The Role of Cellular Lipid in the Resistance of Gram-positive Bacteria to Penicillins

BY W. B. HUGO AND R. J. STRETTON

Department of Pharmacy, The University, Nottingham

(Received 6 August 1965)

SUMMARY

It has been shown that when the Gram-positive micro-organisms *Bacillus subtilis*, *Staphylococcus aureus* and *Streptococcus faecalis* were grown under conditions in which their cellular lipid was increased a corresponding increase in their resistance to the penicillins benzylpenicillin, cloxacillin, methicillin and quinacillin was produced. Likewise cell-wall lipid depletion caused a corresponding increase in sensitivity.

INTRODUCTION

Amongst the so-called semi-synthetic penicillins produced by treating a suitable acyl derivative with 6-aminopenicillanic acid the recently described 3-carboxy-2-quinoxalinyll penicillin or quinacillin possesses some unusual properties. Thus, whereas quinacillin is active against staphylococci it is, unlike other penicillins both natural and semi-synthetic, relatively inactive against streptococci, *Sarcina lutea* and members of the genus *Bacillus*. Gram-negative organisms were completely resistant to quinacillin (Richards, Housley & Spooner, 1963). In a previous study Hugo & Stretton (1964) showed that quinacillin had a typical penicillin-like action in that it promoted the accumulation of acetylamino sugars in growing cultures of *Staphylococcus aureus* strain Oxford.

Distribution studies using water and chloroform at various pH values showed that, whereas between pH 5.5 and 7.0 benzylpenicillin and phenoxymethylpenicillin were appreciably soluble in chloroform, quinacillin was undetectable in the chloroform layer until pH 2.0 was reached. This finding led to a study of the role of cellular lipids on the sensitivity of bacteria to quinacillin and, for comparison, to benzylpenicillin, methicillin and cloxacillin. The experimental approach adopted consisted in measuring the minimum inhibitory concentration of four penicillins against three species of Gram-positive bacteria in which the lipid content had been varied by appropriate treatments.

METHODS

Organisms. *Staphylococcus aureus* strain Oxford, penicillin-sensitive; NCTC 8452, phage type 80, penicillinase producer; strain 100110, isolated at Queen Mary's Hospital for Children, Carshalton, and reported to be methicillin-resistant; *Bacillus subtilis*, Marburg strain, NCTC3601; *Streptococcus faecalis*, NCIB8553.

Materials. Quinacillin was a sample kindly given by Boots Pure Drug Co. Ltd., cloxacillin and methicillin were a gift from Beecham's Research Laboratories Ltd.

Benzylpenicillin was a commercial sample. All other chemicals used were of A.R. B.P., or B.P.C. quality when available or of laboratory reagent grade. Two samples of lipase were used: one of animal origin (pancreatic lipase, steapsin, L. Light and Co. Ltd.) and one of plant origin (wheat-germ lipase, British Drug Houses Ltd.). Silica gel for thin-layer chromatography was as supplied by E. Merck A.G. Darmstadt. A more detailed chromatographic investigation was made by using Whatman no. 1 paper impregnated with silicic acid (S.G.81).

Media and conditions of growth. Culture media were prepared from Oxoid material with appropriate additions, sterilized 10 lb/30 min., final pH, 7.2.

The nutrient broth used contained (g./l.): peptone (Oxoid no. 1) 10, meat extract (Lemco, Oxoid) 5, sodium chloride 5; to provide adequate growth for *Streptococcus faecalis* yeast extract 5 was added. To these media the following single additions were made as required: (a) glycerol to a final concentration of 3%, (b) sodium acetate 0.5%, (c) sodium oleate 20 µg./ml., (d) coenzyme A 1300 µM, (e) acriflavine 10^{-5} M, (f) flavine mononucleotide 10^{-4} M, (g) pancreatic lipase 0.01 mg./ml., (h) wheat-germ lipase 0.10 mg./ml. When a solid medium was required Ion-agar no. 2, 1.2%, was incorporated.

Media (a)–(c) were used to enhance the lipid content of the organism, (d)–(h) were designed for diminishing lipid content. All incubation was at 37°.

For lipase treatment, in addition to growth in the presence of the enzyme, washed suspensions were treated for 2 hr at 37° with the enzyme at a concentration of 0.01 (pancreatic) and 0.1 (wheat germ) mg./ml. before examination for possible lipid depletion.

Determination of minimum inhibitory concentrations. The minimum inhibitory concentration (MIC) was determined using a tube dilution method with a final inoculum size of 10^3 organisms/ml. in the test system. All readings were taken exactly 24 hr from commencement of incubation. The media used were the basal media with the addition, where appropriate, of reagents to maintain the depleted or enhanced lipid content.

Determination of lipid. 1. *Total lipid by extraction.* Organisms grown on solid medium for 18 hr were harvested by washing with distilled water, centrifuged at low speed to remove agar and the bacteria collected by centrifugation at 8000g and thrice washed by the same procedure. The washed bacteria were dried on an aluminium dish to constant weight in a partial vacuum over phosphoric oxide, transferred quantitatively to a Soxhlet apparatus and extracted with chloroform + methanol (2 + 1 by vol.). All solvents used with bacterial lipid contained 0.01% butylated hydroxytoluene as an antioxidant. The extract so obtained was quantitatively transferred to an aluminium dish, the solvent allowed to evaporate spontaneously and residual solvent allowed to evaporate in a desiccator under vacuum over phosphoric oxide.

2. *Chromatography of the lipid extracts.* Preliminary identification of the components of the extracted lipid was made by thin-layer chromatography on silica gel using chloroform + methanol + water (14 + 6 + 1) for phosphatid components and *n*-hexane + ether + acetic acid (50 + 50 + 1) for non-phosphatide fractions (Nichols, 1964). Visualization of the spots in all cases was with iodine vapour (Whitehouse, Bresler & Staple, 1958). Standards were run at the same time as an aid to identification. A more detailed chromatographic investigation was made by on silicic-

acid-impregnated paper developed with di-isobutylketone + acetic acid + water (40 + 20 + 3 by vol.; Marinetti, 1964).

Identification of the various components was accomplished by the use of appropriate spray reagents: periodate-Schiff's reagent for vicinal-glycol-containing lipids, Dragendorff's reagent for choline, ninhydrin for amino lipids and rhodamine 6G (0.05 % in 96 % ethanol) followed by examination under ultraviolet light, a procedure which detects lipids generally, but produces different colours according to the type of lipid present. Thus: yellow, lysolecithin; orange, phosphatidyl- and lysophosphatidylethanolamine, neutral lipid; blue, lysophosphatidylserine, phosphatidylglycerol, bis (phosphatidic) acid and phosphatidic acid.

Phosphatidylserine (Koch-Light Ltd.), phosphatidyl ethanolamine (Sigma Chemical Co.) and lecithin, 95-100 % (British Drug Houses Ltd.), were used as markers or standards. The two latter compounds although not chromatographically pure contained a major component in excess which was taken as the authentic spot. Published R_f values were also used as a further aid to identification (Matches, Walker & Ayres, 1964; Kates, Adams & Martin, 1964).

Detection of lipid by electrophoresis. Dyar & Ordal (1946), Dyar (1948) and Hill, James & Maxted (1963) showed that if the electrophoretic mobility of bacteria. suspensions was measured and compared with the mobility in the presence of an anionic surface-active agent such as sodium dodecyl sulphate (SDS) an increase in negative mobility was observed which could be related quantitatively to surface lipid. Measurements were made using the cell and circuit described by McQuillen (1952). The bacteria, after washing three times in buffer, were suspended in phosphate ($\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$) buffer pH 7.2 ($I = 0.01$) at a final concentration of 0.01 mg. dry wt./ml. Mobilities were measured in this system as described by McQuillen and in the same system containing sodium dodecyl sulphate 10^{-6} , 10^{-5} and 10^{-4}M . Where there was a tendency for the bacteria to form clumps the suspension was shaken for a short time with 2 mm. diameter glass beads to obtain a suspension of single organisms necessary for the test.

The overall reproducibility of duplicate mobility measurements made on the same day was $\pm 1\%$ and on different days $\pm 3\%$ ($P = 0.05$). Samples in which the mobility differed by $\pm 10\%$ were taken as being significantly different. The lipid content of the bacteria, determined by extraction and/or by mobility measurements in the presence of SDS, increased with each subculture in the presence of glycerol, reaching a maximum at between 10 and 15 subcultures. A subculture régime of 10 was adopted for all experiments.

Bacteria in which the lipid content had been decreased by lipase or enhanced by glycerol reverted to their normal value after one subculture in the basal medium. Consequently, all measurements on penicillin inhibition were made in the presence of either lipase or glycerol, with appropriate controls which indicated that these additions did not themselves affect penicillin action or growth.

RESULTS

Addition of glycerol 3% to the basic culture medium resulted in an increase in cellular lipids with all the organisms listed under Methods. Treatment with lipase reduced the lipid content of the bacteria. Pancreatic lipase at the concentration used,

Table 1. *Effect of cellular lipid on the resistance of Gram-positive bacteria to penicillins*

Organism	Treatment	Lipid content (%, w/w, of whole dried bacteria)	Mobility (μ sec. ⁻¹ V. ⁻¹ cm. ⁻¹ in the presence of 10 ⁻¹ M sodium dodecyl sulphate)	Minimum inhibitory concentration (μ g./ml.)			
				Quinacillin	Benzylpenicillin	Methicillin	Cloxacillin
<i>Staphylococcus aureus</i> (Oxford)	Control	6.4	— 2.84	0.3	0.03	8.0	0.35
	Grown with glycerol	18.3	— 4.56	20.0	2.0	20.0	8.0
	Lipase treated	6.4	— 2.40	0.1	0.01	6.0	0.25
	Grown with oleate	6.5	.	1.0	0.5	9.0	0.5
<i>S. aureus</i> NCTC 8452	Control	6.5	— 3.21	0.7	*	1.0	0.5
	Grown with glycerol	7.0	— 3.68	2.0	*	2.0	0.8
	Grown with oleate	.	.	1.5	*	1.5	0.75
<i>S. aureus</i> 100110	Control	.	— 3.69	5.0	100	26	1.0
	Grown with glycerol	.	— 3.68	5.0	100	26	1.0
	Lipase treated	.	— 3.0	3.5	100	24	0.8
<i>Streptococcus faecalis</i>	Control	5.3	— 2.42	110	3.2	45	40
	Grown with glycerol	5.9	— 3.01	350	10.0	100	200
<i>Bacillus subtilis</i>	Control	8.6	— 4.56	8.0	0.6	0.4	0.01
	Grown with glycerol	19.0	— 5.48	40	5.0	1.0	0.03
	Lipase treated	8.3	— 3.45	5.0	0.3	0.2	0.008

* Destroyed by penicillinase.

0.01 mg./ml., was not inhibitory, but was effective in diminishing cellular lipid; wheat-germ lipase inhibited the growth of *Staphylococcus aureus* unless the concentration was decreased to 0.05 %. In the case of staphylococci producing an extra-cellular lipase cellular lipid could be increased by adding a lipase inhibitor to the growth medium. It was found that sodium oleate, 20 µg./ml., inhibited pancreatic lipase and the lipase present in the culture medium in which *S. aureus* (Oxford) had been grown. When this amount of sodium oleate was added to culture media in which *S. aureus* (Oxford) or 8452 were growing, the lipid content and resistance to penicillins was increased. *Staphylococcus aureus* 100110 did not produce an extra-cellular lipase.

Robinson, Brady & Bradley (1963) reported that acriflavine, coenzyme A and flavine mononucleotide inhibited the biosynthesis of fatty acids in enzymes from cat brain and liver. In our hands these reagents at appropriate concentrations had no detectable effect on the lipid content of the bacterial species used in this study.

Acetate was less effective than glycerol in inducing cellular lipid and was, in fact, inhibitory towards the growth of *Staphylococcus aureus* even at concentrations of 0.5 %. Without exception, but in varying degrees, increasing cellular lipid resulted in an increase in the dose of penicillin required to inhibit growth whereas decrease in lipid produced precisely the opposite effect (Table 1).

DISCUSSION

Quinacillin has been shown to cause the accumulation of *N*-acetyl amino sugars at concentrations which inhibit cell growth (Hugo & Stretton, 1964). However, the different pattern of solubility of quinacillin in chloroform when compared with benzylpenicillin or phenoxymethylpenicillin led to the suggestion that cellular lipid may be a factor in determining the susceptibility of bacteria to penicillins or the toxicity of penicillins towards bacteria. Lipid enhancement resulted in a marked increase in the dose to inhibit growth of all the penicillins tested; lipid depletion caused a less well marked but none the less significant increase in sensitivity. Changes in lipid content were also reflected in the appearance of the culture. In general, with increase in lipid content pellicle formation was either induced or, where already present as in *Bacillus subtilis*, became thicker and more wax-like. Treatment with lipase abolished pellicle formation in this organism. It was not possible to attribute increase in penicillin resistance to any particular lipid component, since all appeared to increase equally, as assessed by chromatography, during growth in glycerol.

Some evidence that the cellular location of the lipid was the controlling factor was obtained from experiments in which lipid extracted from fattened bacteria was added to cultures of non-fattened bacteria of the same species to give, as near as could be achieved, the same overall lipid content in the system. In these circumstances no increase in resistance to the penicillins could be detected.

The role of drug lipid solubility as measured by chloroform buffer distribution seems to be of small importance as compared with the marked general effect of cell lipid in penicillin resistance. Support for the general hypothesis on the role of lipid comes from a consideration of the difference in sensitivity of Gram-positive and Gram-negative bacteria to penicillin. The former are, in general, sensitive, the

latter are not, yet the mode of action is the same (Gale, 1964); but, whereas Gram-negative cell walls contain a large amount of lipid (up to 25 %), Gram-positive cell walls normally contain little or none (Salton, 1964).

It is possible that an increase or decrease in cellular lipid of the type reported in this paper might occur *in vivo* leading to a change in resistance to the penicillins; in this connexion it is interesting to note that Hill *et al.* (1963) have shown that freshly isolated pathogenic streptococci possess more cell lipid than laboratory strains. Both serum glycerol and possibly serum lipases may play a role in changing the sensitivity of the Gram-positive bacteria to the penicillins.

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