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SOME PHYSICAL INVESTIGATIONS OF THE BEHAVIOUR OF BACTERIAL SURFACES

X. THE OCCURRENCE OF LIPID IN THE STREPTOCOCCAL CELL WALL

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SUMMARY

1. The incorporation of glycerol, sodium oleate, or sodium acetate in the growth medium resulted in an increase in the amount of lipid in the cell wall of a number of strains of *Streptococcus pyogenes*.

2. This increase in lipid was detected electrophoretically by an increase in the mobility of the cells in the presence of sodium dodecyl sulphate, and was confirmed by solvent extraction.

3. The amount of lipid in cells grown in glycerol medium varied during the growth cycle.

4. Chemical groups sensitive to alkaline phosphatase (EC 3.1.3.1) were present on the surface of cells grown in Todd–Hewitt medium to which glycerol or oleate had been added, but not on cells grown in this medium alone.

5. Two groups of naturally occurring *Streptococcus pyogenes* strains are exceptional in that they possess large amounts of cell-wall lipid under ordinary conditions of growth. These include (a) tetracycline-resistant strains, in which the rate of production of lipid is unusually high, and (b) strains isolated from impetigo lesions, which are tetracycline-sensitive and produce no extracellular lipase.

INTRODUCTION

The production of acetone- and ether-soluble lipid by bacteria has not been studied extensively, but it has been stated that lipid accounts for 20–25% of the cell-wall material of Gram-negative bacteria and less than 5% of the wall of Gram-positive bacteria[1]. A small number of strains of *Streptococcus pyogenes* examined by extraction methods contained little or no cell-wall lipid[1,2].

There are scattered references to the dependence of the amount of cell-wall lipid on growth conditions, but this fact has not been fully investigated. LARSON AND

Abbreviations: SDS, sodium dodecyl sulphate; M, "matt", and G, "glossy" refer to the possession and lack of M protein antigen, respectively; the colonial appearance after 24 h on horse-blood agar and the presence of M antigen are related.

LARSON[3] observed that the addition of glycerol to the growth medium resulted in an increase in the amount of lipid extractable from the cells with acetone and ether; the increase was from 8 to 40% for *Staphylococcus albus*, from 10 to 20% for *Escherichia coli*, and from 9 to 34% for *Bacillus megaterium*. DYAR AND ORDAL[4,5] showed that the lipid content of one strain of *Staphylococcus aureus* was increased by the addition of glycerol to the growth medium. They detected this increase both by solvent extraction[4] and by an increase in electrophoretic mobility in the presence of SDS[5]. The latter method was used by LERCHE[6] to show the presence of lipid on one of two other strains of *Staphylococcus aureus*. The hydrophobic alkyl chain of SDS is soluble in the lipid on the cell wall, leaving the negative sulphate ion at the surface. Thus an increase of the negative charge of a bacterial cell (*i.e.* an increase in the negative value of the electrophoretic mobility) in the presence of SDS indicates the presence of surface lipid. At SDS concentrations exceeding 0.5 mM non-specific adsorption occurs on to almost all surfaces; this can lead to erroneous conclusions.

In all these instances the lipid content was increased merely by growth in a medium containing added glycerol. The lipid content of some organisms increases during growth in the presence of specific bacteriostatic or bactericidal agents, *e.g.* detergents[7] or crystal violet[8].

In addition to increases which occur under laboratory conditions, it has been reported that naturally occurring antibiotic-resistant strains of *Staphylococcus aureus*[9,10] and of some Gram-negative bacteria[11] have an increased lipid content. The increase in antibiotic resistance of three strains of *Staphylococcus aureus* was correlated with the amount of cephalin present[9].

In this work the effect of the nature of the growth medium on the amount of cell-wall lipid of strains of Group-A streptococcus has been studied. The occurrence of large quantities of cell-wall lipid in naturally occurring strains grown in normal medium was also investigated and this was correlated with the amount of extracellular lipase (EC 3.1.1.3).

METHODS

Growth media

Todd–Hewitt broth[12], a beef extract containing glucose and buffer, was used as the basic medium; "glycerol medium", "oleate medium", and "acetate medium" consisted of Todd–Hewitt broth to which had been added 3% (v/v) glycerol, 0.002% (w/v) sodium oleate, and 5% (w/v) sodium acetate, respectively. Although sodium oleate was inhibitory to streptococcal growth at higher concentrations, the concentration used had no effect on the rate of growth of the organisms.

Strains

The strains studied, using the previous terminology[12,13], were type 2 M and 2 G (matt and glossy variants of NCTC 8322), type 3M (strain D58X/11), type 6M (NCTC 8302), type 12G (glossy variant of strain 1130), type 12/416 (strain 416) and type 22/63T (NCTC 8330), together with 58 strains recently isolated in the Streptococcus Reference Laboratory.

Lipid extraction and fractionation

Cell walls were prepared from a 24-h culture by Mickle disintegration of washed suspensions of organisms in distilled water[14]. The cell-wall material was washed three

times in physiological saline, treated with RNAase (EC 2.7.7.16) (B.D.H.) at a concentration of 1 mg/ml of suspension, washed a further three times in saline, dried *in vacuo* over P_2O_5 and weighed. The lipid was extracted from a weighed amount (200 mg) of the wall material by refluxing with chloroform–methanol (2:1) for 8 h and then with ether for 8 h. The insoluble material was removed by centrifugation and the solvent distilled off from the supernatant. The last traces of solvent were removed in a vacuum dessicator over P_2O_5 and the residual lipid was weighed.

The weighed lipid was dissolved in ether and the phosphatide lipid precipitated by adding 30 vol. of acetone. The precipitate was allowed to stand at 4° for 24 h, and then removed by centrifugation, leaving the neutral lipid in the supernatant. The neutral lipid was recovered by distilling off the solvent and drying over P_2O_5 *in vacuo*.

Treatment with alkaline phosphatase

Washed cells from a 24-h culture were treated with hyaluronidase (EC 4.2.99.1)[12], washed three times in phosphate buffer solution (pH 7.0, I 0.012) and then suspended in barbiturate solution (pH 7.9, I 0.02) containing crystalline alkaline phosphatase (EC 3.1.3.1) (B.D.H.), of final concentration 0.0005% at 37° for 1 h.

Measurement of electrophoretic mobility

The electrophoretic mobility was measured by the method of GITTENS AND JAMES[15]. The washing procedure and the buffer solutions used for measurement were those described previously[12]. When buffer solutions of extreme pH value were used it was necessary to be sure that the surface of the cells was not permanently damaged by this treatment. After suspension in the buffer solution of extreme pH, the cells were washed twice in buffer solution (pH 7.1) and the mobility measured at this pH. When the mobility was significantly different from that of control cells in this buffer solution it was assumed that surface disorganisation had taken place, and the results at that pH were not accepted.

All the bacterial populations examined were electrophoretically homogeneous (confidence limit for a single mean at $P = 0.05$ is $\pm$ 3%), and hence only mean values are quoted. Samples in which the mobility values differ by 10% are, on statistical grounds, significantly different.

The use of SDS solution

Bacterial surface lipid was detected by studying the variation of mobility of the cells with the concentration of SDS[5] in the suspension medium. Cells harvested at 24 h were washed three times in phosphate buffer solution (pH 7.0, I 0.012) and then divided into four parts for suspension in phosphate buffer solution and in solutions of 10^{-6} M, 10^{-5} M, and 10^{-4} M SDS in phosphate buffer solution, respectively. These concentrations of SDS were not high enough to alter the pH, ionic strength or conductivity of the buffer solution. The results of this test for the presence of lipid were consistent with those obtained by solvent extraction. It was found that at concentrations of SDS exceeding 0.5 mM non-specific adsorption occurred on to the surface of *Strep. pyogenes*; this gave false results.

Test for lipase in culture supernatant

The supernatant from 5 ml of 24-h growth culture was adjusted to pH 8 with NaOH solution. To 1 ml of culture supernatant was added 0.1 ml of tributyrin and

2 drops of B.D.H. "Universal" indicator. The lipolytic activity was detected visually; the reaction was considered complete when the colour of the indicator changed from green to red. This usually took 3–5 days at 37° with streptococcal supernatants.

When testing the effect of sodium oleate on the lipase activity at other pH values, a modification of this procedure was used. To 1 ml of culture supernatant containing sodium oleate, 0.1 ml of tributyrin was added. Two controls, one containing no tributyrin and the other containing heat-inactivated culture supernatant were included, and the three tubes were incubated for the required time (usually 7–8 days). The volume of 0.2 N NaOH solution required to bring each solution to pH 7 was then determined. In general, the two control tubes gave identical titres. The difference between the volume of NaOH needed to neutralise the test solution and the control was a measure of the lipase activity.

RESULTS

Detection of surface lipid electrophoretically

The mobility of 24-h cells of the matt variants of types 2, 3 and 6 and the glossy variants of types 2 and 22/63T grown in Todd–Hewitt medium was determined in the standard phosphate buffer solution containing SDS. The mobility of cells of all these strains was constant within the limits of experimental error at concentrations in the range 0 to 10^{-4}M SDS (Table I), demonstrating the absence of surface lipid. However, the negative value of the mobility of the same strains after ten subcultures in glycerol medium increased with increasing SDS concentration (Table I). Thus, growth in glycerol medium led to the formation of surface lipid on the walls of these strains. The

TABLE I

THE EFFECT OF GROWTH IN DIFFERENT MEDIA ON THE ELECTROPHORETIC MOBILITY MEASURED IN THE PRESENCE OF SDS

Type	*Growth medium*	*Electrophoretic mobility* ($cm^2 \cdot V^{-1} \cdot sec^{-1} \times 10^4$)			
	SDS concentration (M)	*0*	10^{-6}	10^{-5}	10^{-4}
2M	Todd–Hewitt	−1.28	−1.25	−1.26	−1.26
	Glycerol	−1.16	−1.36	−1.46	−1.65
	Acetate	−1.26	−1.41	—	−1.70
	Oleate	−1.25	−1.33	−1.52	−1.64
2G	Todd–Hewitt	−1.32	−1.28	−1.27	−1.36
	Glycerol	−1.26	−1.49	−1.60	−1.80
	Acetate	−1.42	−1.71	—	−1.88
	Oleate	−1.22	−1.40	−1.59	−1.84
3M	Todd–Hewitt	−1.53	−1.45	−1.50	−1.44
	Glycerol	−1.21	−1.45	−1.62	−1.74
	Acetate	−1.45	−1.64	−1.79	−1.88
	Oleate	−1.45	−1.58	—	−1.89
6M	Todd–Hewitt	−1.20	−1.20	−1.20	−1.18
	Glycerol	−1.17	−1.27	−1.47	−1.78
	Oleate	−1.22	−1.33	−1.58	−1.88
22/63T	Todd–Hewitt	−1.79	−1.77	−1.77	−1.77
	Glycerol	−1.69	−1.87	−2.06	−2.28

supernatants of cultures grown in glycerol medium had similar lipase activities to those grown in Todd–Hewitt medium. The increase in the amount of cell-wall lipid appeared, therefore, to be due to a stimulation of the lipid production and not to a decrease in the lipase activity.

Since it is believed that substrates for lipid synthesis are first converted to acetate, the addition of sodium acetate in place of glycerol should produce the same result, if the acetate acts only as a substrate for lipid synthesis. After ten subcultures in acetate medium the mobility of the cells again increased with SDS concentration; the proportional increase in mobility was, however, lower than for cells grown in glycerol medium (40–60% increase in glycerol, 33% increase in acetate).

The amount of cell-wall lipid should also be increased by inhibiting the activity of extracellular lipase. At a concentration of 0.002%, sodium oleate does not effect the growth rate or the final amount of growth, but it reduces the lipase activity by 80% at pH 7.1, and completely inhibits it at pH 5.3. After ten subcultures through oleate medium, the mobility of the cells increased with increasing SDS concentration, again indicating the presence of surface lipid (Table I).

Repeated growth of the cells in these different media produced small changes in their mobility value when measured in the phosphate buffer solution, in the absence of SDS. With one exception, the changes were not significant. It was also observed that although the mobility of cells grown in Todd–Hewitt medium was constant for the strain and independent of the batch of medium, the mobility of cells grown in the presence of glycerol, acetate and oleate varied with the batch of medium. The important conclusions are that for cells of a given strain grown in a specified medium the mobility of the cells becomes more negative with increasing SDS concentration (*i.e.* in Table I only horizontal comparisons are strictly valid), and that the proportional increase in mobility in SDS is independent of the original mobility in the absence of SDS.

In a more detailed study of type-6M organisms it was found that the increased lipid content was attained during the first five subcultures in glycerol medium and remained constant up to 15 subcultures. This increased lipid content was not a stable character of the organisms for after one subculture in the absence of glycerol little lipid could be detected. Cells grown in oleate, retained their lipid after one subculture in the absence of oleate, but not after three.

The extraction of lipid from the walls of cells grown in various media

Little extractable material (about 1% of the dry weight) was detected in the cell wall of types 2M, 6M, and 22/63T grown in Todd–Hewitt medium (Table II). After

TABLE II

THE TOTAL LIPID CONTENT OF CELL WALLS OF *Strep. pyogenes* GROWN IN VARIOUS MEDIA

Type / Medium	Total cell-wall lipid (%)		
	Todd–Hewitt	Glycerol	Oleate
2M	0.7	21.0	22.2
6M	1.4	25.4	—
22/63T	1.1	25.5	—

ten subcultures in glycerol medium, the cell walls contained approx. 25% lipid; preliminary studies indicated that it was mainly neutral lipid.

Cell-wall preparations of type-2M organisms which had been subcultured ten times in oleate medium contained 22% lipid. This material, though mainly neutral lipid, appeared to contain a greater proportion of phosphatide lipid than was found in the material from the cells grown in glycerol medium.

The variation of the mobility of cells grown in glycerol medium, when measured in 0.1 mM SDS buffer solution, with the culture age

The amount of lipid present on the surface of *Strep. pyogenes* grown in glycerol medium represents a balance between the rate of production of lipid and the activity of the extracellular lipase. As with the hyaluronidase–hyaluronic acid equilibrium[12], it might be expected to vary with the age of the culture. Two 50-ml samples were aseptically removed from a culture of cells of type 22/63T in glycerol medium at intervals during the growth cycle (0–24 h). The cells in both samples were washed and treated with hyaluronidase (1 I.U./ml for 1 h at 37°), and then washed three times in phosphate buffer solution; those in one sample were finally resuspended in phosphate buffer solution and those in the other in 0.1 mM SDS buffer solution. The mobility values of the cells in the two suspensions were determined. The growth curve was plotted by determining the absorbancy at 600 mμ of samples removed periodically from the growing culture[12].

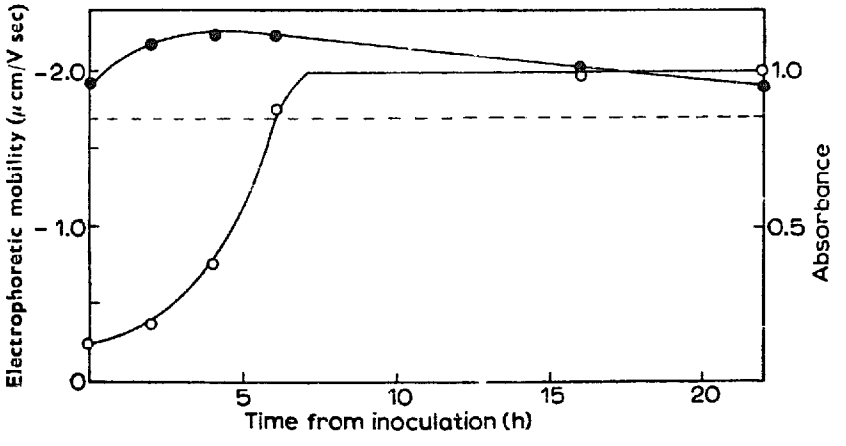

Fig. 1. The variation of the mobility of cells of type 22/63T grown in glycerol medium with the culture age. ●—●, mobility in 0.1 mM SDS solution; – – –, mobility in phosphate buffer solution; O—O, growth curve.

The mobility in phosphate buffer solution remained constant throughout the growth cycle. The mobility in 0.1 mM SDS buffer solution increased significantly to a maximum towards the end of the logarithmic growth phase and then decreased to the original value after 24 h (Fig. 1). The increase in mobility (approx. 15%) during the phase of active growth indicates that, even in glycerol medium, the rate of production of lipid is not much greater than the rate of its destruction by lipase.

A similar variation of the mobility in 0.1 mM SDS buffer solution with age was obtained with cells of the matt variant of type 6 when grown in glycerol medium.

The variation of the mobility of cells grown in various media with the pH of the suspension medium

The growth from 500 ml of a 24-h culture was harvested by centrifugation, treated with hyaluronidase, washed twice in the standard phosphate buffer solution and divided into a number of portions. The cells in each portion were subsequently washed twice in the appropriate barbiturate buffer solution of known pH (I 0.02) and resuspended in that solution. The mean mobility of the cells at each pH value was determined. The pH–mobility curves of cells of the M variant of type 6 grown in normal, glycerol and oleate medium were coincident at pH $>$ 5.5, but the isopotential points (*i.e.* the pH of zero charge) of the three surfaces were different; 4.6 in Todd–Hewitt, 4.0 in glycerol and 3.6 in oleate medium (Figs. 2–4). Since this displacement might have been due to the presence of surface phosphate groups[16] the effect of alkaline phosphatase was examined.

Cells harvested by centrifugation from the various media (*i.e.* Todd–Hewitt, glycerol and oleate) were treated with hyaluronidase and then with alkaline phosphatase before washing and resuspension in the appropriate buffer solutions. The pH–mobility curve of cells grown in Todd–Hewitt medium and treated with alkaline phosphatase was coincident with that of normal cells (Fig. 2). In contrast, the pH–mobility curves of the phosphatase-treated cells grown in glycerol and oleate were displaced from the previous curves and were coincident at pH values exceeding 5.5 (Figs. 3 and 4). Although the isopotential point, irrespective of the original value for the untreated cells was now constant at pH 4.6, the value for cells grown in Todd–Hewitt medium, the plateau mobility value of the cells grown in glycerol and oleate was less than that of normal cells. These results indicated the presence of phosphate groups on the surface of cells grown in glycerol or oleate medium.

The natural occurrence of strains of Strep. pyogenes containing large amounts of cell-wall lipid

Cells resistant to bacteriostatic or bactericidal agents (*e.g.* crystal violet and antibiotics) possess large quantities of cell-wall lipid[7–10]. Streptococci are extremely sensitive to most antibiotics, but a number of strains resistant to tetracycline have been isolated recently in Great Britain and America[17,18]. Tests for lipid were therefore made on a collection of recently isolated strains of *Strep. pyogenes* some of which were resistant to and some sensitive to tetracycline. The mobility of 32 out of 34 tetracycline-resistant strains (Table III) increased with SDS concentration, indicating the presence of surface lipid. From the correlation, observed earlier, between the electrophoretic mobility in the presence of various concentrations of SDS and the total extractable lipid (see also ref. 4), it was possible to assess the lipid content of these naturally occurring strains from electrophoretic studies. The total extractable lipid of 2 of these strains, selected at random, was in agreement with the values given in Table III. It was shown by the electrophoretic technique that 17 out of 18 tetracycline-sensitive strains had no surface lipid, and in the remaining strain it constituted only 5–10% of the cell wall. A strain of *Strep. pyogenes*, type 12G (ref. 12) which had been shown in earlier studies to have a low iso-potential point, was also tetracycline-sensitive and possessed a small amount of surface lipid. All strains tested, both tetracycline-sensitive and -resistant, produced lipase of about the same activity. Typical

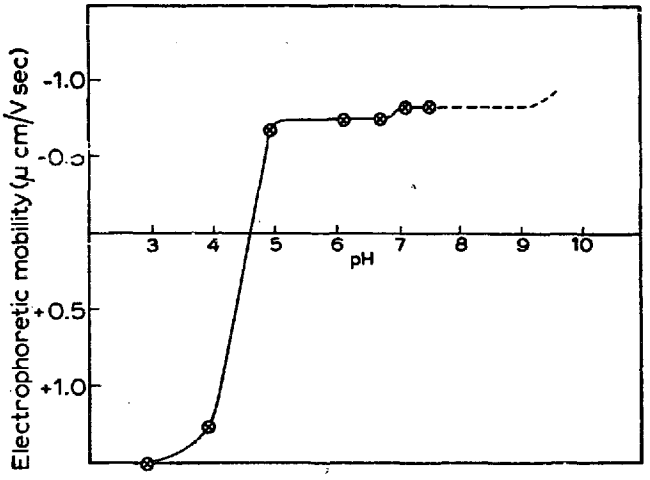

Fig. 2. pH–mobility curves of 24-h cells of type 6M grown in Todd–Hewitt medium. ×—×, normal cells; O—O, cells after treatment with alkaline phosphatase.

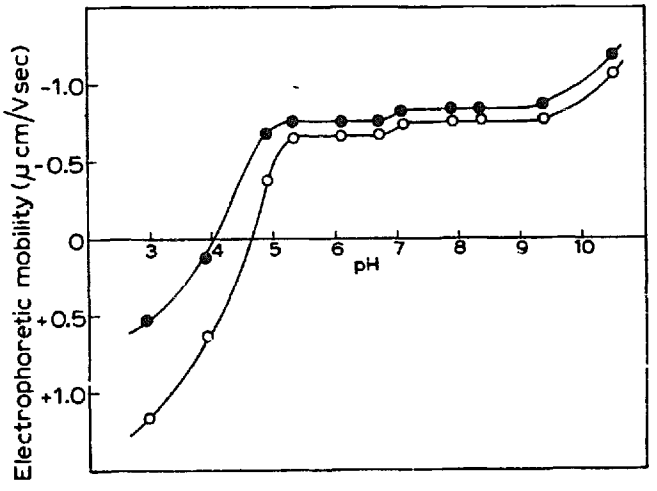

Fig. 3. pH–mobility curves of 24-h cells of type 6M grown in glycerol medium. ●—●, normal cells; O—O, cells after treatment with alkaline phosphatase.

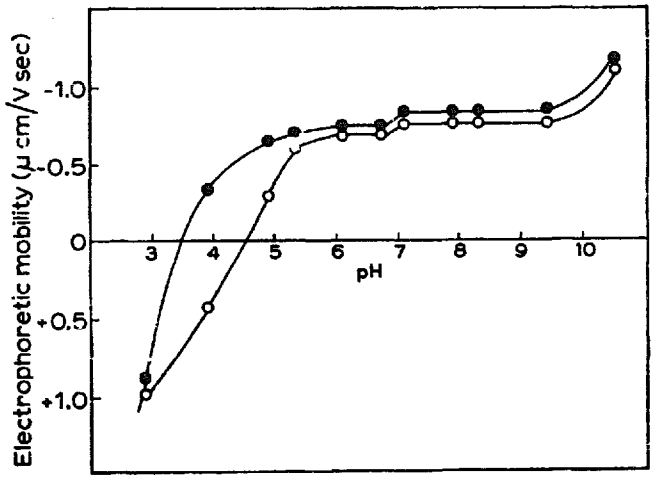

Fig. 4. pH–mobility curves of 24-h cells of type 6M grown in oleate medium. ●—●, normal cells; O—O, cells after treatment with alkaline phosphatase.

results for 8 tetracycline-resistant and 8 tetracycline-sensitive strains, together with the values for the 2 aberrant strains of each group, are shown in Table IV.

MAXTED[19] observed that strains of *Strep. pyogenes* isolated from the skin lesions of patients with impetigo, which have a characteristic antigenic structure[20], produce no detectable lipase. This was confirmed for 6 impetigo strains, 3 of which had T-agglutination pattern 3/13/B3264 and 3 the pattern 5/11/12/27/44. All 6 were tetracycline-sensitive. Two of these strains were examined by solvent extraction and by the electrophoretic method; their cell walls contained about 20% lipid. The increased lipid content of these impetigo strains is, therefore, associated with a lack of extracellular lipase. A more extensive study of the cell wall composition of streptococci from impetigo might be of interest.

TABLE III

A COMPARISON OF THE LIPID CONTENT OF THE TETRACYCLINE-RESISTANT AND -SENSITIVE STRAINS STUDIED

*Lipid content (%)**	*Tetracycline-resistant*	*Tetracycline-sensitive*
15–25	7	0
10–15	7	0
2–10	18	2
< 2	2	22

* Deduced from the study of the variation of mobility with SDS concentration (see also ref. 4).

TABLE IV

THE CORRELATION OF THE SENSITIVITY TO TETRACYCLINE OF *Strep. pyogenes* AND THE LIPID CONTENT

Strain	*Serological type*			*Mobility ($cm^2 \cdot V^{-1} \cdot sec^{-1} \times 10^4$) in the presence of SDS*			*Sensitivity to tetracycline**
	T	*R*	*M*	10^{-6} *M*	10^{-5} *M*	10^{-4} *M*	
340	2/28	28	—	−1.27	−1.40	−1.98	R
416	12	—	—	−1.61	−1.71	−2.37	R
2270	12	—	12	−1.12	−1.15	−1.80	R
513	4/28	28	—	−1.18	−1.38	−1.67	R
455	—	28	—	−0.94	−0.97	−1.45	R
408	4/28	28	—	−0.94	−0.99	−1.59	R
2324	12	—	12	−0.97	−1.06	−1.33	R
2256	12	—	—	−1.08	−1.11	−1.37	R
2318	2	—	—	−1.24	−1.26	−1.18	R
312	3/B3264	—	—	−1.00	−0.99	−1.02	R
325	12	—	—	−1.05	−1.01	−1.03	S
360	2	—	—	−1.07	−1.08	−1.14	S
492	1	—	1	−1.17	−1.13	−1.14	S
374	12	—	—	−1.00	−1.00	−1.02	S
504	4/28	28	—	−0.97	−0.93	−0.98	S
531	3/13	—	—	−0.90	−0.92	−0.92	S
197	12	—	12	—	−0.93	−0.93	S
2557	12	—	12	−0.90	−0.90	−0.91	S
34	12	—	—	−1.04	−1.02	−1.13	S
12G	12	—	—	−1.42	−1.38	−1.93	S

* Tested with paper discs containing 5 μg tetracycline, R = absence of inhibition.

DISCUSSION

It is now widely accepted that the lipid content of the cell walls of Gram-positive bacteria is less than 5%, whereas that of Gram-negative bacteria is 20–25%. This difference has even been used as the basis for the explanation of the Gram stain[21], although several workers[3,5,6] have shown that the amount of cell-wall lipid depends on the composition of the growth medium. The organisms studied here, whether containing large or insignificant amounts of cell lipid, were always Gram-positive.

In agreement with previous observations[1,2], the lipid content of the cell walls of representative strains of *Strep. pyogenes* grown under normal conditions (*i.e.* in Todd–Hewitt medium) was about 1%. When these organisms were repeatedly subcultured in a medium containing added glycerol or sodium acetate the cell-wall lipid increased to 20–25%; the lipase activity of the culture supernatants, however, was the same as that in Todd–Hewitt alone. A similar increase in the lipid content occurred when the organisms were grown in the presence of sodium oleate, but in this instance the accumulation was due to the inhibition of the extra-cellular lipase. It is therefore apparent that the amount of cell-wall lipid can be increased either by promoting the production of lipid or by inhibiting the lipase.

The total cell-wall lipid was determined by the solvent extraction method, while the presence of surface lipid was demonstrated by the increase in the negative value of the electrophoretic mobility of the cells in the presence of SDS (Table I). The correlation between the total extractable lipid and the change of mobility in SDS for the strains studied by both methods made it possible to assess the total lipid content of cell walls of other strains from electrokinetic studies alone (Table III).

The amount of cell-wall lipid of organisms growing in glycerol medium varied throughout the growth cycle; this probably reflected a disturbance of the lipid–lipase equilibrium. During the logarithmic growth phase there was a small, but significant increase in the negative value of the mobility of the cells in the presence of 0.1 mM SDS (Fig. 1). This suggested that the rate of production of lipid, stimulated by the glycerol, exceeded the rate of its destruction by lipase, at this time. In the stationary phase, when lipid production had ceased, the substrate was slowly destroyed by the enzyme.

The stimulation of lipid production by the different growth conditions resulted in the formation of both neutral and phosphatide lipid, although more phosphatide lipid was produced in the presence of sodium oleate than in the presence of glycerol. The lower iso-potential point of cells (type 6M) grown in oleate (3.6) and glycerol (4.0) compared with that of normal cells (4.6), which can be attributed to the presence of surface phosphate groups (pK 1.8, ref. 16), further supports the fractionation results. Confirmation of the presence of surface phosphate groups was obtained by treatment of the various cells with alkaline phosphatase. The pH–mobility curves (Figs. 3 and 4) for the phosphatase-treated surfaces of cells grown in glycerol and oleate were now coincident at pH values exceeding 5.5, suggesting that the surfaces were indistinguishable electrokinetically. Further the iso-potential points of these surfaces were identical with that of normal cells (4.6). Although the general shape of these curves was similar to that of the normal cells, (Fig. 2) indicating the presence of similar ionogenic groups, the plateau values were less than that of the normal cells. It was thus apparent that the lipid present on the surface of cells grown in glycerol or oleate-grown cells covered a small proportion of the surface carboxyl groups.

During the course of this work a number of recently isolated strains of *Strep. pyogenes* were examined, and it was found that some of them had a high lipid content, even when grown in Todd–Hewitt medium (Table IV). The strains with the high lipid content could be divided into two distinct classes according to the way in which the lipid–lipase equilibrium had apparently been disturbed. In streptococci of the first class, which included only strains resistant to the antibiotic tetracycline, the activity of the extra-cellular lipase in the culture supernatants was normal. The behaviour of these strains in an ordinary nutrient medium, such as Todd–Hewitt broth, thus resembles that of normal strains of streptococci grown in glycerol medium. Most tetracycline-sensitive strains had the normal 1% lipid in their cell walls. It is possible, therefore, that the changes in metabolism which are associated with resistance to tetracycline may also result in a stimulation of the production of lipid.

The second class of strains with high lipid content were isolated only from impetigo lesions. These strains, grown in Todd–Hewitt medium, produced no detectable lipase and thus resemble normal strains grown in the presence of sodium oleate.

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