Cellular Lipid and the Antistaphylococcal Activity of Phenols

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SUMMARY

Staphylococcus aureus (OXFORD) when grown in the presence of glycerol increased its cellular lipid content to the extent of 18 % calculated on a cell dry-weight basis. When challenged by a homologous series of 4-*n*-alkyl phenols (H- to C₆H₁₃-) an increase in resistance was noted with the glycerol grown (fattened) cocci after the 4-*n*-butyl (C₄H₉-) phenol. This phenomenon was investigated by means of electrophoresis of single cocci, the measurement of drug uptake, and the effect on leakage of cytoplasmic constituents as well as by the assessment of minimum inhibitory concentrations. Electron micrographs of thin sections of normal and fattened cocci were also examined.

INTRODUCTION

Hugo & Stretton (1966*a, b*) showed that when the lipid content of certain Gram-positive organisms was increased by growth in the presence of glycerol, these fattened organisms became more resistant to the penicillins, quinacillin, benzylpenicillin, methicillin and cloxacillin; in some cases, when normal lipid content of the organisms was decreased by treatment with lipase, the resistance decreased. It was decided to investigate this phenomenon further but to challenge both fattened and normal organisms with a homologous series of compounds, the 4-alkyl phenols, because the physical properties of this series might be expected to vary in a regular and determinable or determined pattern. Previous findings had shown that the lipid content of the Oxford staphylococcus could be increased threefold on growth in the presence of glycerol; accordingly, this organism was used. Some of the results reported here were briefly reported elsewhere (Hugo & Franklin, 1966).

METHODS

Organism. The organism used throughout this work was *Staphylococcus aureus*, strain OXFORD.

Materials. 4-Methylphenol (*p*-cresol) and oleyl alcohol were of laboratory reagent grade (British Drug Houses Ltd.); *p*-*n*-butylphenol and *p*-*n*-pentyl (amyl) phenol were gifts from Boots Pure Drug Co. Ltd., Nottingham; the remaining phenols were from Kodak Ltd., Kirby, Liverpool. All other chemicals were of analytical reagent quality.

The phenols were purified by distillation, yielding white crystalline products and were stored in sealed amber glass ampoules, from which samples were removed as required. Melting points (uncorrected) of the products so purified were: phenol 43°, 4-methylphenol (*p*-cresol) 34°, 4-ethylphenol 47–48°, 4-*n*-propylphenol 21–22°, 4-*n*-butylphenol 22°, 4-*sec*.-butylphenol 59°, 4-*n*-pentylphenol (amylphenol) 87–89°, 4-*tert*.-amylphenol 92–93°, 4-*n*-hexylphenol 23–24°.

Media and conditions of growth. Culture media were prepared from Oxoid material and contained (g./l.): peptone (Oxoid no. 1) 10, meat extract (Lemco, Oxoid) 5, sodium chloride 5, and, where appropriate, glycerol 30. Sterilization was done at 115° for 30 min.; final pH 7.4.

Buffer solutions. Phosphate buffer was prepared by mixing appropriate quantities of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ and $\text{KH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, giving a range from pH 5.4 to 8.0. Tris buffer was used when cellular inorganic phosphate was being investigated. Carbonate + bicarbonate buffer was used in one experiment for the range pH 8–10.6.

Determination of minimum inhibitory concentrations. A tube dilution method was used with inoculum to give finally 10^6 cocci/ml. Results were read after 3 days.

Total lipid and electrophoretic mobilities of cocci. These were determined as previously described (Hugo & Stretton, 1966a).

Analytical determination of phenols. This was by the colorimetric method of Lykken, Treseder & Zahn (1946), which involves the conversion of the phenols to nitrosophenols and the determination of the coloured nitrosophenol spectroscopically with a Unicam SP 600 (Cambridge Instruments Ltd.). For each phenol, a calibration curve was constructed from which the phenol content of unknown solutions was calculated. When phenols were to be determined in aqueous solution uncontaminated by cellular constituents the appropriate D value ($\ln I_0/I$) was determined in the ultraviolet region at the previously determined λ_{max} using the SP 500 spectrophotometer (Cambridge Instruments Ltd.). The λ_{max} values lay between 270 and 280 m μ .

Water solubility of phenols. Water was saturated with the appropriate phenol which was determined spectroscopically. From the optical density of the saturated solution and a solution of known concentration at the appropriate λ_{max} the water solubilities were calculated. Values obtained (g./100 ml.) were: phenol, 9.37; 4-methylphenol, 2.54; 4-ethylphenol, 0.72; 4-propylphenol 0.36; 4-*n*-butylphenol, 0.10; 4-*n*-pentylphenol, 0.044; 4-*n*-hexylphenol, 0.045; 4-*sec*.-butylphenol, 0.12, 4-*tert*.-amylphenol, 0.033.

Distribution coefficients. These were obtained as between oleyl alcohol and 0.067 M-phosphate buffer by adding known volumes of the alcohol and the phenol in the buffer and rotating in a stoppered tube at room temperature until equilibrium was established. A sample of the aqueous layer was assayed for its phenol content spectrophotometrically and the partition coefficient calculated from the following expression:

$$\text{distribution coefficient} = \frac{\text{Volume of aqueous phase} \times \text{final concentration in oily phase}}{\text{Volume of oily phase} \times \text{final concentration in the aqueous phase}}$$

The phosphate buffer held the pH value at 7.4 but was of a sufficiently high dilution to ensure that the ions did not contribute significantly to the distribution. Phenol concentrations in the initial aqueous layer were of the same order as encountered in the antibacterial studies. Because of the high solubility of the higher members in the oily phase, their concentration in the aqueous phase was too low for reproducible results to be obtained.

Determination of uptake of phenols by Staphylococcus aureus. To 18 ml. of the phenol solution was added 2 ml. of the bacterial suspension (equiv. 10 mg. dry wt./ml.) in 0.067 M-tris buffer (pH 7.4). The mixtures were maintained in a water bath at 37° and at appropriate times the bacteria were removed by centrifugation twice (15 min. each) at 9000g and the residual phenol determined by the colorimetric method.

Determination of phenol-induced leakage of cellular constituents. Determination of leakage of cellular material absorbing at 260 m μ is complicated by the 270–280 m μ absorbing peak of the phenols. However, by extraction of the supernatant liquid remaining after centrifugation of phenol-treated cocci with three 25 ml. amounts of chloroform, the phenols (except phenol itself) were removed and the aqueous layer after warming to remove dissolved chloroform was examined for material absorbing at 260 m μ . The adequacy of this procedure was tested by mixing the supernatant fluid from boiled cocci, of which the 260 m μ absorbing material had been determined, with a solution of each of the phenols in turn, subjecting this mixture to the extraction procedure and redetermining the 260 m μ absorbing material; almost quantitative recoveries were obtained. Leakage of cytoplasmic constituents was also investigated by determining inorganic phosphate in the cell-free supernatant fluid by the method of Fiske & SubbaRow (1925). Phenols, at the concentrations used, did not interfere in this determination.

Preparation of thin sections of cocci and electron microscopy. This was done according to the method described by Hamilton & Stubbs (1967), with the glutaraldehyde + OsO₄ fixative.

RESULTS

Growth of cocci in glycerol and their lipid content

Ten serial subcultures of the Oxford staphylococcus in the culture medium containing 3 % glycerol resulted in a total lipid content of 18.8 ± 0.8 ; the lipid content of cocci grown under similar circumstances but without glycerol was $6.5 \% \pm 0.4$, in good agreement with the findings of Hugo & Stretton (1966*a, b*). Growth curves showed that the presence of glycerol had no significant effect on the log phase or the mean generation time of this organism but the final yield of organisms was higher with glycerol. Because fattened cocci rapidly revert to their normal lipid content after one subculture in the absence of glycerol, all experiments with fattened cocci involving growth were done in the presence of glycerol, 3 % (Fig. 1).

Table 1. *Minimum inhibitory concentrations for control and fattened cocci of Staphylococcus aureus (OXFORD)*

	Control cocci m.i.c. (μ g./ml.)	Fattened cocci m.i.c. (μ g./ml.)
Phenol	2200	2200
Cresol	1300	1300
Ethylphenol	640	640
Propylphenol	390	390
<i>n</i> -Butylphenol	85	90
<i>n</i> -Amylphenol	38	44
<i>n</i> -Hexylphenol	42	52
<i>sec.</i> -Butylphenol	140	150
<i>tert.</i> -Amylphenol	55	110

Minimum inhibitory concentrations of phenols

These are presented in Table 1. The final inoculum was 10^6 cocci/ml. As the tubes inoculated with fattened cocci must contain 3 % glycerol and in view of the findings of Cooper (1948) that glycerol in concentrations of 30–40 % depresses the antibacterial

effects of phenols to the extent of 20–40 %, experiments were made on the effect of 3 % glycerol on the minimum inhibitory concentration (m.i.c.) values of the phenols used. At this concentration, glycerol had no detectable effect.

Uptake of phenols by normal and fattened cocci

Preliminary experiments showed that equilibrium between cocci and the phenol was rapidly attained and was virtually complete in 5 min. The data obtained is shown in Table 2, where it can be seen that the fattened cocci took up slightly less of the phenols tested than did the controls.

Table 2. *The uptake of phenols by fattened and unfattened (normal) cocci of Staphylococcus aureus (OXFORD)*

Phenol	Uptake of phenol, $\mu\text{g}/\text{mg}$ dry wt. cells	
	Fattened cells	Unfattened cells
Propyl	42.9	46.4
<i>n</i> -Butyl	22.2	27.3
<i>n</i> -Amyl	34.8	49.9
<i>sec.</i> -Butyl	84.8	96.3
<i>tert.</i> -Amyl	41.8	50.8

Table 3. *Staphylococcus aureus (OXFORD). Increase in electrophoretic mobilities as compared with mobilities in buffer of control cocci (a) and fattened cocci (b) in the presence of various concentrations of nine *p*-alkyl phenols in phosphate buffer (pH 7.4, $I = 0.01$)*

Concentration ($\mu\text{g}/\text{ml.}$)	Phenol			4-Methylphenol			4-Ethylphenol		
	1000	2000	3000	1000	1500	2000	400	600	800
Increase in mobilities ($\mu/\text{sec.}/\text{V.}/\text{cm.}$)	{(a) 0.07 0.29 0.33			{(a) 0.52 0.55 0.60			{(a) 0.55 0.70 0.82		
	{(b) 0.51 0.64 0.69			{(b) 0.76 0.77 0.84			{(b) 0.80 0.92 1.04		
Concentration ($\mu\text{g}/\text{ml.}$)	4-Propylphenol			4-Butylphenol			4-Pentylphenol		
	200	400	500	50	100	150	20	50	80
Increase in mobility ($\mu/\text{sec.}/\text{V.}/\text{cm.}$)	{(a) 0.56 0.67 0.94			{(a) 0.79 1.04 1.48			{(a) 0.52 0.72 1.17		
	{(b) 0.80 0.90 1.16			{(b) 1.02 1.46 1.82			{(b) 0.76 1.13 1.43		
Concentration ($\mu\text{g}/\text{ml.}$)	4-Hexylphenol			4- <i>sec.</i> -Butylphenol			4- <i>tert.</i> -Pentylphenol		
	30	60	75	100	150	200	50	100	125
Increase in mobility ($\mu/\text{sec.}/\text{V.}/\text{cm.}$)	{(a) 1.15 1.43 1.53			{(a) 0.78 0.90 1.07			{(a) 0.56 0.75 0.96		
	{(b) 1.46 1.66 1.86			{(b) 1.05 1.17 1.31			{(b) 0.79 0.99 1.28		

Electrophoretic mobilities

Mobilities in the presence of sodium dodecylsulphate (SDS). Data obtained in the presence and absence of SDS (10^{-6} , 10^{-5} , 10^{-4} M) gave figures of a very similar order to that obtained by Hugo & Stretton (1966a) for the Oxford staphylococcus and confirmed the build-up of surface lipid in the presence of glycerol and its rapid depletion after one subculture in its absence (Fig. 1).

Mobilities of fattened and unfattened cocci in the absence of added agent. The

absolute mobility, $\mu/\text{sec.}/V./\text{cm.}$ measured on the same day of fattened cocci was 2.002 and of control cells, 2.239, a difference of 10.2%.

Mobilities of fattened and unfattened cells in the presence of phenols. Mobilities were measured at three concentrations of each phenol. The data are presented in Table 3, where it can be seen that whereas the phenols in general increase the negative mobility of the cocci the increase was greater with fattened cocci. The effect of increasing chain length on the mobility differences is shown in Fig. 2. Similar figures were obtained at different initial concentrations of the phenol.

Fig. 1

Fig. 2

Fig. 3

Fig. 1. The build-up of cellular lipid in *Staphylococcus aureus* (OXFORD) on 10 successive subcultures in nutrient broth containing 3% glycerol and its depletion after one subculture in the absence of glycerol. Total lipid obtained by extraction, $\bigcirc-\bigcirc-\bigcirc$; electrophoretic mobility in the presence of 10^{-4} M-SDS, $\bullet-\bullet-\bullet$.

Fig. 2. Effect of chain length (number of carbon atoms in the 4-alkyl phenol substituent) on the electrophoretic mobility of *Staphylococcus aureus* (OXFORD) cocci. Fattened cocci, $\bullet-\bullet-\bullet$; control cocci, $\bigcirc-\bigcirc-\bigcirc$.

Fig. 3. The effect of pH value on the electrophoretic mobilities of *Staphylococcus aureus* (OXFORD) cocci in the presence and absence of 4-ethylphenol (400 $\mu\text{g.}/\text{ml.}$). Control cocci in buffer, $\bigcirc-\cdots-\bigcirc$; fattened cocci in buffer, $\bullet-\cdots-\bullet$; control cocci in ethylphenol solution, $\bigcirc-\bigcirc-\bigcirc$; fattened cocci in ethylphenol solution, $\bullet-\bullet-\bullet$.

Effect of pH on the mobilities of cocci in the presence of phenols. For the range pH 5–8 phosphate buffer was used and for pH 8–10.6 carbonate+bicarbonate buffer. Phenol solution, buffer solution and coccal suspensions were mixed so that the final ionic strength of the system was 0.01, and the coccal density equiv. 0.1 mg. dry wt./ml. Similar patterns were found for each of the phenols. The data for *p*-ethylphenol are shown in Fig. 3. This experiment indicated the necessity for controlling the pH value of the system during electrophoretic studies, for small changes in pH value over the range pH 6–8 result in marked changes in mobilities.

Phenol-induced leakage of cellular constituents from fattened and unfattened cocci

When the effect of time on leakage of 260 $m\mu$ absorbing material and inorganic phosphate was examined, it was found that a rapid initial leakage of both constituents occurred within 2.5 min. In the case of the lower members of the phenol series (up to the propyl compound) it was complete in 30 min.; in the remainder it slowly increased

up to 180 min. When the data for fattened and unfattened cocci are compared it can be seen that there was a rather greater release of these materials from the unfattened cocci. This is illustrated in Table 4, where the leakage of the two cytoplasmic constituents after 15 min. contact with the phenols is presented.

Fig. 4

Fig. 5

Fig. 4. *Staphylococcus aureus* (OXFORD). Effect of chain length (number of carbon atoms) in 4-alkyl substituent on: electrophoretic mobility, x—x; m.i.c. plotted reciprocally, ●—●—●; leakage of 260 mμ absorbing material, ○—○; water solubility, +—+—+.

Fig. 5. *Staphylococcus aureus* (OXFORD). Effect of chain length on the difference in observed effect as between control and fattened cocci as measured by: m.i.c. plotted reciprocally, x—x; electrophoretic mobility, ○—○; leakage of 260 mμ absorbing material, ●—●.

Table 4. *Staphylococcus aureus* OXFORD. The release of inorganic phosphate (Pi) and 260 mμ absorbing material from normal and fattened cocci after treatment for 15 min. with phenols at the concentrations stated

	Concn. phenol (μg./ml.)	μg Pi/mg, dry wt. cocci		ln I ⁰ /I (260 mμ)/mg. dry wt. cocci	
		Control	Fattened	Control	Fattened
Phenol	500	0.44	0.41	—	—
4-Methylphenol	250	0.32	0.18	0.24	0.13
4-Ethylphenol	120	0.33	0.28	0.13	0.11
4-Propylphenol	200	0.28	0.22	0.21	0.20
4-Butylphenol	100	0.53	0.36	0.33	0.25
4-Pentylphenol	80	2.78	2.07	0.50	0.30
4-Hexylphenol	60	3.33	3.07	0.66	0.43
4-sec.-Butylphenol	200	0.63	0.60	0.45	0.41
4-tert.-Pentylphenol	100	0.80	0.60	0.40	0.27

Electron micrographs

Thin sections of the Oxford staphylococcus grown for 10 generations in the presence of glycerol showed a distinct thickening of the extra-cytoplasmic layers (Plate 1). The thickening appeared to be confined to the walls and was especially pronounced in the septum separating the dividing cocci where presumably active cell-wall synthesis was occurring.

General

The inter-relationship of the several criteria and properties used in this work to evaluate coccal interaction are convincingly seen when the results are plotted on one graph as a function of the number of carbon atoms in the 4-primary alkyl substituent. In the first such plot (Fig. 4) the general effect of increasing the number of carbon atoms on the antibacterial and auxiliary activities and properties of phenols upon normal cocci is shown. It is clear that the side chain effect makes itself felt after the C₃ (propyl) phenol.

The influence of cellular lipid on the m.i.c., mobility and cytoplasmic integrity as it changes with chain length of the phenols was shown by plotting differences in response at each experimental point as between normal and fattened cocci against alkyl chain length (Fig. 5). Once again the differences become marked after the -C₃ compound, suggesting that lipid begins to exert its protective effect when the phenol tends to become more lipid soluble.

DISCUSSION

It is apparent from evidence from various sources that variation in the level of cellular lipid may be one of the factors involved in bacterial resistance (Chaplin, 1952; Church, Halvorson & Ramsey, 1956; Lowick & James, 1957; Vaczi & Farkas, 1961; Ivanov, Markov, Golowinsky & Charisanova, 1964; Truby & Bennett, 1961).

The use of a homologous series of phenols, such as the 4-normal alkyl phenols, a family of compounds whose properties change in a fairly regular way, with regard to water solubility and distribution coefficient between an aqueous and oily phase, might prove to be an ideal tool to test systematically the effect, if any, of cellular lipid upon resistance.

In considering the data presented in this paper it is reasonable to suppose that the phenols will distribute themselves between the lipid and aqueous components in the cell, in accordance with the normal partition laws, and in the case of fattened cells presumably these phenols will be partially distributed in the deposited lipid.

As the length of the normal alkyl side chain at position 4 increases, not only does the solubility in water decrease and the solubility in lipid increase, but the molecule tends to become polar and consequently surface active. This in turn means that it tends to orientate at an oil/water interface if such an interface is available. In fact changes in response to the phenols as between fattened and normal cells first show themselves distinctly with *n*-butylphenol. In the case of the fattened cell it is likely that, as lipid solubility increases, phenol is immobilized with the alkyl side chain immersed in the cell lipid and the phenolic hydroxy group projecting into the aqueous environment and it could well be that many drug molecules are thus trapped and sensitive areas in the cytoplasmic membrane protected.

This hypothesis receives support from the experimental data obtained. Thus, the smaller amount of material which leaks from fattened as against unfattened cells, which begins to be significant after the butyl compound, is compatible with the hypothesis that the membrane or sensitive areas in it are in some way protected. This, in turn, would explain why, when ability to reproduce (the m.i.c. value) is being measured, the fattened cells are less susceptible to the same dose of drug (above the C₄ compound) than are the control cells, for several groups of workers have equated

one toxic manifestation of non-specific antibacterial drugs with their ability to impair the integrity of the cytoplasmic membrane (Gale & Taylor, 1947; Salton, 1951; Beckett, Patki & Robinson, 1959; Hugo & Longworth, 1964).

The phenols at equivalent concentration cause an apparent increase in the mobility of the fattened cells as compared with the control cells which is here first noticeable with the C2 compound but again shows a marked difference after the C4. With the C4 compound and thereafter, more molecules are held at the cell surface with the phenolic group projecting into the aqueous fluid in which the cells are suspended. There will be a tendency for these to have a slightly greater negative charge at the surface hence the increased velocity towards the positive electrode for the same applied potential; indeed the phenols here are playing a similar role to that of SDS used as a detector of surface lipid, although of course their degree of ionization is much less.

It can be seen from Fig. 2 that differences between mobilities for fattened and control cells begin to show themselves at the C2 compound; electrophoresis is probably the most sensitive of the methods used in this work to detect drug/cell interaction. It is possible that small differences in response to all the phenols occur, but are only really apparent when they assume an order of magnitude detectable by the experimental method being used. It could well be that m.i.c. measurements, measurements of leakage of metabolites and drug uptake determinations are not of sufficient sensitivity to demonstrate any differences with lower alkyl phenols.

Finally the hypothesis of drug trapping will explain why fattened cells appear to take up less drug than do control cells, a finding which may appear at first sight to contradict the other data obtained. For the experiments used to determine drug uptake cannot distinguish between surface adsorption and general absorption; with the lipid present in the cell, fewer drug molecules penetrate into the cell and the surface becomes saturated, whereas with a normal cell many of the drug molecules taken up will pass into the interior of the cell as well as being held near the surface.

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Fig. 1

Fig. 2

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EXPLANATION OF PLATE

Fig. 1. Thin section of *Staphylococcus aureus* (OXFORD) grown in nutrient broth. $\times 30,000$.

Fig. 2. Thin section of *Staphylococcus aureus* (OXFORD) after growth for ten generations in nutrient broth containing 3 % glycerol. $\times 30,000$.