Inhibition of the Biosynthesis of Cell-Wall Mucopeptides by the Penicillins

A STUDY OF PENICILLIN-SENSITIVE AND PENICILLIN-RESISTANT STRAINS OF *STAPHYLOCOCCUS AUREUS*

By H. J. ROGERS AND J. JELJASZEWICZ*

National Institute for Medical Research, The Ridgeway, Mill Hill, London, N.W. 7

(Received 13 April 1961)

Previous work has shown that benzylpenicillin can inhibit cell-wall-mucopeptide synthesis by staphylococci (Park, 1958; Mandelstam & Rogers, 1959). The precise relationship, however, between the concentrations of the antibiotic required to do this and those necessary to prevent growth of the organisms has not been examined in detail. In the work described by Mandelstam & Rogers (1959), a 'naturally' resistant strain of the organism was used (i.e. strain 524/SC). It was originally isolated from a human patient before re-isolation by single-cell selection (Rogers, 1953). Such 'naturally resistant' strains produce inducible penicillinase, and although the organism was grown in the absence of penicillin and examined in the presence of chloramphenicol in this earlier work (Mandelstam & Rogers, 1959), residual penicillinase was likely to have been present in the suspensions. As a consequence no attempt was made to examine the precise concentration of benzylpenicillin required to inhibit mucopeptide synthesis. Staphylococci selected in the laboratory from media containing penicillin, however, do not produce detectable amounts of penicillinase (Barber, 1953). If the antibiotic action of benzylpenicillin on both sensitive and resistant strains is due to inhibition of mucopeptide synthesis, then one would expect the lethal concentrations to coincide with those required to cause significant inhibition of mucopeptide synthesis.

Two new penicillins derived from 6-aminopenicillanic acid (Batchelor, Doyle, Nayler & Rolinson, 1959) with different side chains have now been described (Stewart, Harrison & Holt, 1960; Rolinson, Stevens, Batchelor, Wood & Chain, 1960). Again, if the antibiotic action of these compounds is due to their ability to inhibit mucopeptide synthesis, the lethal concentrations and those required to inhibit mucopeptide synthesis should bear a constant relationship. The effects upon mucopeptide synthesis of various concentrations of the new penicillins and of benzylpenicillin have now been

examined, with the sensitive *Staphylococcus aureus* strain Oxford, and a resistant substrain derived from it. The results obtained provide strong evidence in favour of the hypothesis, first put forward by Park & Strominger (1957), that the primary action of penicillin is upon the formation of cell wall. Although the position with Gram-negative forms may be more complicated, it seems probable that the hypothesis is correct for the Gram-positive staphylococci. The effect of one of the new penicillins, sodium 6-(2:6-dimethoxybenzamido)penicillinate (AB 1241, Methicillin, Celbenin), upon mucopeptide synthesis by the penicillinase-producing *S. aureus* strain 524/SC has also been examined. If the presence of penicillinase in the cells influences the inhibition produced by benzylpenicillin (Mandelstam & Rogers, 1959), 6-(2:6-dimethoxybenzamido)penicillin should be relatively more effective against this strain than the benzyl derivative. This penicillin is not readily hydrolysed by staphylococcal penicillinase (Rolinson *et al.* 1960; Knox, 1960).

METHODS AND MATERIALS

Strains of organism. *S. aureus* strain Oxford in a dried form was obtained from Mr J. Lightbown of this Institute. Strain 524/SC was as used previously (Mandelstam & Rogers, 1959).

Testing of penicillin sensitivity and selection of penicillin-resistant substrain. A subculture of *S. aureus* Oxford in broth (see below) was tested for sensitivity to penicillin by inoculating (a) a series of flasks of Hedley-Wright broth (Rogers, 1953) containing increasing amounts of penicillin; (b) a series of Petri dishes containing the same medium solidified with 2% of agar and containing increasing concentrations of benzylpenicillin. Growth was found to be inhibited by 0.036 μg . of benzylpenicillin/ml. but not by 0.024 μg /ml. on agar plates, and by 0.12 μg /ml. but not by 0.06 μg /ml. when growing in broth. In order to select penicillin-resistant substrains, two techniques were used: (a) the gradient plate technique of Szybalski & Bryerson (1952) and (b) the selection of colonies from heavily inoculated agar plates containing sufficient penicillin almost to inhibit growth completely. As an example, a plate containing, say, 0.036 μg . of penicillin/ml. was inoculated with the original sensitive strain and a few colonies appeared

* Present address: Department of Microbiology, Medical Academy, Al. Stalingradzka 3, Poznan, Poland.

(growth up to 76 hr. was necessary at some stages of the selection). One of these was selected and a culture grown from it in liquid medium containing 0.036 $\mu\text{g.}$ of penicillin/ml. This culture was subcultured on a plate containing a slightly higher concentration of the antibiotic, and so on. By these processes, substrains resistant to concentrations of penicillin of 2.1 $\mu\text{g./ml.}$ up to 30 $\mu\text{g./ml.}$ were obtained. For the present work only the one resistant to 2.1 $\mu\text{g./ml.}$ (i.e. growing in 2.1 $\mu\text{g./ml.}$ but not in 2.4 $\mu\text{g./ml.}$) was used. This strain very readily reverted to penicillin sensitivity, and growth through two or three subcultures in the absence of antibiotic considerably decreased its resistance. During the course of the work the resistance of the strain declined. The strain was then reselected from agar plates containing 1.8 $\mu\text{g.}$ of penicillin/ml. When the reselected strain was tested it was found to grow in 24 hr. broth containing 9.5 $\mu\text{g.}$ of penicillin/ml., but not 19.0 $\mu\text{g./ml.}$ The first resistant substrain isolated will be distinguished as no. 1 from the re-isolated substrain, to be called no. 2.

Penicillin. Commercial benzylpenicillin (Glaxo Laboratories Ltd.) in phials containing 10^6 i.u. (i.e. 60 mg.) was used. For critical experiments the precise strength of solutions made from this material was tested by comparison with solutions of the British Standard Penicillin, having a potency of 1.680 i.u./ $\mu\text{g.}$ The latter was given to us by Mr J. Lightbown of this Institute. 6-(α -Phenoxypropion-amido)penicillanic acid (Broxil), 6-(2:6-dimethoxybenz-amido)penicillin (AB 1241, Methicillin, Celbenin) and 6-aminopenicillanic acid were obtained through the courtesy of Dr M. R. Pollock and Dr D. L. Swallow.

Broth medium. This was Hedley-Wright broth, containing 2.5% of sodium β -glycerophosphate as previously described (Rogers, 1953). This medium was substituted for the casein-hydrolysate growth medium previously used, because growth of the resistant substrains in the casein hydrolysate was very slow and did not occur at all when benzylpenicillin at the concentration at which the substrain had been selected was included to preclude back-mutation to sensitivity. It was subsequently shown that back-mutation did not occur in sufficient cells during the short growth period used to influence the results, and the antibiotic was therefore omitted from the broth medium. Even in broth medium and in the absence of the antibiotic, growth of the resistant substrains had a considerable lag period and frequently stopped much sooner than that of the normal strain. The technique for growing the resistant strains was standardized as follows. Cultures of the organism grown on agar containing 1.8 $\mu\text{g.}$ of benzylpenicillin/ml. for 24 hr. were dried by the Stamp (1947) technique. A new dried culture was then used for each experiment. It was inoculated into 10 ml. of Hedley-Wright broth medium containing 1.8 $\mu\text{g.}$ of benzylpenicillin/ml., and incubated with shaking at 35° for 18 hr. This culture (5.0 ml.) was then added to 200 ml. of the same medium without penicillin and incubated under the same conditions for 7 hr., cooled and stored at 0–4° overnight. The next morning it was diluted with an equal volume of broth and incubated until the extinction of the culture had doubled. After this the suspension was prepared as previously described.

Conditions for cell-wall biosynthesis. Suspensions were prepared and incubated as previously described (Mandelstam & Rogers, 1958, 1959; Rogers & Perkins, 1960); all four of the mucopeptide amino acids and chloramphenicol

(50 $\mu\text{g./ml.}$) were always present unless otherwise stated. Penicillin solutions, when required, were added along with the other ingredients before addition of the cell suspension. Incubation was for 90 min., unless otherwise stated, at 35°, and the flasks were shaken in order to aerate the suspensions.

Preparation of mixed trichloroacetic acid precipitates and cell walls. These were prepared as previously described (Mandelstam & Rogers, 1958, 1959; Rogers & Perkins, 1960).

Analytical methods, including radioactivity determinations. These were as previously described (Mandelstam & Rogers, 1958, 1959; Rogers & Perkins, 1960); 1000 counts above background were always collected. It was, however, not found possible to measure cell-wall synthesis by the increase in extinction of the suspensions when strain Oxford was used.

Radioactive amino acids. [^{14}C]Glycine, [^{14}C]alanine, [^{14}C]glutamic acid and generally ^{14}C -labelled lysine were obtained from The Radiochemical Centre, Amersham, and initially diluted with unlabelled amino acids so that 1 mg. $\equiv$ 1 μC ; these materials were then diluted a further 10-fold with the corresponding unlabelled compound in the test system.

RESULTS

Effect of age of the culture

In all the previous work (Mandelstam & Rogers, 1958, 1959; Rogers & Perkins, 1960), the cells used for preparing suspensions to study cell-wall synthesis were obtained from rapidly growing cultures in casein-hydrolysate media. Both *S. aureus* strain 524/SC and the original Oxford strain grew rapidly in casein-hydrolysate media, thus allowing completion of the experiment in a reasonable time. The selected benzylpenicillin-resistant substrains, however (see Methods and Materials section), grew very slowly and often to a very low final culture density in casein hydrolysate. At one stage in the work it was thought that in order to obtain enough organisms and to perform the experiments in a reasonable period of time, it might be possible to use cultures incubated overnight, i.e. cultures in which cells had ceased to multiply rapidly. To test this possibility a comparison was made between suspensions prepared from cultures of the normal Oxford strain in which the organisms were growing rapidly (Mandelstam & Rogers, 1958) and from cultures in which the cells had ceased to multiply. Broth medium (cf. Methods and Materials section) was used. Suspensions were prepared in the usual way and their ability to incorporate [^{14}C]glycine was measured in the presence and absence of two concentrations of penicillin (Table 1). The cells from overnight cultures differed from those obtained by the standard procedure, which employed cells that had been growing rapidly, in two important ways. The total incorporation of the amino acid in the period of incubation and hence mucopeptide synthesis was less, and the higher concentrations of benzylpenicillin were less inhibitory to

Table 1. *Effect of benzylpenicillin upon staphylococci from cultures of different ages*

Strain: *Staphylococcus aureus* Oxford, normal. A, Strain incubated for 24 hr. at 37° in Hedley-Wright broth supplemented with glucose and sodium β -glycerophosphate. B, Strain incubated for about 4 hr. to the density of 1 mg./ml.; it was left overnight in the cold, warmed, the same volume of medium was added and incubated to density 1 mg./ml. The suspensions of washed organisms were adjusted to the density of 1 mg./ml. to study incorporation of [1-¹⁴C]glycine. The radioactivity at infinite thickness of hot trichloroacetic acid precipitates is given.

Time of incubation: 90 min.

Benzyl- penicillin (μ g./ml.)	Counts/min.		Inhibition (%)	
	A	B	A	B
0.012	68.4	143.1	15.6	1.6
0.024	65.5	130.0	19.2	10.6
0.048	54.5	90.0	32.8	38.1
0.060	50.6	77.8	37.6	46.5
0.30	47.7	54.8	41.2	62.3
0.60	43.7	55.5	46.1	61.8
1.2	40.6	44.4	49.9	69.5
0	81.0	145.2	—	—

the process. Since one of the purposes of the present work was to compare the effects of benzylpenicillin upon the sensitive and resistant substrains, all further work was done with rapidly growing cells (i.e. cells treated by the standard procedure). This experiment stresses the need for careful control of the growth conditions for the micro-organisms in studies of mucopeptide biosynthesis.

Effect of benzylpenicillin on incorporation of cell-wall amino acids

The cell suspensions of both strains were prepared in the usual way from cultures of exactly the same age. Both suspensions in phosphate-glucose solution were added to the usual mixture of the four mucopeptide amino acids, penicillin and chloramphenicol. In one experiment the glycine was labelled, and in the other glutamic acid was labelled. Concentrations of benzylpenicillin including those to which the cells were sensitive when growing in liquid and solid media (i.e. about 0.03–0.06 μ g./ml. for the sensitive strains and 2.4–12.0 μ g./ml. for the resistant strain) were employed. After incubation mixed hot trichloroacetic acid precipitates were prepared and their radioactivity was measured at infinite thickness. Resistant sub-strain no. 1 was used.

The incorporation by the sensitive strain of labelled glutamic acid is significantly inhibited by doses of benzylpenicillin in the range 0.018–0.06 μ g./ml. (Fig. 1). The difference between duplicate tests within a single experiment has been found to be about 10% over a large number of

Fig. 1. Inhibition of the incorporation of [1-¹⁴C]glutamic acid into the material insoluble in hot trichloroacetic acid prepared from suspensions of the sensitive (●) and resistant (no. 2) substrain (○) of *S. aureus* strain Oxford. The suspensions had been incubated under the conditions set out in the Methods and Materials section. In the absence of penicillin the radioactivity of the preparations from both strains was equivalent to about 80 counts/min.

experiments. Similar results were obtained for the incorporation of glycine.

Comparison of the behaviour of the resistant and sensitive substrains (Fig. 1) clearly shows the difference between them. At a concentration of 0.06 μ g./ml. or more of benzylpenicillin the two strains differ markedly for the incorporation of [1-¹⁴C]glutamic acid. At a concentration of 0.6 μ g./ml. of antibiotic only the resistant strain grew. Even when the concentration of benzylpenicillin was increased to 6.0 μ g./ml., glutamic acid incorporation by the resistant strain was inhibited by only 40% and glycine incorporation by 60%. At 24.0 μ g./ml. glycine incorporation was decreased by 70%, an inhibition still somewhat less than that caused by 1.2 μ g./ml. with the sensitive strain.

Table 2 shows the effect of three concentrations of benzylpenicillin on the incorporation by the sensitive substrain of each of the four amino acids present in the mucopeptide; at a concentration equivalent to 0.03 μ g./ml., inhibition of incorporation of all four amino acids was between 23 and 36%.

When the concentration of antibiotic was increased 100-fold, to 3.0 μ g./ml., the incorporation of the amino acids by the sensitive strain was not completely stopped. In the earlier experiments this seemed to apply particularly to the incorporation of [1-¹⁴C]alanine. When these experiments were repeated, however, some months later, rather higher degrees of inhibition were obtained, as for example in Expts. 3 and 4 of Table 2, and the difference between the behaviour with this amino acid and the others was then less marked. The incomplete

Table 2. *Comparative inhibition of the incorporation of the four cell-wall amino acids into cell-wall mucopeptide by benzylpenicillin acting on the sensitive substrain*

The techniques were as given in the Methods and Materials section. The radioactivity of hot trichloroacetic acid precipitates was measured.

Concn. of penicillin ($\mu\text{g./ml.}$)	Expt. no.	Alanine		Glutamic acid		Glycine		Lysine	
		Counts/min.	Inhibition (%)	Counts/min.	Inhibition (%)	Counts/min.	Inhibition (%)	Counts/min.	Inhibition (%)
0	1	148	—	72	—	98	—	119	—
0.03	1	114	23	46	36	73	25	86	28
1.2	1	62	58	20	72	25	74	52	56
3.0	1	66	53	19	74	—	—	49	59
0	2	170	—	—	—	106	—	—	—
3.0	2	61	64	—	—	20	81	—	—
0	3	150	—	—	—	—	—	—	—
3.0	3	36	76	—	—	—	—	—	—
0	3	258*	—	—	—	—	—	—	—
3.0	3	76*	71	—	—	—	—	—	—

* Approximately twice the usual concentration of [$1\text{-}^{14}\text{C}$]alanine was used in this experiment.

inhibition with higher doses of antibiotic is largely due to a short initial period of rapid incorporation which is dealt with more fully in the next section of this paper.

Time-course of incorporation of amino acids by the sensitive strain in the presence of penicillin

In these experiments separate flasks containing a suspension of the sensitive cells in a solution of the usual four amino acids (one of which was labelled), chloramphenicol, glucose and phosphate, with or without benzylpenicillin, were incubated at 35° with shaking. Pairs of flasks were removed at the times indicated by the points on Fig. 2, which illustrates the results obtained for glutamic acid incorporation. Most of the incorporation which took place in the presence of the higher concentrations of antibiotic occurred during the first 10–20 min., and there was only a very slow continuous deposition after this. If this period of rapid incorporation is ignored, the subsequent inhibitions of mucopeptide synthesis deduced from Fig. 2 are 47, 72 and 96 % respectively for concentrations of 0.06, 0.12 and 12.0 $\mu\text{g.}$ of benzylpenicillin/ml.

Comparison of mixed trichloroacetic acid precipitates with cell-wall preparations

Eight separate flasks containing the usual components for studying mucopeptide synthesis were set up: four contained 1.2 $\mu\text{g.}$ of benzylpenicillin/ml. and four served as controls. A different amino acid was labelled in each of the four pairs. Thus a comparison could be made of the effect of 1.2 $\mu\text{g.}$ of benzylpenicillin/ml. upon the incorporation of each of the four mucopeptide amino acids. The sensitive strain of *S. aureus* Oxford was used. At the end of the usual 90 min. incubation, the suspensions were cooled, a sample was withdrawn for

Fig. 2. Influence of various concentrations of benzylpenicillin upon incorporation of [$1\text{-}^{14}\text{C}$]glutamic acid into the material insoluble in hot trichloroacetic acid prepared from suspensions of the sensitive strain of *S. aureus* Oxford. The suspensions were incubated under the usual conditions for cell-wall-mucopeptide synthesis. The flasks contained: ●, no penicillin, this served as the control; ○, 0.12 $\mu\text{g.}$ of benzylpenicillin/ml.; ▲, 1.2 $\mu\text{g.}$ of benzylpenicillin/ml.; △, 12 $\mu\text{g.}$ of benzylpenicillin/ml.

the preparation of mixed trichloroacetic acid precipitates and the remainder was centrifuged, the cells were washed and the cell-wall preparations made as before (Mandelstam & Rogers, 1959). The radioactivity in all the preparations was measured at infinite thickness.

As can be seen from Table 3, in most instances the agreement between the degree of inhibition of incorporation into the cell-wall preparations and into the material insoluble in hot trichloroacetic acid, which also includes the proteins of the cells, was within experimental error. Thus it seems justified to conclude, as might be expected on the basis of earlier work (Mandelstam & Rogers, 1958, 1959), that results obtained with the mixed trichloroacetic acid precipitates are a good indication of incorporation into mucopeptide and hence of the cell-wall synthesis. However, some caution may still be necessary, because in two experiments with the higher concentration of antibiotic of 3.0 $\mu\text{g./ml.}$ it was found that the incorporation of [^{14}C]-alanine into cell-wall preparations was inhibited to a significantly greater extent than into material insoluble in hot trichloroacetic acid (see Table 3, Expts. 2 and 3). The reasons for this are at present under investigation.

Composition of the cell wall of the resistant substrain

Cell walls were prepared from the sensitive and resistant strains by the method of Cummins &

Harris (1956) and analysed by the usual methods (Table 4). The differences between the results for the two strains are chiefly a higher content of glutamic acid and a lower value for muramic acid in the batches of wall from the sensitive organism. However, over a period of years, variation of the analytical values obtained for different batches of wall preparations from the sensitive strain have been greater than the differences shown in Table 4 between the sensitive and resistant forms. For example, the molecular proportions for the amino acids previously quoted by Rogers & Perkins (1959) for *S. aureus* strain Oxford show such a greater difference from the present results for the same strain (sensitive strain in Table 4). Similar variation has previously been reported for *S. aureus* strain 524/SC (Mandelstam & Rogers, 1959). It is therefore doubted whether the walls from the sensitive and resistant strains can be said to differ significantly in composition. The total sum of the amino acids and amino sugars, allowing for the water of hydrolysis, accounts for about 80–85% of the dry weight of the preparations from both strains.

Table 3. *Effect of penicillin on the incorporation of radioactively labelled amino acids into cell-wall preparations and into material insoluble in hot trichloroacetic acid*

The sensitive strain of *S. aureus* Oxford was used. Methods were as described in the Methods and Materials section.

Amino acid	Expt. no.	Dose of benzyl-penicillin ($\mu\text{g./ml.}$)	Inhibition (%)	
			Cell-wall preparation	Hot acid precipitate
Alanine	1	1.2	48	47
Glutamic acid	1		63	69.7
Glycine	1		89	77
Lysine	1		42	45
Alanine	2	3.0	91	76
Alanine	3		85	71

Table 4. *Analyses of cell-wall preparations from the resistant (no. 2) and sensitive strains of Staphylococcus aureus Oxford*

The analytical methods are as stated in the Methods and Materials section, and the molar proportions of the components are given in relation to glutamic acid taken as 1.00. The two sets of figures given for the sensitive strain are on cell walls prepared from different batches of cells.

	Sensitive strain		Resistant strain (no. 2)	
	%	mol.prop.	%	mol.prop.
Alanine	10.2, 13.2	1.4, 1.71	11.2	1.94
Lysine	10.4, 9.4	0.87, 0.71	9.9	1.04
Glutamic acid	11.8, 12.8	1.00, 1.00	9.5	1.00
Glycine	15.4, 20.2	2.50, 3.10	16.8	3.43
Glucosamine	17.2, 15.2	1.19, 0.99	14.8	1.27
N-Acetylglucosamine	21.2, 18.8	—	18.3	—
Muramic acid	14.4, 14.8	0.70, 0.69	17.4	1.06
N-Acetylmuramic acid	17.2, 17.4	—	20.4	—
Total	86.2, 91.6	—	86.1	—
Total – water of hydrolysis	81.3, 84.7	—	80.0	—

Inhibition by the 'new penicillins' upon normal and resistant substrains of strain Oxford

The effects of Methicillin, Broxil and 6-aminopenicillanic acid upon mucopeptide synthesis were examined by the same techniques as those already described, the sensitive and resistant substrains being used (Table 5). With the sensitive strain, the ratios of the concentrations of the new penicillins to that of benzylpenicillin required to give 60–70 % inhibition of mucopeptide synthesis are: Methicillin about 60–100, Broxil about 1 and 6-aminopenicillanic acid about 1000. The corresponding ratios for the concentrations required for prevention of growth are quoted by Garrod (1960) as about 60 and 1, for Celbenin and Broxil, whereas the corresponding ratio for 6-aminopenicillanic acid would appear to be about 1–2000 (unpublished observation). Thus there is rather close similarity between the relative effects of the four antibiotics on mucopeptide biosynthesis and growth. Considering now the resistant substrain, the ratios, in the same order as given above, are less than 6, rather less than 1 and greater than 100. Methicillin is very much more effective against the benzylpenicillin-resistant strain than would be expected, with the result that the ratio of the amount of Methicillin to that of benzylpenicillin required to inhibit mucopeptide synthesis is less than with the sensitive strain. It appears that organisms which are resistant to benzylpenicillin are not resistant to Methicillin, as far as mucopeptide synthesis is concerned.

Effect of Methicillin upon protein and nucleic acid formation by the sensitive Staphylococcus aureus Oxford

In view of the failure of cells which are resistant to benzylpenicillin to be resistant to Methicillin, it seemed necessary to check that the new antibiotic did not have some more widespread attack on the cell than inhibition of mucopeptide synthesis. To test this, two series of experiments were done to examine the effect of the antibiotic on protein and nucleic acid synthesis.

Protein synthesis. Cells were incubated in the usual system used for studying mucopeptide synthesis with the addition of 0.2 mg. of L-[¹⁴C]phenylalanine (containing 0.05 μ C of radioactivity)/ml. and 0.2 mg. of acid-hydrolysed casein (supplemented by tryptophan; Rogers, 1954)/ml.; chloramphenicol was omitted from all except one flask. This flask served to show that the incorporation measured in the others was in fact due to protein synthesis. The radioactivity of the material insoluble in 5 % trichloroacetic acid at 90° was measured at infinite thickness.

Nucleic acid synthesis. Cells were incubated in the usual system used for studying mucopeptide synthesis. After incubation for varying periods they were washed twice with cold 5 % trichloroacetic acid, the washings were discarded and the deposit was extracted at 90° for 15 min. with 5 % trichloroacetic acid. The trichloroacetic acid was removed from the supernatants by extraction four times with equal volumes of ether, and the ether

Table 5. *Effect of the new penicillins upon cell-wall biosynthesis by the sensitive and resistant substrains of Staphylococcus aureus Oxford*

The resistant substrain was no. 2. Conditions for studying [¹⁴C]glutamic acid incorporation were as described in the Methods and Materials section.

	Concn. (μ g./ml.)	Inhibition (%)		Lethal dose* for sensitive strain (μ g./ml.)
		Sensitive strain	Resistant strain	
Benzylpenicillin	0.3	70	—	0.02–0.032
	3.0	—	35	
Methicillin	1.0	8	—	1–2.0
	2.0	19	—	
	5.0	51	—	
	10	51	46	
	20	59	69	
	50	71	—	
Broxil	100	71	85	0.01–0.032
	0.16	51	—	
	0.32	61	—	
6-Aminopenicillanic acid	0.2	3	—	~ 60
	20	28	—	
	200	—	38	
	300	72	—	
	400	—	39	

* Results taken from Garrod (1960), Knox (1960) and Rolinson, Stevens, Batchelor, Wood & Chain (1960).

removed by a stream of air. Finally the extracts were made up to 10 ml. and the extinction at 260 m μ of suitable dilutions was measured. In all the experiments, flasks which contained 60 μ g. of benzylpenicillin/ml. and 100 μ g. of Methicillin/ml. were compared with control flasks which contained no antibiotic.

The results were entirely negative in that, at these concentrations, neither antibiotic had any influence either upon the incorporation of [14 C]-phenylalanine or upon the formation of nucleic acids. The incorporation of [14 C]phenylalanine into the material insoluble in hot trichloroacetic acid was inhibited 96% by 50 μ g. of chloramphenicol/ml. Chloramphenicol has no effect upon mucopeptide synthesis (Mandelstam & Rogers, 1958; Hancock & Park, 1958).

Effects of benzylpenicillin and Methicillin upon cell-wall formation by strain 524/SC

The organisms were grown and incorporation of [14 C]glutamic acid into the material insoluble in hot trichloroacetic acid in the presence of the antibiotics was studied as previously described (Mandelstam & Rogers, 1958, 1959). Fig. 3 shows the results obtained. It will be noted that, as was found

Fig. 3. Effect of different concentrations of benzylpenicillin (O) and Methicillin (●) on the incorporation of [14 C]-glutamic acid into the material insoluble in hot trichloroacetic acid prepared from suspensions of *S. aureus* strain 524/SC after incubation under the usual conditions for the formation of cell-wall mucopeptides.

before (Mandelstam & Rogers, 1959), inhibition of cell-wall formation by benzylpenicillin is far from complete, and even when the concentration of the antibiotic is increased very much, inhibition is not greatly increased. With Methicillin, however, inhibition up to 80–90% occurs at concentrations of Methicillin similar to those required for this effect upon the sensitive Oxford strain.

DISCUSSION

The results described in this paper are all consistent with the hypothesis originally put forward by Park & Strominger (1957), that the primary site of action of benzylpenicillin is the mechanism which forms the cell walls in staphylococci. When penicillin-resistant cells are selected on artificial media, the mucopeptide-forming system in such organisms is strikingly less sensitive to benzylpenicillin than that in cells from the original culture. The reason for the small amount of mucopeptide formation by the sensitive strain which still occurs even in the presence of relatively high doses of the antibiotic (i.e. inhibition after 90 min. incubation of the system never appears to be more than about 70–80% complete) is made clear by an examination of the time-course of incorporation. During the first 10–15 min. of incubation, penicillin has relatively little effect upon the synthesizing system; therefore with concentrations of benzylpenicillin of 0.6 μ g./ml. and more, inhibition is almost complete. Although the conditions for the assay of the effects of antibiotics upon growth of the organisms and of those for the present tests for mucopeptide synthesis are so very different, the concentrations of benzylpenicillin required to affect both are of the same order for both sensitive and laboratory-selected resistant strains. Such a comparison becomes more impressive when penicillins with different side chains are compared. The relative effectiveness of the substances for the two manifestations of the antibiotics are remarkably similar. The one apparent anomaly is that, with the cells made resistant to benzylpenicillin, mucopeptide synthesis is still sensitive to Methicillin. Such a lack of correlation is to be expected with strain 524/SC, since resistance of this latter strain is due to the production of penicillinase, and Methicillin is much less readily hydrolysed by penicillinase. With strains made resistant in the laboratory by selection, this explanation should not hold, since their resistance is not to be explained by the production of penicillinase. However, Barber (1961) found that strains selected to grow in the presence of benzylpenicillin show little or no ability to grow in higher concentrations of Methicillin. Hence even this apparent contradiction is not a real one as far as the relative effects of Methicillin on growth and

cell-wall-mucopeptide formation are concerned. Within the limitations of the examination made, the mechanism of action of Methicillin appears to be the same as that of benzylpenicillin. Of three major biosynthetic processes in the cell (namely those for proteins, nucleic acids and cell-wall mucopeptides), only the formation of the mucopeptides is affected by high concentrations of the antibiotic. This conclusion is similar to that drawn by Gooder & Maxted (1961) for streptococci.

In earlier work (Mandelstam & Rogers, 1959) it was reasoned that because the strain 524/SC had been grown in the absence of benzylpenicillin, the cells then washed well and incubated in the presence of chloramphenicol so that no more enzyme could be formed, penicillinase was not likely to play any part in the effect of the antibiotic on mucopeptide synthesis. Nevertheless it was found that mucopeptide formation continued, although at a reduced rate, for about 1 hr. in the presence of very high concentrations of penicillin before ceasing altogether. It was then argued that this must be due to the presence of a penicillin-resistant pathway for synthesis in the organisms. Moreover, the mucopeptide deposited in the presence of penicillin was different from that formed in the absence of the antibiotic. It has now been shown that when Methicillin is used, which is hydrolysed much less easily by penicillinase than is benzylpenicillin, inhibition of mucopeptide formation reaches values of 80–90% with concentrations of the antibiotic similar to those required for inhibition of sensitive strains. Hence it is possible that the previous results were due to the presence of penicillinase in the cells. However, in view of the results for the action of Methicillin upon laboratory-selected resistant mutants of strain Oxford, caution in the total rejection of the previous idea should be exercised. The behaviour of these resistant mutants is similar to that of 524/SC. Both strains require high concentrations of benzylpenicillin to effect inhibition of mucopeptide synthesis, but both are sensitive to Methicillin, yet the selected mutants of strain Oxford do not produce penicillinase, whereas, of course, 524/SC does. Hence the difference between the effects of benzylpenicillin and Methicillin upon 524/SC need not be necessarily related to the presence of penicillinase.

The hypothesis that the primary site of the lethal action of penicillin is the system responsible for mucopeptide biosynthesis has been challenged by the experiments of Hugo & Russell (1960*a, b*) and by those of Meadow (1960). The observations of the former authors are: (a) that 6-aminopenicillanic acid is much more effective in causing spheroplast formation than in killing a variety of species of Gram-negative organisms, and (b) that in the presence of large amounts of benzylpenicillin acting

on *Escherichia coli*, the sum of the number of spheroplasts and the number of viable organisms is only about 40% of the original total number of viable organisms before the addition of the antibiotics, and it appeared unlikely that this loss was wholly due to lysis of spheroplasts. No complete answer is, of course, available to enable this work to be understood in the light of the results obtained in the present paper. However, the concentrations of benzylpenicillin used by Hugo & Russell (1960*a, b*) are of the order of 10^3 – 10^4 times those required to inhibit mucopeptide formation by the penicillin-sensitive *S. aureus*. It may be asked whether at such high concentrations the antibiotic might not have more widespread side effects on the cells than it does at the concentrations required to kill sensitive organisms. Also, the formation of spheroplasts is necessarily itself a secondary effect of penicillin, since it involves first an inhibition of mucopeptide formation and secondly some method either for removing part of the wall or so altering its physical properties that it ceases to be rigid; it is doubted whether a simple bursting of the wall at its seams, as a result of expansion of internal contents against a static wall, is a likely mechanism of spheroplast formation. The most convincing evidence, however, for some different effect of high concentrations of penicillin on Gram-negative forms is the work of Meadow (1960). This author found no effect of penicillin on the incorporation of $\alpha\epsilon$ -diamino- ^{14}C pimelic acid into *E. coli* under conditions where growth was strongly inhibited and spheroplasts were formed. This amino acid is usually confined in the cell to the wall mucopeptides. Again the concentration of penicillin necessary was very high (150 $\mu\text{g./ml.}$, unpublished results from Dr Pauline Meadow), and one might conclude from this work that benzylpenicillin at high concentrations has harmful effects on Gram-negative cells other than by inhibiting mucopeptide biosynthesis. However, Nathenson & Strominger (1961) have claimed that benzylpenicillin inhibits the incorporation of tritiated $\alpha\epsilon$ -diaminopimelic acid into *E. coli* cell walls by 72%.

The evidence for Gram-negative cells may be confused, but for staphylococci it is completely consistent with the hypothesis that the site of the lethal action of the penicillins is the system responsible for the formation of the cell-wall mucopeptides.

SUMMARY

1. The concentration of benzylpenicillin required to inhibit cell-wall-mucopeptide formation by *Staphylococcus aureus* strain Oxford is of the same order as that required to prevent growth.

2. Benzylpenicillin-resistant cells selected on artificial media from the original culture require

very much higher concentrations of the antibiotic to inhibit cell-wall-mucopeptide formation.

3. The composition of the cell walls of the sensitive and resistant cells is the same.

4. The inhibitory effects upon mucopeptide synthesis by sensitive cells of sodium 6-(2:6-dimethoxybenzamido)penicillin (Methicillin, Celbenin, AB 1241), 6-(α -phenoxypropionamido)penicillanic acid (Broxil) and 6-aminopenicillanic acid are as predicted from their relative potencies tested as antibiotics.

5. Cells made resistant to benzylpenicillin do not require increased concentrations of sodium 6-(2:6-dimethoxybenzamido)penicillin to inhibit mucopeptide synthesis.

6. Sodium 6-(2:6-dimethoxybenzamido)penicillin, like benzylpenicillin, does not inhibit the formation of either proteins or nucleic acids by *Staphylococcus aureus*.

7. Sodium 6-(2:6-dimethoxybenzamido)penicillin inhibits almost completely mucopeptide synthesis by the penicillinase-producing strain *Staphylococcus aureus* 524/SC. The concentration required is similar to that needed by the sensitive strain Oxford.

We wish to thank Mr I. Mathison for continuous technical assistance during this work, and Dr H. R. Perkins for the chemical analyses of the cell-wall preparations.

REFERENCES

- Barber, M. (1953). *J. gen. Microbiol.* **8**, 111.
 Barber, M. (1961). *J. clin. Path.* (in the Press).
 Batchelor, F. R., Doyle, F. P., Nayler, J. H. C. & Rolinson, G. N. (1959). *Nature, Lond.*, **183**, 257.
 Cummins, C. S. & Harris, H. (1956). *J. gen. Microbiol.* **14**, 583.
 Garrod, L. P. (1960). *Brit. med. J.* **ii**, 1695.
 Gooder, H. & Maxted, W. R. (1961). *Brit. med. J.* **i**, 205.
 Hancock, R. & Park, J. T. (1958). *Nature, Lond.*, **181**, 1050.
 Hugo, W. B. & Russell, A. D. (1960a). *Nature, Lond.*, **188**, 875.
 Hugo, W. B. & Russell, A. D. (1960b). *J. Bact.* **80**, 436.
 Knox, R. (1960). *Brit. med. J.* **ii**, 690.
 Mandelstam, J. & Rogers, H. J. (1958). *Nature, Lond.*, **181**, 956.
 Mandelstam, J. & Rogers, H. J. (1959). *Biochem. J.* **72**, 654.
 Meadow, P. (1960). *Biochem. J.* **76**, 8p.
 Nathenson, S. G. & Strominger, J. L. (1961). *J. Pharmacol.* **131**, 1.
 Park, J. T. (1958). *Biochem. J.* **70**, 2p.
 Park, J. T. & Strominger, J. (1957). *Science*, **125**, 99.
 Rogers, H. J. (1953). *J. Path. Bact.* **66**, 545.
 Rogers, H. J. (1954). *J. gen. Microbiol.* **10**, 209.
 Rogers, H. J. & Perkins, H. R. (1959). *Nature, Lond.*, **184**, 520.
 Rogers, H. J. & Perkins, H. R. (1960). *Biochem. J.* **77**, 448.
 Rolinson, G. N., Stevens, S., Batchelor, F. R., Wood, J. C. & Chain, E. B. (1960). *Lancet*, **ii**, 564.
 Stamp, Lord (1947). *J. gen. Microbiol.* **1**, 251.
 Stewart, G. T., Harrison, P. N. & Holt, R. J. (1960). *Brit. med. J.* **ii**, 694.
 Szybalski, W. & Bryerson, V. (1952). *J. Bact.* **64**, 489.

Biochem. J. (1961) **81**, 584

The Effects of Corn Oil on the Amounts of Cholesterol and the Excretion of Sterol in the Rat

By T. GERSON AND F. B. SHORLAND

Fats Research Laboratory, D.S.I.R., Wellington, New Zealand

AND YVONNE ADAMS

Nutrition Research Department, Medical Research Council, University of Otago Medical School, Dunedin, New Zealand

(Received 1 May 1961)

Using chicks as experimental animals, Peterson (1951) discovered that the addition of plant sterols to a cholesterol-containing diet decreased the concentration of cholesterol in the plasma. This effect was attributed by Peterson, Shneour, Peek & Gaffey (1953) to an inhibition of the formation of cholesterol esters in the mucosa by plant sterols, including β -sitosterol, present in soya-bean oil. Another mechanism proposed by Glover & Green (1955) for the inhibition of dietary-cholesterol absorption involved a blockage of the sites of

absorption of cholesterol in the intestinal mucosa by β -sitosterol and other plant sterols. However, the results of Beveridge, Connell, Mayer & Haust (1958), showing that the concentration of cholesterol in human plasma was lowered by adding corn oil and β -sitosterol to a cholesterol-free diet, cannot be explained fully on the basis of either of the mechanisms cited above. If the limiting factor was ester formation, the absorption of dietary and biliary cholesterol should result in an increase of free cholesterol relative to ester cholesterol in the