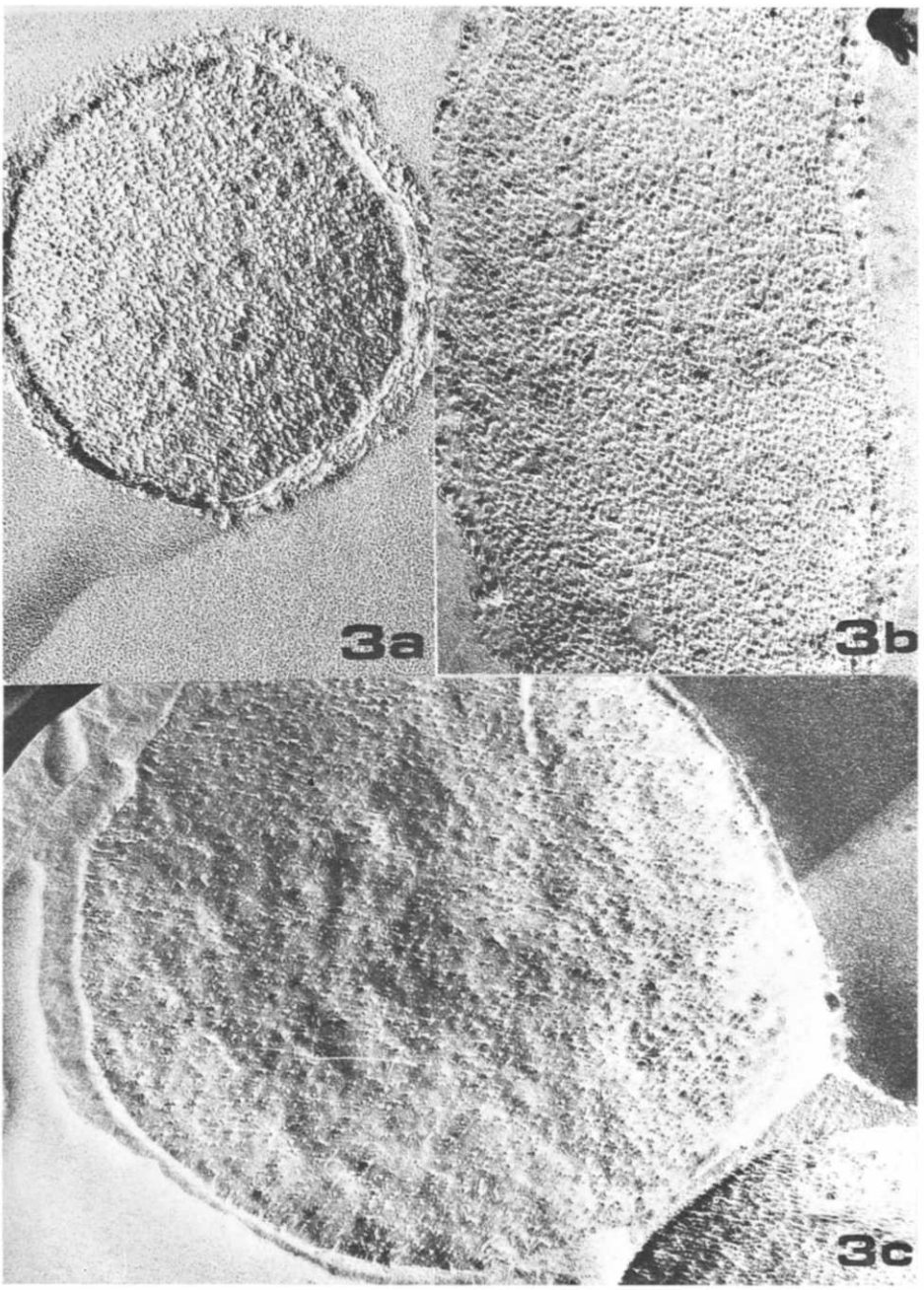

Fig 3 Freeze-fracture faces of membranes of *S aureus* (a), *B subtilis* (b) and *A laidlawii* grown on fatty acids prepared from *S aureus* phospholipids and quenched from −5 °C (c) The particle distribution in a and b is typical for membranes quenched from a temperature between −10 and 30 °C Magnification 120 000 ×

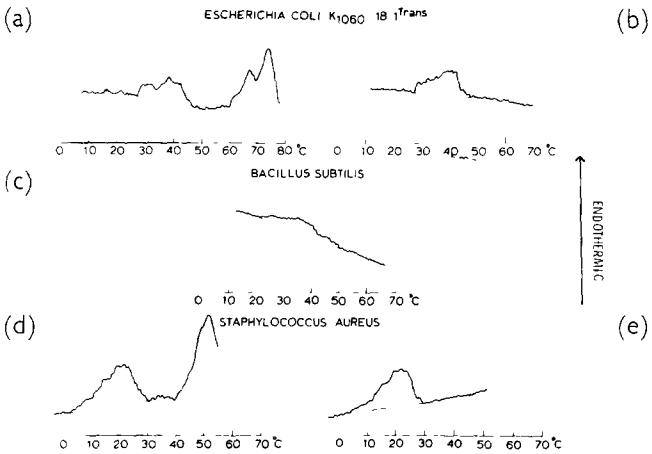

Fig 4 Differential calorimetric scans of some bacterial membranes Membranes from *E coli* K_{1060} cells grown on elaidic acid before (a) and after (b) protein denaturation, membranes from *B subtilis* grown with glucose (pH 5 4) (c), and membranes from *S aureus* cells grown without glucose (pH 6 5) before (d) and after (e) protein denaturation

particles in the membranes of *S. aureus* and *B. subtilis.* Figs 3a and 3b show that no aggregation could be observed even when the cells were cooled to −10 °C. On the other hand it could clearly be demonstrated that lipid phase transitions occur in the membranes of these cells In Fig 4 differential scanning calorimetry scans show that the lipid transition in *S aureus* occurs between 4 and 31 °C and in *B subtilis* between 11 and 40 °C Fig. 5b shows kinks in the Arrhenius plots of membrane bound enzymes. From comparison of the calorimetric and "enzymatic" transitions it may be concluded that the kinks in the Arrhenius plots occur somewhere in the middle of the differential scanning calorimetry transition (Figs 4 and 5). In seeking the reason for the non-appearance of the particle aggregation we realised that both *S. aureus* and *B subtilis* contain a high amount of branched chain fatty acids. Subsequent studies on *B. cereus* and *B megaterium*, containing branched chain acids, also failed to show aggregation of the particles

In order to prove that the branching of the fatty acids is indeed the reason for the non-appearance of particle aggregation, we extracted the phospholipids from *S aureus* and prepared fatty acids from them which were added to the growth medium of *A. laidlawii* B. As shown in Table I the branched fatty acid chains were incorporated to a high extent in the membrane lipids of *A. laidlawii*. It is shown in Fig 3c that at −5 °C, in contrast to cells with straight fatty acids no aggregation of particles could be observed in the membranes of these modified *A laidlawii* cells. It has been suggested that the presence of branched chain fatty acids allows supercooling [24]. However, the differential scanning calorimetry analysis clearly demonstrates reversible transitions (Fig 4b) thus indicating that this phenomenon, does not occur. It may be that the branched-chain lipids in the gel phase are orientated in a different manner to the normal straight-chain lipids Evidence for such a difference could be obtained from an X-ray diffraction pattern. X-ray measurements on *A. laidlawii*, *E. coli* [13, 14, 25] and liposomes [26] have shown that the liquid–crystalline state of the membrane

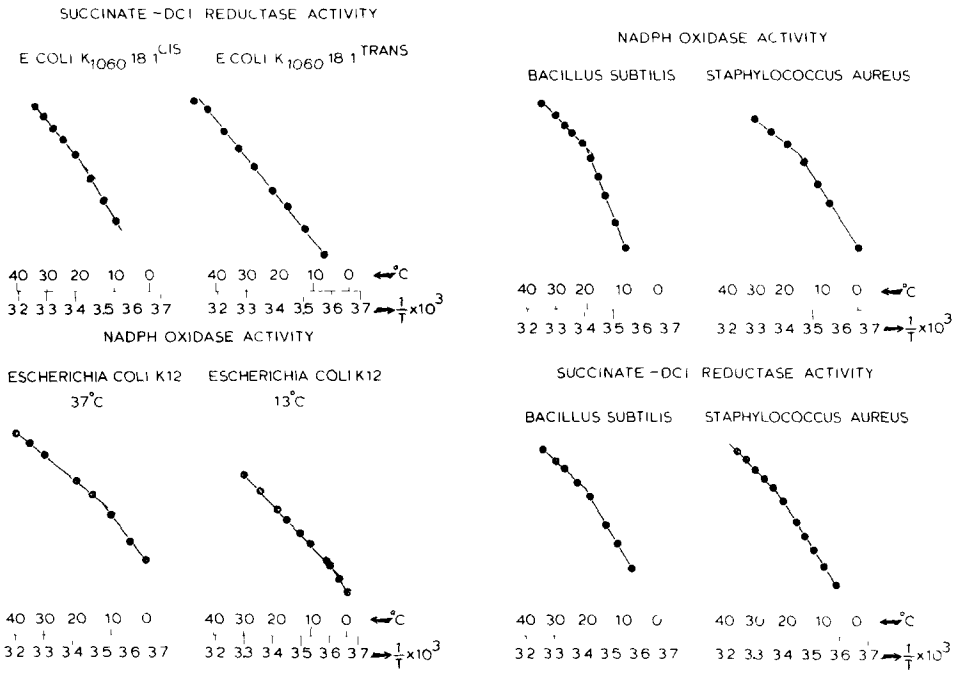

Fig 5 (a) The temperature dependency of NADPH oxidase and succinate–DCIP reductase activities in *E coli* membranes (b) The temperature dependency of NADPH oxidase and succinate–DCIP reductase activities in membranes of *S aureus* and *B subtilis*

TABLE I

FATTY ACID COMPOSITION OF *A LAIDLAWII* GROWN ON FATTY ACIDS PREPARED FROM *S AUREUS* PHOSPHOLIPIDS

Data are expressed as percentages of the total fatty acids *S aureus* phospholipids were saponified for 10 min with 0 6 g KOH in 5 ml triethyleneglycol at 160 °C After acidification of the reaction mixture, the fatty acids were extracted with diethyl ether *A laidlawii* cells were grown as described before [10] in the medium supplemented with 0 12 mM *S aureus* fatty acids

Fatty acid		Composition of the supplemented fatty acids prepared from *S aureus* phospholipids	Fatty acid composition of *A laidlawii* lipids
	12 0	+	1 3
Iso	14 0	0 9	5 8
	14 0	–	6 1
Iso	15 0	+	+
Anteiso	15 0	54 9	37 7
	15 0	+	–
	16 0	2 2	9 3
Iso	17 0	1 7	+
Anteiso	17 0	22 9	20 2
	18 0	5 9	3 1
	18 1		1 8
Anteiso	19 0	4 1	–
	20 0	4 2	6 3
Unidentified		3 2	8 4

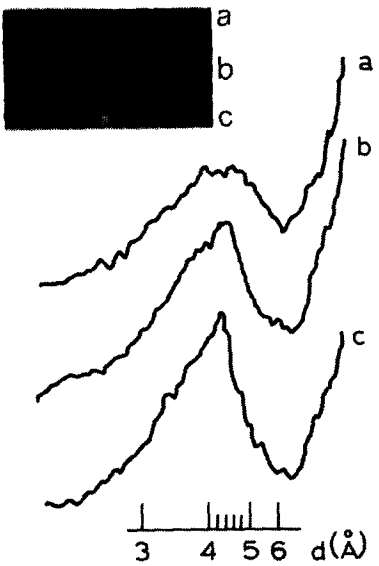

Fig 6 X-ray diffraction pattern of the lipids extracted from *S aureus* membranes The sample has been cooled gradually from 23 to −20 °C A funnel-shaped X-ray pattern was recorded during cooling from 23 to −20 °C Densitometer tracings were made at different times of the X-ray diagram corresponding with a = 20 °C, b = 0 °C and c = −15 °C.

lipids is characterised by a broad reflection around 4.5–4 6 Å and the gel state by a sharp reflection at 4.2 Å, which represents a hexagonal packing of the chains. Fig. 6 shows the X-ray diffraction pattern when the membrane lipids of *S. aureus* were scanned between 23 and −20 °C. At 20 °C the broad reflection at 4 4 Å reflects the state of disorder in the lipid phase. Below the transition the reflection is sharpened, however, a repeat distance of 4.4 Å instead of 4 2 Å was apparent in this membrane. Therefore it can be concluded that the branched chains are more loosely packed than straight chains in the gel phase. This difference in packing may be the explanation for the fact that membrane particles are not squeezed out of the membrane lipids during the phase transition

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